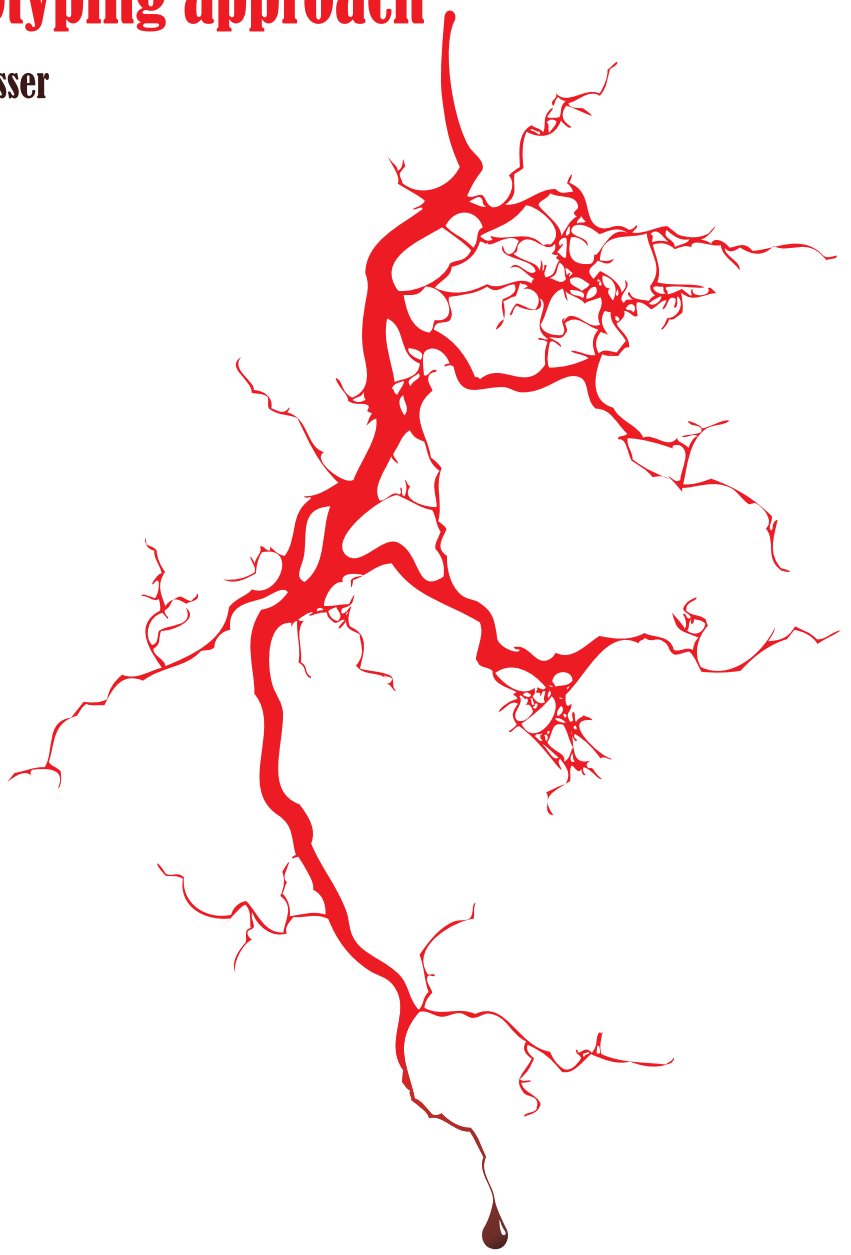
High fat challenges and detection of early perturbations in endothelial health

The use of a comprehensive phenotyping approach

Diederik Esser



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ABSTRACT

Background: Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality worldwide. One of the pathophysiology's that play a pivotal role in the development and progression of CVD is a dysfunctional endothelium. An important lifestyle risk factor for endothelial dysfunction is the diet and several nutrients have been classified to be either beneficial or harmful for the endothelium. Although CVD usually affects middle-aged or older adults, the onset of endothelial dysfunction begins in early life, emphasising the need for primary prevention. We therefore aimed to identify markers of early perturbations in endothelial health by using dietary stressors, e.g. high fat (HF) challenge test. Thereafter we aimed to evaluate if the potential early markers are reversible and can be improved after an intervention with a dietary anti-stressor.

Methods: First we validated the HF challenge test as a tool to trigger the endothelial response capacity. For that purpose, we compared the postprandial response after a HF shake with an average breakfast shake in young healthy men by assessing several plasma markers and functional measures of endothelial function. To identify new markers for early perturbations in endothelial health and to optimized the HF challenge test we applied three HF challenges differing in fatty acid type in two populations of middle-aged men, i.e. one at high- and one at low risk for developing CVD and characterized the postprandial response by applying high-throughput metabolomic and transcriptomic tools next to an extensive phenotyping of vascular function and vascular health parameters. Lastly, we evaluated if the, in the studies above, identified potential early biomarker profile is reversible and can be improved after an intervention with a dietary anti-stressor by means of a high flavanol chocolate intervention.

Results: In young men, we observed that a HF challenge decreased flow mediated dilation (FMD), but this decrease was also found after the consumption of an average breakfast shake. IL-8 concentrations were more pronouncedly increased after HF shake consumption compared to an average breakfast control shake. In middle-aged men, a HF challenge decreased the augmentation index (AIX) and elicited an activated state of cellular adherence in the circulation as determined by increased plasma soluble adhesion molecules, increased leukocyte cell surface integrin and selectin expression and increased number of leukocytes. A challenge high in mono-unsaturated fatty acids (MUFAs) elicited the highest postprandial triglyceride (TG) concentrations and the most pronounced effects on AIX. By applying high-throughput metabolomic tools, we observed that oxylipin profiles were affected by the HF challenge and that these changes were depended on dietary fatty acid composition. Application of transcriptome profiling revealed that changes in peripheral blood mononuclear cell (PBMC) gene expression

profiles after a HF challenge test were different between lean and obese subjects, with the most deviating effect after MUFA intake. The saturated fatty acid (SFA) shake decreased the expression of genes involved in cholesterol uptake and cholesterol biosynthesis and increased expression of genes involved in cholesterol efflux. MUFA increased expression of inflammatory genes and of peroxisome proliferator-activated receptor α (PPARα) target genes involved in β-oxidation. 4-week daily intake of a dietary anti-stressor, e.g. dark chocolate, increased fasting FMD and decreased AIX, and elicited a less activated state of cellular adherence, as determined by a decrease in plasma soluble adhesion molecules, a decrease in leukocyte cell surface integrin and selectin expression and a decrease in the number of leukocytes.

Conclusions: In this thesis we extensively characterized the postprandial response to a HF challenge in human subjects with different disease risk profiles and optimized the HF challenge test. We identified MUFAs as most potent fatty acids to trigger the vascular and cellular response capacity, which makes it the optimal fatty acid type to use in a HF challenge test. We demonstrated that besides functional measures of vascular function, also plasma and cellular factors involved in leukocyte adhesion to the endothelium are adversely affected by dietary stressors and are beneficially affected by a dietary anti-stressor. Therefore, we conclude that endothelial health can be more comprehensively measured by means of a biomarker profile consisting not only of the vascular function measures FMD and AIX, but also of a subset of soluble adhesion molecules in the plasma, leukocyte counts and cell surface integrin and selectin expression. To identify potential new leads for biomarkers, we applied whole genome gene expression profiling, combined with the HF challenge test which enabled us to detect small differences in health status. Furthermore, we identified metabolic and inflammatory pathways that are specifically affected by either MUFAs or SFAs. These findings increased our understanding on how a SFA or MUFA challenge exert their distinct effects on stress related and metabolic compensatory cellular processes and provided us with new potential leads to detect early perturbations in endothelial health.

Contents

Chapter 1

Introduction

Cardiovascular disease

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality worldwide. CVD includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), hypertension and peripheral artery disease. CVD has a strong genetic component, but lifestyle factors such as diet and exercise play also an important role in the onset and progression of these diseases. CVD is causally linked to obesity and type 2 diabetes mellitus. The incidence of CVD, obesity and type 2 diabetes mellitus is increasing rapidly and accounts for a considerable part of health care costs.

CVD is caused by disorders of the heart and blood vessels. The underlying pathological feature of most of these disorders is atherosclerosis. Atherosclerosis is a slowly progressing chronic disease which is characterised by the formation of atherosclerotic plaques in blood vessels. These plaques consists of necrotic cores, calcified regions, accumulated modified lipids, migrated smooth muscle cells (SMC), foam cells, endothelial cells, and leukocytes. Atherosclerosis is initiated by inflammatory processes in response to accumulated low-density lipoprotein (LDL) in the artery wall [1]. One of the pathophysiology's that play a pivotal role in the development and progression of atherosclerosis is a dysfunctional endothelium [2]. Although CVD usually affects middle-aged adults, the onset of endothelial dysfunction and subsequently atherosclerosis begins in early life, emphasising the need for primary prevention.

The endothelium

The endothelium is the thin layer of cells that lines the interior surface of all blood vessels. Vascular endothelial cells line the entire circulatory system, from the heart to the smallest capillaries and cover a surface area of approximately 1 to 7 m^2. The endothelium is involved in many aspects of vascular biology. It acts as a physical barrier, plays an important role in inflammation and coagulation and it is involved in the dilation and constriction of blood vessels, hence controlling vascular tone and heamodynamics [3].

Role of the endothelium in vascular heamodynamics

The way in which the endothelium regulates vascular tone is by secretion of mediators that enables vascular smooth muscle cells to contract or relax, leading to the constriction or dilation of blood vessels [3]. Nitric oxide (NO) plays a key role in vasodilatation. NO has a very short half-life and is continuously synthesized by the enzyme endothelial nitric oxide synthase

(eNOS) from the amino acid L-arginine requiring oxygen and electrons carried by NADPH [4]. The principle physiological stimulation for the synthesis and release of NO is the shear stress of blood flow [5]. The synthesis and release of NO can also be induced by a variety of agonist, including acetylcholine and bradykinin [6]. After being released in the sub-endothelial space, NO is able to stimulate soluble guanylyl cyclase in vascular smooth muscle cells to produce cyclic guanosine monophosphate (cGMP). This increase in cGMP activates GMP-dependent kinases, which dephosphorylates myosin light chain kinase, leading to relaxation. This relaxation of vascular smooth muscle cells subsequently dilate blood vessels and induce an increase in blood flow [7,8].

Role of the endothelium in coagulation

The endothelium plays also an important role in facilitating blood fluidity and blood flow. One of the ways endothelial cells maintain blood fluidity and blood flow is by creating an anti-thrombotic surface. The endothelium inhibits platelet adhesion and coagulation by releasing antithrombotic factors into the lumen, such as tissue factor pathway inhibitors (TFPIs), thrombomodulin, NO and prostaglandin I2 (PGI_2) [3]. However, upon activation by physical forces or by specific molecules such as, thrombin, endotoxins, several cytokines and oxidized lipoproteins, endothelial cells can secrete pro-thrombotic factors such as fibrinogen, P-selectin and von Willebrand factor (vWF), into the blood stream. The increase in pro-thrombotic factors may enhance the formation of fibrin clots and may diminish blood fluidity and blood flow [3].

Role of the endothelium in inflammation

Endothelial cells play also an important role in inflammation. Endothelial cells quiescence leukocytes by the release of NO and by suppressing the release of pro-inflammatory cytokines [9]. Upon activation, endothelial cells can secrete pro-inflammatory cytokines such as P-selectin, E-selectin, IL-6, IL-8 and cell-adhesion molecules (ICAMs), into the lumen. In addition, activated endothelial cells up-regulate cell surface expression of adhesion molecules such as E-selectin, vascular cell adhesion molecule 1 (VCAM1) and intra cellular adhesion molecule 1 (ICAM1) by the transcriptional regulation of NF-κB and activator protein 1 (AP1). These pro-inflammatory cytokines and adhesion molecules are able to activate and recruit leukocytes towards sites of infection or injury.

Un-activated leukocytes have low affinity to the endothelium, but upon activation, leukocytes increase the expression of selectins and integrins on their outer membranes [10-12]. Leukocyte

selectins and integrins are able to interact with cell adhesion molecules (CAMs) present on the endothelium. During the first steps of leukocyte-endothelial adhesion, low-affinity selectins such as L-selectin (CD62L) on leukocytes create weak bonds with P-selectin and E-selectin on the endothelium. These weak bonds causing leukocytes to slow their velocity and to roll along the endothelial surface under shear forces. During the second step of leukocyte-endothelial adhesion, leukocytes are more firmly bound to ICAMs and VCAMs on the endothelium by high-affinity integrins such as CD11a/CD18 (LFA-1), CD11b/CD18 (MAC-1/CR3) and CD11c/CD18. This firm adhesion is causing the leukocytes to arrest. During the final steps, these firmly bound leukocytes can transmigrate through the endothelium and infiltrate inflamed tissues [13]. Besides being important in response to infection or injury, the recruitment and trans-endothelial migration of leukocytes are also the initial steps of atherosclerosis [13,14].

Reduction in endothelial NO by oxidative stress

A reduced bioavailability of NO in endothelial cells is considered to be the main cause of endothelial dysfunction. A diminished bioavailability of NO causes constriction of arteries and facilitates vascular inflammation and coagulation. Several factors can account for the reduction in bioactive NO, including a decreased activity of the enzyme eNOS, depletion of the eNOS substrate L-arginine or the depletion of NO by reactive oxidant species (ROS). ROS are able to inactivate NO by scavenging NO to form peroxynitrite (ONOO-), itself a potent oxidant. In addition, ROS can oxidize tetrahydrobiopterin (BH_4), a cofactor needed by eNOS for the production of NO. Oxidizing BH_4 leads to uncoupling of this cofactor from eNOS [15]. Uncoupled eNOS produces ROS instead of NO, which will enhance oxidative stress, resulting in a vicious cycle. ROS are highly produced by leukocytes and endothelial cells under inflammatory conditions [16].

Nutritional impact on endothelial function

The endothelium and leukocytes are continuously exposed to a changing environment. An example of a significant change in environment occurs after successive food intake. It is therefore not surprizing that the diet plays an essential role in the aetiology of cardiovascular disorders. Several nutrients, such as high loads of saturated fat and sugar, have been classified as harmful stressors for the endothelium, whereas others have been classified as beneficial. The latter are also referred to as anti-stressors.

Dietary stressors for the endothelium

An example of a nutritional stressor to the endothelium is a high fat (HF) load. The intake of HF can temporary decrease vascular function and induce a low-grade pro-inflammatory response [17,18]. Elevated postprandial triglyceride levels are considered as a risk factor for CVD [19,20]. The ability to respond to a dietary stressor by means of a HF challenge, may reflect the flexibility of endothelial cells and leukocytes. A HF meal challenge can therefore be a useful tool to detect early perturbations in the endothelium or leukocytes, even before this can be measured under fasting conditions [21,22].

The exact impact and underlying mechanism behind this effect remains to be determined. One of the current hypothesis is that the elevated plasma triglyceride rich lipoproteins (TRLs) after a HF meal activate endothelial cells and leukocytes [20,34,35]. On endothelial cells, TRLs can stimulate the expression and secretion of ICAMs and other pro-inflammatory cytokines into the blood [11,23]. On leukocytes, TRLs can up-regulate the expression of integrins and selectins on their outer membranes and stimulate leukocytes to secrete pro-inflammatory interleukins, soluble adhesion molecules and coagulation factors [10-12]. Furthermore, postprandial TRLs increase neutrophil counts, with a concomitant production of pro-inflammatory cytokines and oxidative stress [24,25]. The combination of activated endothelial cells, increased leukocyte adherence and a pro-inflammatory milieu, will reduce the bioavailability of NO, subsequently diminishing endothelial function.

Dietary fatty acids and endothelial function

The impact of a HF challenge on endothelial health may be affected by the type of fat in the challenge. One way dietary fatty acids may exert their effect on endothelial health is by the modulation of gene transcription. Fatty acids are able to modulate gene transcription by regulating the activity of transcription factors, including nuclear receptors such as sterol regulatory element-binding proteins (SREBPs), liver X receptor (LXR) and the peroxisome proliferator-activated receptors (PPARs) [26]. Changing the activity of these transcription factors can alter the expression of genes involved in important processes of cardiovascular health. As an example, SREBPs regulates the transcription of genes involved in the biosynthesis of cholesterol and fatty acids [27], LXR regulate the transcription of genes involved in cholesterol storage, transport and catabolism [28,29] and PPARs are important regulators of lipid metabolism, but play also a role in the anti-inflammatory response [30-32]. The affinity of fatty acids for a transcription factor can dependent on their molecular structure. For example, mouse studies have shown that the best activators for PPARs are long-chained polyunsaturated fatty acids [33].

Dietary fatty acids may also modulate the postprandial inflammatory response by the formation of oxylipins. These fatty acid metabolites are involved in both pro- and anti-inflammatory processes [34]. Oxylipins are known for their autocrine and paracrine effects, but recent evidence suggests that oxylipins may also have systemic endocrine effects [35].

Dietary anti-stressors for the endothelium

There is increasing epidemiological evidence that a high intake of fruit and vegetables has a beneficial effects on CVD risk [36,37]. Fruit and vegetables may exert parts of their positive cardiovascular health effects by their flavonoid content. An important subgroup of the flavonoid family are flavanols [38]. Besides being highly present in fruits and vegetables, flavanols are also highly present in food products such as red wine and tea, and in particular in cacao or cacao derived products such as dark chocolate [38]. Studies are accumulating, that despite the fact that dark chocolate also contains high amounts of saturated fat and sugar, it still exerts positive cardiovascular health effects [39]. The underlying mechanisms on how cacao flavanols affect endothelial cells are not completely understood, but the beneficial cardiovascular health effects are most likely due to the increase in bioavailability of NO. Cacao flavanols may increase activity and gene expression of eNOS, the enzyme responsible for endothelial NO production [40]. Another described beneficial effect of cacao flavanols is on decreasing vascular arginase activity, thereby increasing local levels of L-arginine, the precursor for the synthesis of NO [41]. In addition, it has been suggested that cacao flavanols may improve endothelial function acutely by diminishing the breakdown of NO by free radicals through inhibition of NADPH oxidase [42]. Besides the direct effects of cacao flavanols on the endothelium, several studies suggest that cacao flavanols may also have anti-inflammatory and anti-thrombotic properties, decrease insulin resistance and have a positive effect on blood lipids [40,43]. These above described effects of cacao flavanols are mainly based on animal, *in vitro* or human *ex vivo* studies. Several human intervention studies have demonstrated that short term daily intake of flavanol enriched dark chocolate or cacao powder can improve endothelium-dependent vasodilatation, as determined by an increase in flow mediated dilation (FMD). Recently, the European Food and Safety Authority (EFSA) approved the health claim that cocoa flavanols consumption helps to maintain endothelium-dependent vasodilation [44].

Early detection of endothelial dysfunction

Endothelial dysfunction in its early stage is still reversible, which makes early detection of endothelial dysfunction of great importance. Identifying early and sensitive markers of endothelial function is therefore an important target for disease prevention.

Functional measures of endothelial dysfunction

The major limitation in studying the endothelium is the limited accessibility of this organ. To study endothelial function *in vivo* in a pre-clinical setting, one is restricted to non-invasive methodologies. To date, most research on endothelial function comprises the vascular measurement flow mediated dilation (FMD). The assessment of FMD was firstly introduced by Celermajer *et al.* in 1992 [45] and up till now it is still the most accepted and established method to study endothelial function. The method assesses the ability of vascular endothelial cells to respond to changes in shear stress. A meta-analyses investigating the association between FMD on the relative risk of cardiovascular events, found that each 1% increase in FMD is associated with a relative risk of cardiovascular events of 0.87 (95%CI: 0.83, 0.91) [46]. In addition, the EFSA considers that a sustained increase in FMD under fasting conditions in response to an intervention is a beneficial physiological effect [44]. However, the technique is relatively time consuming, technically challenging and shows relatively large within-subject variation. The question remains if other functional measures of endothelial function are available that are easier to use and shows less within-subject variation.

Comprehensive phenotyping

Comprehensive phenotyping of individuals before and after an intervention of dietary stressors, may be a conductive strategy to identify early changes in endothelial function. The examination of leukocyte function together with a wide range of plasma markers involved in cellular adherence of leukocytes to the endothelium, coagulation and systemic inflammation, paralleled by examination of vascular function will provide a more complete picture of the impact of an intervention on endothelial health. Furthermore, the application of largely unbiased high-throughput techniques, in particular transcriptomics but also metabolomics, may not only increase our understanding on how certain nutrients are able to affect inflammatory and metabolic processes and hence endothelial health but may also help to identify new early biomarker for endothelial dysfunction. As blood leukocytes play an important role in inflammation and can interact with the endothelium, peripheral blood mononuclear cells

(PBMCs) are a potential valuable source of biological material for transcriptomics analyses. PBMCs are a mixture of T-cells, B-cells and monocytes and as they are present in blood they are exposed to physiological changes including changes in nutrient level and inflammation [47]. As endothelial cell are not accessible and PMBCs are easy to collect, they form an important biological material to study endothelial heath *in vivo*.

Outline thesis

In the current thesis we used HF challenges to identify early markers for reduced endothelial function. To identify new early markers for endothelial perturbations we first needed to evaluate our stressor model, i.e. the high fat challenge test and evaluated several measures of vascular function. Therefore, we compared in **chapter 2** the postprandial response after a high fat/ high energy shake with an average breakfast shake in young healthy men by determining several measures of vascular function and plasma markers of endothelial function. After selection of the most promising vascular function techniques we tried to optimize the high fat challenge by comparing three different FA, and to identify new early markers for endothelial health we applied these three HF challenge tests in two populations of middle-aged men, i.e. one at high and one at low risk for developing CVD. To identify early markers for endothelial health, the postprandial HF response was comprehensively characterized by measuring a combination of vascular function, plasma markers and leukocyte cell surface markers (**chapter 3**), and by applying more sensitive techniques, such as plasma metabolomics (**chapter 4**) and transcriptome analysis of PBMCs (**chapter 5**). To evaluate if the early markers are reversible after an intervention with a dietary anti-stressor, we investigated in chapter 6 the effects of acute intake and of 4-week daily high flavanol chocolate and normal flavanol chocolate consumption on FMD, heamodynamics, arterial stiffness, leukocyte numbers, plasma cytokines and leukocyte cell surfaces molecules in healthy overweight middle-aged men. In addition we investigated if a high or normal flavanol chocolate background is able to affect the response capacity towards a HF challenge. An overall discussion of **chapters 2 to 6** will be described in **chapter 7**.

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Chapter 2

Vascular and inflammatory high fat meal responses in young healthy men; a discriminative role of IL-8 observed in a randomized trial

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ABSTRACT

Background: High fat meal challenges are known to induce postprandial low-grade inflammation and endothelial dysfunction. This assumption is largely based on studies performed in older populations or in populations with a progressed disease state and an appropriate control meal is often lacking. Young healthy individuals might be more resilient to such challenges. We therefore aimed to characterize the vascular and inflammatory response after a high fat meal in young healthy individuals.

Methods: In a double-blind randomized cross-over intervention study, we used a comprehensive phenotyping approach to determine the vascular and inflammatory response after consumption of a high fat/ high energy (HF/HE) shake and after an average breakfast shake in 20 young healthy subjects. Both interventions were performed three times.

Results: Many features of the vascular postprandial response, such as FMD, arterial stiffness and micro-vascular skin blood flow were not different between shakes. HF/HE shake consumption was associated with a more pronounced increase in blood pressure, heart rate, plasma concentrations of IL-8 and PBMCs gene expression of IL-8 and CD54 (ICAM-1), whereas plasma concentrations of sVCAM1 were decreased compared to an average breakfast.

Conclusions: Whereas no difference in postprandial response were observed on classical markers of endothelial function, we did observe differences between consumption of a HF/HE and an average breakfast meal on blood pressure and IL-8 in young healthy volunteers. IL-8 might play an important role in dealing with high fat challenges and might be an early marker for endothelial stress, a stage preceding endothelial dysfunction.

INTRODUCTION

A lifestyle factor known to be important in development and progression of cardiovascular disease (CVD) is diet. Although several dietary components and patterns have been related to vascular function, the postprandial response has gained specific attention since it has been associated with an impaired vascular function, low grade inflammation and increased cardiovascular risk [1-3]. Postprandial effects on vascular function and inflammation are reversible and temporally, but can be of importance since most individuals are in the postprandial state the greater part of the day [4-8]. Most studies that investigated the postprandial vascular response used flow mediated dilation (FMD) as measure of vascular function. Vascular function can also be assessed by other measures, of which some have been applied in postprandial studies [9-12]. Postprandial challenges often used in relation to CVD are high fat (HF) meals, as these atherogenic meals provide a direct source of stress [13]. However, many previous studies only investigated a small part of the postprandial response or were performed in older individuals or in populations with a progressed disease state, such as diabetes, metabolic syndrome, hypertension or cardiovascular disease. The postprandial impact of high fat/high energy (HF/HE) meals on both vascular function and inflammation in healthy young subjects has been less well studied. In addition, many postprandial studies compared HF/HE meals with water consumption, with other macronutrients or only with baseline recordings. In these studies the question remains whether observed postprandial changes are due to the high fat content itself, to a common meal effect, or to circadian rhythm influences. Hence, it is not completely clear whether the HF/HE meal-induced changes in vascular function and inflammation are solely caused by the HF content or by the meal itself and whether this temporary impairment is also elicited in younger healthy individuals. To address these points, we examined the postprandial response after a HF/HE shake in young healthy men on several measures of vascular function and blood markers of endothelial function and inflammation, and compared this response with an average breakfast milkshake. Since most functional measures of vascular function are known to have large variations in reproducibility, the effects of both shakes were studied three times within the same individual.

MATERIALS AND METHODS

Ethics statement

All subjects gave written informed consent and the study was approved by the Medical Ethics Committee of Wageningen. The study was conducted according to the principles of the

Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO) and registered at ClinicalTrials.gov (Identifier: NCT00766623).

Subjects

Twenty healthy male volunteers of Western European descent, between 18 and 27 years, were recruited. Exclusion criteria were a body mass index (BMI) <18 or >28 kg/m^2, urine glucose concentrations >0.25 g/l, fasting blood glucose <3 or >5.5 mmol/L or blood Hb values <8.4 mmol/L and smoking. Furthermore, subjects were excluded if they were diagnosed with any long-term medical condition or high blood pressure (systolic BP>140 mmHg and/or diastolic BP>90 mmHg).

Study design

The study was a double-blind randomized cross-over intervention study in which participants visited the university six times in total; three times to obtain postprandial responses on a HF/HE shake and three times to obtain postprandial responses on an average breakfast shake. The latter was used to acquire a common postprandial response and shakes were therefore not isocaloric. A one-week washout period was the minimum between consecutive study days. Shakes were assigned alternately over the study days and order of start was randomly assigned. A research assistant generated the random allocation sequence. Shakes were given a code and both subjects and researchers were blinded to the intervention. Prior to each study day, subjects consumed a standardized low fat evening meal, were refrained from alcohol or strenuous exercise and were not allowed to eat or drink anything except water after 08.00 pm. For each subject, starting time of every study day was kept constant.

A study day was executed as follows: upon arrival, a cannula was placed, baseline fasted blood samples were collected baseline vascular measurements were done. Subsequently, the subject received either a HF/HE or an average breakfast shake. Postprandial vascular measurements and blood samples were taken 3 and 6 hours after milkshake consumption. These time points were chosen because in previous studies, a maximal FMD response was observed 3 hours after high fat meal consumption and the FMD measure was back to baseline values 6 hours postprandially [14,15]. Throughout the study day, subjects were not allowed to eat or drink anything except water.

Shakes

The HF/HE shake consisted of 53%(w/v) fresh cream, 3%(w/v) sugar and 44%(w/v) water and reflected a macronutrient composition of 6g protein, 95g fat (of which 54g saturated), 22g carbohydrates and represented a total energy content of 3,992KJ. The average breakfast shake was, based on macronutrient composition, comparable to a breakfast as averagely consumed by young men in the Netherlands [16]. This average breakfast shake consisted of 43%(w/v) full cream milk, 48%(w/v) full cream yoghurt, 4%(w/v) lemonade, 4%(w/v) fantomalt (Nutricia B.V., the Netherlands) and 1%(w/v) wheat fiber and reflected a macronutrient composition of 17g protein, 14.5g fat, (of which 9g saturated), 49.5g carbohydrates and 2.3g fiber and represented a total energy content of 1674KJ (NEVO 2006). Both shakes had a total volume of 500ml.

Metabolic parameters

Plasma triacylglycerol (TG), free fatty acids (FFA), insulin and glucose concentrations were assessed at baseline and 1, 2, 3, 4, and 6 hours after milkshake consumption and were measured by a hospital laboratory (SHO, Velp, the Netherlands).

Functional measures of vascular function

Measurements of vascular function included micro-vascular skin blood flow, arterial stiffness and FMD. Measures were performed in above mentioned order and whole data was acquired at baseline and 3 and 6 hours after shake consumption. All measurements were performed in supine position after 10 minutes rest, in a quiet temperature controlled room at moderate light intensity.

Blood pressure

Blood pressure (BP) and heart rate (HR) were assessed automatically (DINAMAP® PRO 100) during the functional measurements with a 5 minute interval.

Iontophoresis laser Doppler

Micro-vascular skin blood flow was assessed by laser Doppler iontophoresis [17]. Briefly, two ion chambers (MIC-ION6, Moor Instruments, UK) on the volar aspect of the forearm were filled with 1% sodium nitroprusside (SNP) (Sigma - 31444-50G) or 1% acetylcholine (Ach) (Sigma - A6625-25G) solution and iontophoretically administered (MIC2™, Moor Instruments,

UK). Skin blood flow was recorded by laser Doppler (MoorFLPI, Moor Instruments, UK) and expressed as incremental area under the curve (AUC) calculated from mean flux outcomes plotted against total recorded time period (NCSS software v07.1.4).

Pulse wave analysis of the radial artery

Arterial stiffness was assessed by pulse wave analysis (PWA) of the radial artery by applanation tonometry (SphygmoCor®CP System, ATcor Medical). In short, a pressure-sensitive probe was placed on the radial artery to generate a pulse pressure wave (Sphygmocor software v8.0). In combination with the brachial blood pressure measurements we deduced central aortic pressures and the heart rate corrected augmentation index (AIX) [18].

Flow-mediated dilation

FMD was performed according to techniques described by the International Brachial Artery Reactivity Task Force [19]. In short, after baseline recordings, a pressure cuff on the forearm was inflated and kept constant at a pressure of 200mmHg for 5 min. Thereafter, the cuff was released and records of the artery were made every 20 seconds for 4 minutes (Picus, ART.LAB v2.1, Esaote benelux bv.). FMD was computed as maximum vessel diameter after cuff release divided by baseline and expressed in percentage. A nitroglycerin dose was administrated sublingually by spray at the end of each day.

Blood measures

Plasma markers

Baseline and postprandial plasma cytokine concentrations were determined once for each shake and analyzed on preformatted arrays on a SECTOR Imager 2400 reader (Meso Scale Diagnostics, LLC) as described previously [20].

PBMC gene expression

Peripheral blood mononuclear cells (PBMCs) were isolated by BD Vacutainer Cell Preparation Tubes. RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantified (Nanodrop ND 1000, Nanodrop technologies, Wilmington, Delaware USA) and quality was determined (Agilent 2100 Bioanalyser, Agilent Technologies, South Queensferry, UK). RNA with a RIN score >7 was thereafter reverse transcribed (cDNA synthesis kit, Promega, Leiden, the Netherlands) and analyzed by qPCR (SensiMix SYBR No-ROX, Bioline, London, UK) on a CFX384 Real-Time System (C1000 Thermal Cycler, Biorad, Veenendaal, The Netherlands).

Primer sequences were chosen based on the sequences available in Primer3 (v. 0.4.0). Data was normalized by the housekeeping gene hUPO.

Statistics

Study outcomes of all three testing days are expressed as pooled mean and SD was calculated by the root mean squared error. Statistical comparisons were performed by linear mixed models for repeated measures (PASW statistics 17.0.3), using 'diet', 'time point' and 'diet x time point' as fixed effects and subject as random effect. Postprandial responses on plasma cytokines were determined once for each shake and baseline values were included as covariate in the model if they were of significant influence and Studentized residuals >3, obtained from the mixed model, were considered outliers and removed from the model. A value of $P<0.05$ was considered significant.

RESULTS

Subjects characteristics

Twenty volunteers entered the study. Eighteen completed all six study days, one volunteer completed four study days and one completed two study days (**Figure 2.1**). Baseline characteristics of the subjects are listed in **Table 2.1**.

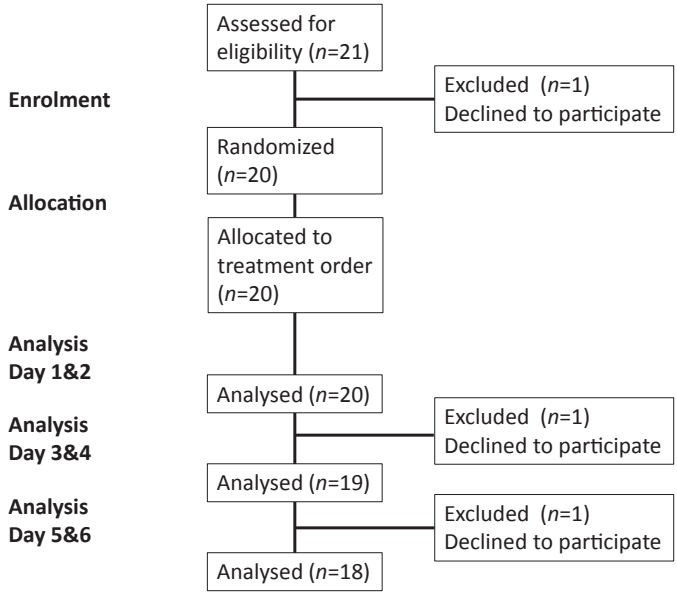

Figure 2.1 CONSORT flow diagram.

Table 2.1 Baseline characteristics of the participants

	Mean ± SD (*n*=20)
Age (y)	22 ± 2
Length (cm)	185 ± 7
Weight (kg)	78 ± 10
BMI (kg/m^2)	22.7 ± 2.4
Glucose (mmol/L)	4.9 ± 0.3
TG (mmol/L)	1.1 ± 0.3
FFA (mmol/L)	0.52 ± 0.18
Insulin (uIU/ml)	6.94 ± 3.22
Systolic BP (mmHg)	111 ± 9
Diastolic BP (mmHg)	59 ± 6

Values are expressed as mean ± SD. TG, triglycerides; FFA, free fatty acids; BP, Blood pressure.

Metabolic parameters

Baseline and postprandial changes in TG, FFA, insulin and glucose are listed in **Table 2.2**. A significant difference in response between the HF/HE and the average breakfast shake was observed for all parameters.

Measures of vascular function

Outcomes of all functional vascular measures are listed in **Table 2.3**. Consumption of both shakes resulted in a small but significant postprandial reduction in FMD% at 3 hours that returned to baseline after 6 hours. No significant difference in response between shakes was observed. Consumption of both shakes resulted in an significant postprandial increase in HR and systolic blood pressure (SBP), with a significant higher response after HF/HE shake consumption. No significant postprandial changes were observed for diastolic blood pressure (DBP). Similar to the effects on SBP, HF/HE consumption also resulted in a higher postprandial increase in central aortic pulse pressure. The AIX was decreased for both shakes, with no difference in response between shakes. Micro-vascular blood flow was significant reduced after consumption of both shakes, with no difference in response between shakes. Similar results were observed for both SNP and Ach treatment.

Plasma cytokines

Plasma cytokines levels before and after milkshake consumption are listed in **Table 2.4**. A significant increase in IL-8 was observed after HE/HF shake consumption compared to an

Table 2.2 Baseline and postprandial concentrations of metabolic plasma parameters after high fat/high energy (HF/HE) or average breakfast control shake consumption

		Time						*P*-value		
	Shake	Baseline	1h	2h	3h	4h	6h	Shake	Time	Interaction Shake x Time
TG	HF/HE	1.0 ± 0.2	1.1 ± 0.2	1.5 ± 0.3	1.7 ± 0.3	2.0 ± 0.4	1.5 ± 0.3	<0.001	<0.001	<0.001
(mmol/L)	control	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	0.9 ± 0.2			
Glucose	HF/HE	4.2 ± 0.4	4.0 ± 0.4	3.8 ± 0.3	4.0 ± 0.2	4.1 ± 0.3	3.9 ± 0.3	NS	<0.001	0.019
(mmol/L)	control	4.2 ± 0.3	4.1 ± 0.6	3.6 ± 0.4	4.0 ± 0.2	4.0 ± 0.3	4.0 ± 0.3			
FFA	HF/HE	0.50 ± 0.15	0.32 ± 0.10	0.34 ± 0.06	0.55 ± 0.09	0.67 ± 0.07	0.76 ± 0.10	<0.001	<0.001	<0.001
(mmol/L)	control	0.51 ± 0.17	0.15 ± 0.05	0.17 ± 0.04	0.32 ± 0.09	0.53 ± 0.10	0.78 ± 0.14			
Insulin	HF/HE	6.9 ± 1.8	20.0 ± 11.5	10.2 ± 2.9	7.1 ± 2.5	8.2 ± 2.9	4.8 ± 1.4	<0.001	<0.001	<0.001
(uIU/ml)	control	7.0 ± 2.3	43.3 ± 17.0	10.9 ± 7.6	5.6 ± 1.8	4.4 ± 1.4	3.3 ± 1.1			

Values are pooled mean ± SD of all three study days (n=20). TG, triglycerides; FFA, free fatty acids. *P*-values were calculated using linear mixed models for repeated measures.

average breakfast. Plasma concentrations of SAA and VCAM-1 were significantly decreased after a HE/HF milkshake compared to an average breakfast. Consumption of both shakes resulted in a significant postprandial increase in plasma levels of IL-6, whereas E-selectin and thrombomodulin were decreased postprandially.

Table 2.3 Baseline and postprandial values of vascular function measures after high fat/high energy (HF/HE) or average breakfast control shake consumption

		Time			*P*-value		
	Shake	Baseline	3h	6h	Shake	Time	Interaction Shake x Time
Iontophoresis							
Total Ach (AUC)	HF/HE	2674 ± 982	1328 ± 576	1175 ± 533	NS	<0.001	NS
	control	2464 ± 1030	1457 ± 861	1134 ± 664			
Total SNP (AUC)	HF/HE	2964 ± 823	2189 ± 809	2149 ± 787	NS	<0.001	NS
	control	2731 ± 960	2328 ± 868	1997 ± 1091			
Brachial blood pressure							
Systolic BP (mmHg)	HF/HE	110 ± 5	114 ± 4	116 ± 4	0.001	<0.001	0.003
	control	110 ± 4	111 ± 4	113 ± 4			
Diastolic BP (mmHg)	HF/HE	59 ± 5	58 ± 4	58 ± 4	NS	NS	NS
	control	59 ± 4	58 ± 4	58 ± 4			
Heart rate (BPM)	HF/HE	54 ± 5	58 ± 4	57 ± 4	0.001	<0.001	0.001
	control	55 ± 4	55 ± 4	55 ± 4			
Pulse wave analysis							
Central systolic BP (mmHg)	HF/HE	92 ± 4	93 ± 4	94 ± 3	0.001	NS	NS
	control	92 ± 4	91 ± 3	92 ± 3			
Central pulse pressure (mmHg)	HF/HE	33 ± 3	35 ± 2	36 ± 2	<0.001	<0.001	0.028
	control	33 ± 3	33 ± 2	34 ± 3			
AIX (%)	HF/HE	-13 ± 10	-15 ± 10	-16 ± 6	0.012	NS	NS
	control	-16 ± 7	-17 ± 7	-18 ± 8			
FMD							
Baseline vessel diameter (mm)	HF/HE	4.21 ± 0.15	4.23 ± 0.14	4.22 ± 0.14	NS	NS	NS
	control	4.21 ± 0.14	4.18 ± 0.13	4.19 ± 0.18			
Maximum vessel diameter (mm)	HF/HE	4.42 ± 0.18	4.42 ± 0.15	4.45 ± 0.19	0.025	0.026	NS
	control	4.41 ± 0.17	4.35 ± 0.13	4.41 ± 0.16			
FMD (%)	HF/HE	5.05 ± 2.01	4.54 ± 1.88	5.36 ± 2.55	NS	0.004	NS
	control	4.87 ± 2.50	4.13 ± 2.02	5.25 ± 2.09			

Values are pooled mean ± SD of all three study days (n=20). *P*-values were calculated using linear mixed models for repeated measures. Ach, Acetylcholine; SNP, sodium nitroprusside; AUC, area under the curve; BPM, beats per minute; BP, blood pressure; AIX, augmentation index; FMD, flow mediated dilation.

Table 2.4 Baseline and postprandial changes in inflammatory cytokines after high fat/high energy (HF/HE) or average breakfast control shake consumption

		Time			*P*-value		
	Shake	Baseline	3h	6h	Shake	Time	Interaction Shake x Time
CRP (ng/ml)	HF/HE	464 ± 615	435 ± 573	412 ± 528	NS	NS	NS
	control	461 ± 746	641 ± 1357	769 ±1841			
SAA (ng/ml)	HF/HE	497 ± 277	422 ± 267	382 ± 206	NS	NS	0.022
	control	520 ± 432	532 ± 490	585 ±604			
sICAM-1 (ng/ml)	HF/HE	181 ± 27	179 ± 26	176 ± 30	NS	NS	NS
	control	177 ± 27	179 ± 31	178 ± 32			
sVCAM-1 (ng/ml)	HF/HE	305 ± 37	296 ± 35	296 ± 35	0.003	NS	0.038
	control	294 ± 45	298 ± 49	304 ± 50			
E-selectin (ng/ml)	HF/HE	6.03 ± 1.91	5.66 ± 1.81	5.79 ± 1.87	NS	0.038	NS
	control	6.40 ± 2.52	5.83 ± 1.83	5.81 ± 1.60			
P-selectin (ng/ml)	HF/HE	51.9 ± 16.1	52.0 ± 15.5	53.0 ± 18.8	NS	NS	NS
	control	50.5 ± 11.9	51.4 ± 11.0	49.8 ± 13.2			
sICAM-3 (ng/ml)	HF/HE	1.47 ± 0.28	1.48 ± 0.26	1.52 ± 0.27	NS	0.042	NS
	control	1.52 ± 0.33	1.42 ± 0.18	1.50 ± 0.28			
TM (ng/ml)	HF/HE	2.34 ± 0.42	2.23 ± 0.36	2.21 ± 0.37	NS	<0.001	NS
	control	2.39 ± 0.40	2.28 ± 0.39	2.24 ± 0.37			
IL-1β (pg/ml)	HF/HE	0.47 ± 0.31	0.47 ± 0.28	0.49 ± 0.32	NS	NS	NS
	control	0.38 ± 0.17	0.54 ± 0.48	0.57 ± 0.50			
IL-6 (pg/ml)	HF/HE	0.74 ± 0.24	1.18 ± 0.48	1.38 ± 0.73	NS	<0.001	NS
	control	1.10 ± 1.01	1.40 ± 0.85	2.31 ± 2.31			
IL-8 (pg/ml)	HF/HE	4.08 ± 0.95	4.36 ± 1.18	4.45 ± 1.00	0.002	NS	0.043
	control	4.15 ± 1.20	3.84 ± 0.74	3.78 ± 0.77			
TNFα (pg/ml)	HF/HE	6.12 ± 1.70	6.07 ± 1.96	6.12 ± 1.91	NS	NS	NS
	control	5.90 ± 1.57	6.19 ± 1.75	6.19 ± 1.87			

Values are mean ± SD from the last study days (*n*=20). *P*-values were calculated using linear mixed models for repeated measures. TM, thrombomodulin.

PBMC gene expression

We examined expression changes of a selection of genes (**Table 2.5**), known to be involved in inflammation or endothelial function in PBMCs. HF/HE shake consumption resulted in a higher postprandial up regulation of IL-8 after 3 and 6 hours, and CD54 (ICAM-1) after 3 hours, compared to an average breakfast. Several other inflammatory genes, like TNFα, MCP1 and CD62l, were up-regulated postprandially, with no differences in response between shakes.

Table 2.5 Changes in expression of genes involved in inflammation in PBMCs after high fat/high energy (HF/HE) or average breakfast control shake consumption

		SLR		*P*-value		
	Shake	3h	6h	Shake	Time	Interaction Shake x Time
IL-8	HF/HE	1.23 ± 2.10	1.37 ± 2.56	0.003	<0.001	<0.001
	control	-0.26 ± 2.00	0.68 ± 2.17			
MCP1	HF/HE	0.32 ± 1.20	0.75 ± 1.44	0.049	0.001	NS
	control	-0.03 ± 1.58	0.28 ± 1.61			
TNFα	HF/HE	0.40 ± 0.38	0.29 ± 0.70	NS	<0.001	NS
	control	0.23 ± 0.49	0.22 ± 0.41			
CD62l	HF/HE	0.03 ± 0.39	0.03 ± 0.61	NS	0.035	NS
	control	0.02 ± 0.49	0.19 ±0.49			
CD54/ ICAM-1	HF/HE	0.25 ± 0.62	-0.03 ± 0.64	NS	0.001	0.006
	control	0.08 ± 0.70	0.12 ± 0.64			
IL-1β	HF/HE	0.00 ± 0.70	0.00 ± 0.87	NS	NS	NS
	control	-0.27 ± 0.90	-0.05 ± 1.09			
CD11a	HF/HE	-0.10 ± 0.50	-0.13 ± 0.66	NS	NS	NS
	control	-0.08 ± 0.50	-0.08 ± 0.44			

Changes are expressed as signal to log ratio (SLR) compared to baseline values, values are pooled mean ± SD of all three study days (n=20). *P*-values were calculated using linear mixed models for repeated measures.

As illustrated in **Figure 2.2**, postprandial responses on HR, SBP, FMD and IL-8 gene expression were similar for each repeated testing day.

DISCUSSION

In the current study we used a comprehensive approach to study postprandial effects of a HF/HE meal on vascular function and plasma markers of endothelial function and inflammation in young healthy subjects by comparing it to average breakfast milkshake. HF/HE shake consumption was associated with a more pronounced increase in HR, SBP, plasma IL-8 and PBMC gene expression of IL-8 and CD54 (ICAM-1) compared to average breakfast shake consumption.

Only a limited number of studies on postprandial effects of a HF/HE meal on HR and BP have been performed in young healthy individuals. Ayer *et al.*, showed an increase in HR 3 hours after HF meal consumption in a healthy young population [9]. However no reference

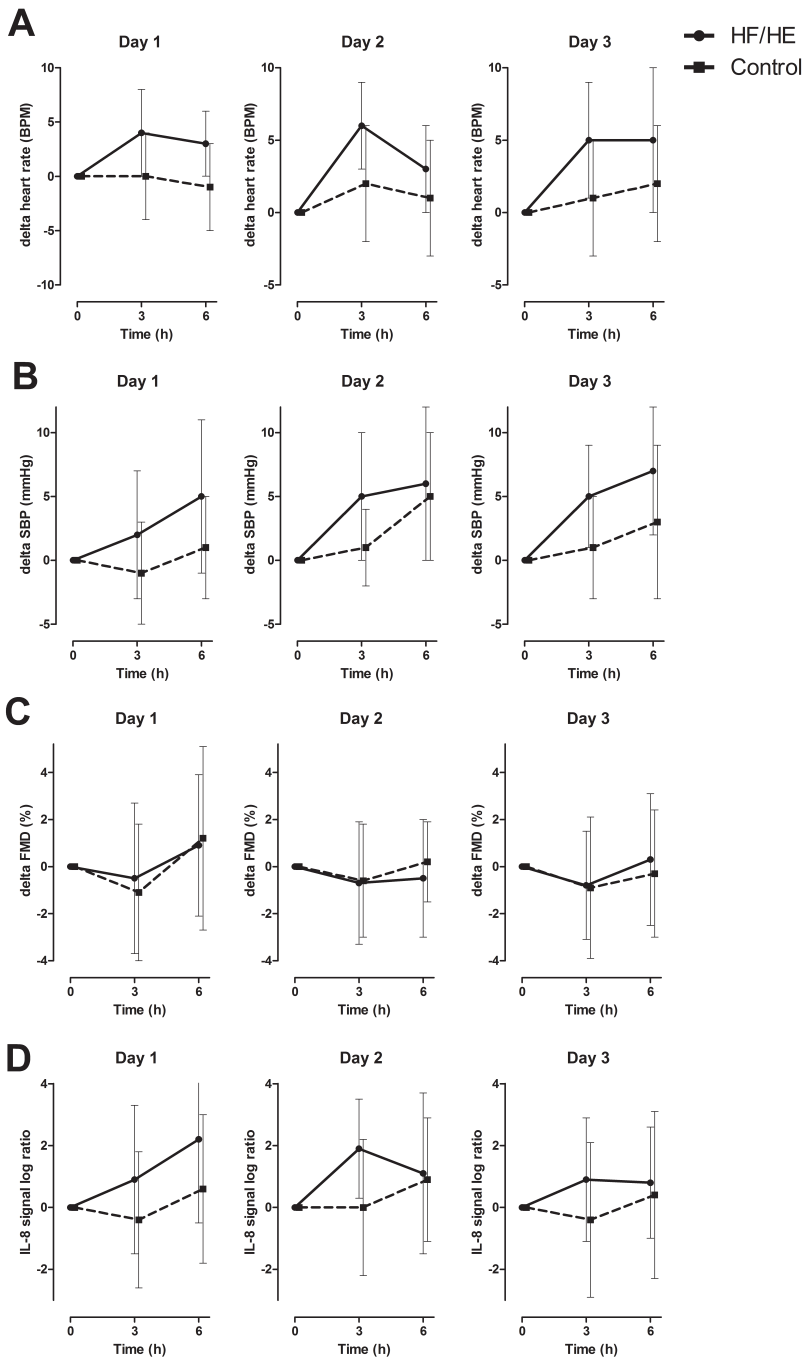

Figure 2.2 Postprandial changes after consumption of high fat/high energy (HF/HE) or average breakfast shake on heart rate (A), systolic blood pressure (B), FMD (C) and PBMC gene expression of IL-8 (D) subdivided for repeated testing days. Values are mean ± SD.

meal was included and it is therefore unclear if the observed effects are caused by HF or by a common postprandial response. With respect to BP, Biston *et al.* observed that ingestion of a mixed meal was associated with a postprandial increase in SBP in young healthy subjects, but they did not compared this response with other meal types [21]. Our observed postprandial increase in BP and HR after both shakes may point towards sympathetic activation after meal intake [22]. Another factor known to affect BP is insulin. By stimulating vasodilatation, insulin is able to reduce BP [23]. The lower BP increase after the average breakfast shake may be due to the higher postprandial insulin concentrations after this shake. However, the insulin peak was increased one hour postprandially, whereas BP was measured three and six hours postprandially.

Although no differences in response between shakes were observed on most vascular measurements, several measures were altered after consumption of both shakes and are therefore caused by consumption of the meal and/or circadian effects. One example is the small but significant postprandial reduction in FMD%. While several previous studies observed a postprandial decrease in FMD after HF consumption, many others found no effect or found an effect with all intervention meals (reviewed by Jackson *et al.*) [5]. Interestingly, many of the studies with no effect were conducted in healthy individuals [9,24,25]. We hypothesize that young healthy individuals are still able to handle a high fat and high energy load in such a way that a similar postprandial endothelial response is observed after an averagely consumed breakfast meal.

Besides a postprandial reduction in FMD, consumption of both shakes also altered the AIX and micro-vascular blood flow. The postprandial decrease in AIX is in line with previous findings [9,10,26]. The mechanism behind this postprandial reduction in AIX has been less well studied, but arterial smooth muscle relaxation in the general circulation in response to nutrient delivery might be an explanation [26]. AIX values were already negative at baseline and an augmented pressure was therefore not present in this study population. The postprandial decrease in AIX did therefore not affect postprandial central pulse pressure outcomes. The blunted vasoactive compound-induced increase in micro-vascular blood flow after consumption of both shakes was observed for both SNP and Ach and is therefore not endothelium dependent. However, vasoactive compounds were repeatedly administrated at the same location within a few hours. We therefore cannot exclude a reduced sensitivity of the vasculature for these compounds, explaining the observed blunted increase in blood flow.

HF/HE shake consumption resulted in an increase in plasma concentrations and PBMC gene expression of IL-8 compared to the average breakfast shake. Postprandial studies on IL-8 measures are limited. Esposito *et al.* found that serum IL-8 concentrations did not change

significantly 4 hours after HF consumption in 30 middle aged diabetic and 30 non-diabetic subjects [27]. However, their HF intervention meal was a mixed meal, which contained besides 52g of fat also 58g of carbohydrates, which is closely to the 49.5g carbohydrates in our average breakfast shake that showed no response on IL-8. Another study that measured plasma concentrations of IL-8 in 8 healthy young men found a non-significant increase two hours after HF meal consumption [14]. However, they did not measure beyond 2 hours postprandially. Although hardly evaluated in postprandial studies, IL-8 is of importance for atherosclerosis development as it is involved in neutrophil activation and recruitment [28] and triggers monocyte adhesion to the vascular endothelium [29]. IL-8 gene expression and production can be regulated by oxidant stress [30,31]. HF/HE consumption may initiate oxidant stress and thereby trigger endothelial cells to produce and release IL-8, where it is stored in Weibel-Palade bodies [32].

The HF/HE specific decrease in plasma levels of sVCAM-1 is not in line with the general prevailing hypothesis that HF meal consumption is associated with an increase in soluble adhesion molecules [2]. However, postprandial studies regarding soluble adhesion molecules have not shown consistent results. Some studies report elevated sICAM-1 and sVCAM-1 levels after HF meal consumption [33,34], whereas many others do not [35-39]. Only one studie found a decrease after HF meal consumption [40]. In general, most studies that did not observe a postprandial increase were performed in younger and often healthy study populations.

Besides the prevailing hypothesis that triglycerides rich particles may increase inflammation, recent human intervention studies demonstrated that high fat/high energy intake can increase circulating endotoxins [41]. These postprandial endotoxins are transported through the gut wall during chylomicron uptake and are able to activate inflammation [42]. Harte *et al.* showed that circulating endotoxins levels show more dramatic postprandial changes in groups with a higher metabolic risk [41]. This might additional explain why in our young healthy study population, several plasma inflammatory cytokines were not altered after HF/HE consumption. To draw a parallel with the measures of vascular function, plasma cytokines were measured at baseline and 3 and 6 hours after shake consumption. As a consequence changes in plasma cytokines that occurred before 3 hours are not detected. It has for example been shown that the cytokines CCL5/RANTES and MCP1 are already changed 1 hour after high fat meal intake [43].

The design used in the current study, with three repeated observations of the intervention within the same individual, allowed to detect small but significant effect sizes, even for measures known for their large variation in reproducibility, such as FMD. In addition, many other previous postprandial studies with high fat challenges used water, fasting or other type

of macronutrients as a reference meal. These studies cannot rule out that observed effects are also elicited by a common meal. To enable distinguishing between a HF/HE and a common meal response, we used an averagely consumed breakfast shake as a control. As a consequence, both shakes were not isocaloric and observed differences in response between shakes cannot solely be described to the high fat content but can also be caused by the high energy content or difference in macronutrient composition. Nevertheless, as individuals are daily exposed to a breakfast, the usage of an average breakfast meal as a control mirrors the real life situation.

Whereas no difference in postprandial response were observed on classical markers of endothelial function, we did observe differences between consumption of a HF/HE and an average breakfast meal on blood pressure and IL-8 in young healthy volunteers. The postprandial increase in blood pressure and plasma IL-8 concentrations after a high fat meal might create a potential harmful environment for the endothelium which in young healthy individuals may not directly affect measures of vascular function, but repeated exposure may on the long run induce endothelial dysfunction. This may be a likely occurrence, since in the western world high fat and high energy meals are regularly consumed. Since IL-8 was one off the only factors different in response between the shakes, it might play a role in dealing with high fat challenges and one of the first factors that may reflect endothelial stress, a stage preceding endothelial dysfunction.

Acknowledgements

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Chapter 3

A high fat SFA, MUFA or n3 PUFA challenge affects the vascular response and initiates an activated state of cellular adherence in lean and obese middle aged men

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ABSTRACT

Background: BMI and fatty-acid type affect postprandial metabolic triglyceride (TG) responses, but if these factors also affect vascular, inflammatory and leukocyte adherence responses remains unclear. We therefore compared those postprandial responses between lean and obese men after three high-fat challenges differing in fatty-acid composition.

Methods: In a cross-over double blind study, 18-lean (BMI 18–25 kg/m^2) and 18-obese (BMI >29kg/m^2) middle-aged men received three isocaloric high-fat milkshakes containing 95g fat (88 energy-%), either high in saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs) or n3 polyunsaturated fatty acids (PUFAs). Hemodynamics, augmentation index (AIX), leukocyte cell-surface adhesion markers and plasma cytokines involved in vascular adherence, coagulation and inflammation were measured before and after shake consumption.

Results: In both groups and after all shakes, AIX decreased, plasma sICAM1, sICAM3, sVCAM1 and IL-8 increased, monocyte CD11a, CD11b and CD621 expression increased, neutrophil CD11a, CD11b and CD621 expression increased and lymphocytes CD62l expression increased. Lymphocyte CD11a and CD11b expression decreased in lean subjects after consumption of all shakes, but did not changed in obese subjects. Obese subjects had a less pronounced decrease in heart rate after the consumption of all shakes. MUFA consumption induced a more pronounced decrease in blood pressure and AIX compared to the other shakes in both lean and obese subjects.

Conclusions: High-fat consumption initiates an activated state of cellular adherence and atherogenic milieu. This response was independent of fatty-acid type consumed or being lean or obese, despite the clear differences in postprandial TG responses between the groups and different shakes. These findings suggests that besides increased TG, other mechanisms are involved in the high-fat consumption induced activated state of cellular adherence.

INTRODUCTION

Elevated postprandial TG concentrations are associated with an increased risk of cardiovascular diseases (CVD) [1,2]. Although a direct relationship between postprandial triglycerides (TG) and elevated CVD risk has been established, the mechanism by which postprandial TG affects cardiovascular health remains unknown [3-5]. One of the current hypothesis is that elevated triglyceride rich lipoproteins (TRLs) in the postprandial state can deteriorate vascular function and inflammation by interacting with endothelial cells and leukocytes [6-8]. First of all, TRLs may stimulate endothelial cells to increase the expression of intercellular adhesion molecules (ICAMs), paralleled by an increase secretion of pro-inflammatory cytokines into the blood [3]. TRLs may also activate leukocytes. Activated leukocytes increase their adherence capacity to endothelial cells by up regulation of integrins and selectins on their outer membranes and by secretion of pro-inflammatory interleukins, soluble adhesion molecules and coagulation factors [9,10]. The combination of activated endothelial cells, increased leukocyte adherence and pro-inflammatory milieu can diminish the bioavailability of nitric oxide (NO) and subsequently diminish vascular function and thereby vascular tone [3,11].

The magnitude of the postprandial TG response is largely dependent on the quantity of fat. High fat meal challenges are therefore often used to determine vascular and inflammatory responses. Postprandial TG responses can be affected by the type of fat and several studies indicate that saturated fatty acids may induce a lower postprandial rise of TG if compared to unsaturated fatty acids [12-14]. However, studies are still inconclusive [15,16] and the exact impact of fatty acid composition on the magnitude of the postprandial TG response remains to be determined. Another important determinant of the magnitude of the postprandial TG response is BMI. Obese subjects can have a perturbed metabolic postprandial response, which is reflected by elevated postprandial plasma TG concentrations if compared to lean individuals [17-19]. Despite the clear effect of type of fat and BMI on the postprandial TG response, it remains unclear if and how these factors affect leukocyte adherence capacity, inflammatory and vascular responses. Based upon findings by our group that polyunsaturated fatty acid (PUFA) consumption induced a postprandial pro-inflammatory gene expression response in blood leukocytes compared to saturated fatty acid (SFA) consumption [20], and the potential increased TG responses after unsaturated fatty acid consumption, we hypothesize that challenges high in unsaturated fatty acids will elicit a more pronounced postprandial inflammatory and vascular responses if compared to challenges high in SFAs. In addition we hypothesize that obese individuals will have perturbed and more pronounced postprandial inflammatory and vascular responses if compared to lean subjects. We therefore compared postprandial responses after HF challenges with different fatty acid composition in lean and obese men on

hemodynamics and arterial stiffness, leukocyte activation and plasma cytokines involved in vascular adherence, coagulation and inflammation.

MATERIALS AND METHODS

Subjects

The study population consisted of 18 lean (predefined BMI 18–25 kg/m^2) and 18 obese (BMI >29 kg/m^2) men between 50–70 years old. All subjects were non-smoking, normoglycemic (WHO criteria) and not diagnosed with any long-term medical condition or high blood pressure (systolic BP> 160 mmHg and/or diastolic BP>100 mmHg). 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 or consumed fish more than four times/wk. A more detailed description of the study population is previously reported [21]. In that study, three groups were included; 18 lean subjects, 18 obese subjects and six obese type 2 diabetic subjects. We excluded the obese diabetic group from the manuscript because of the low number. All subjects gave written informed consent.

Study design

The study was a double blind randomized cross-over intervention study in which participants visited the university three times. On each study day, subjects consumed a milkshake with 95g of fat (88 energy-%), which was either high in SFAs (54 energy-% of total fat), mono-unsaturated fatty acids (MUFAs) (83 energy-% of total fat) or n3 PUFAs (40 energy-% of total fat). Hemodynamics, augmentation index, a measure of arterial stiffness and wave reflection, and plasma cytokines involved in cellular adherence of leukocytes to the endothelium, coagulation and inflammation were assessed during fasting condition at baseline and postprandially 2 and 4h after milkshake consumption. Expression of leukocyte cell surface molecules and relative proportion of neutrophils, monocytes and lymphocytes were measured at baseline and 4h after milkshake consumption. Subjects were randomly assigned to a treatment order and a one-week washout period was the minimum between consecutive study days. On the day prior to each study day, subjects consumed a standardized low fat evening meal, were refrained from alcohol or strenuous exercise and were not allowed to eat or drink anything except water after 08.00h. This study was conducted according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO). The Medical

Ethical Committee of Wageningen University (the Netherlands) approved the study and the study was registered at clinicaltrials.gov as NCT00977262.

Intervention shakes

All three shakes were isocaloric and macronutrient composition differed only in fatty acid composition (**Table 3.1**). The shakes contained low-fat yogurt, low-fat milk, strawberry flavour, 7.5g of sugar and 95g of the test fat. The SFA shake contained 95g palm oil (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) and the MUFA shake contained 95g high-oleic acid sunflower oil (Aldoc BV, Schiedam, The Netherlands). The n3 PUFA shake contained 40g palm oil and 55g Marinol D-40 (Lipid Nutrition, Wormerveer, The Netherlands), of which 40% was docosahexanoic acid (DHA). Vitamin E (165mg Tocoblend L50, Vitablend, Wolvega, The Netherlands) was added to the SFA and MUFA shake to match vitamin E present in Marinol D-40. All three shakes had a volume of 500mL. Nutritional values of the shakes were calculated using the Dutch Nutrient Databank (NEVO 2006) [22].

Hemodynamics and pulse wave analysis

Brachial systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were assessed automatically (DINAMAP® PRO 100) for 10 minutes with a 3 min interval. Central/aortic systolic blood pressure and the heart rate corrected aortic augmentation index (AIX),

Table 3.1 Nutritional values of the three intervention high fat shakes

Nutritional value	SFA	MUFA	n3 PUFA
Energy (kcal)	990	990	990
Protein (g)	10	10	10
Carbohydrates (g)	19	19	19
Fat (g)	95	95	95
Saturated fatty acids (g)	51	8	32
Monounsaturated fatty acids (g)	37	79	25
Polyunsaturated fatty acids (g)	6	8	38
ALA (g)	0	0	0
EPA (g)	0	0	3
DHA (g)	0	0	23
Vitamin E (mg)	165	165	165

Calculated using the Dutch Food Composition table (NEVO 2006). ALA, alpha-linolenic acid (18:3).

a measure of wave reflection and arterial stiffness, were assessed by pulse wave analysis of the radial artery by applanation tonometry (SphygmoCor®CP System, ATcor Medical) [23]. Seven obese subjects were excluded from blood pressure and pulse wave analysis. Five because of the use of blood pressure lowering medication and two because we were not able to deduce a pulse pressure wave due to an irregular heartbeat.

Plasma markers of inflammation

Plasma cytokine concentrations were analysed on preformatted arrays (Meso Scale Diagnostics, LLC) on a SECTOR Imager 2400 reader (Meso Scale Diagnostics, LLC), as described previously [24]. Intra- and interassay CV were: IL-6, 6.6 and 15.1%; IL-8, 3.4 and 4.1%; E-selectin, 2.8 and 7.1%; P-selectin, 3.9 and 5.3%; sICAM3, 3.9 and 8.2%; thrombomodulin, 2.0 and 5.2%; CRP, 4.3 and 3.4%; SAA. 3.0 and 10.9%; sICAM1, 3.2 and 3.9%; and sVCAM1, 2.4 and 3.3%, respectively. Plasma concentrations of von Willebrand factor (vWF) were measured by a custom ELISA, where Dakopatt® capture and conjugated rabbit anti-human vWF polyclonal antibodies were used (Dakopatt®, Denmark). Levels were expressed as a percentage of vWf detected in pooled citrated plasma of healthy volunteers [24]. Intra- and interassay CV for vWf were 2.8 and 3.3%, respectively.

Leukocyte relative cell count and cell surface activation markers

Expression of leukocyte cell surface activation markers were determined at baseline and 4h after shake consumption by flow cytometry (FACSCanto™II, Becton-Dickinson). Whole blood (BD Vacutainer LH) was stained with fluorescent labelled monoclonal antibodies (MAbs) (Becton-Dickinson, The Netherlands) FITC conjugated CD11a, Pacific Blue™ conjugated CD11b, APC conjugated CD62l and PE-Cy™7 conjugated CD45. After staining, FACS lysis solution (Becton-Dickinson, The Netherlands) was added to the samples, washed twice in PBS (Lonza) and dissolved in PBS. Immuno-fluorescence was evaluated for 10,000 events. Neutrophils, monocytes and lymphocytes were identified by their distinct forward and side scattering properties in combination with CD45 cell surface expression (FACSDiva™ Software v6.1.2). Non-specific binding of each label was ruled out by using isotype matched MAbs. Relative neutrophils, monocytes and lymphocytes count, in proportion to the other two populations, are expressed as percentage. Expression of cell surface markers are expressed as Mean Florescence Intensity (MFI) in arbitrary units.

Metabolic markers and TG fatty acid composition

Plasma TG, glucose, insulin and free fatty acids (FFA) were assessed during fasting condition and postprandially 2 and 4h after milkshake consumption. The TG fatty acid composition was determined in pooled serum samples per group (lean n=18 and obese n=18), for each shake at baseline and 4 h postprandially by gas-liquid chromatography as previously described [25]. Metabolic markers and TG fatty acid composition outcomes are included as compliance measures and have been published [21].

Statistical analysis

Statistical analysis was performed by linear mixed models for repeated measures (PASW statistics 18.0.3). Repeated measures were taken into account in the baseline comparisons between groups. Comparisons of postprandial responses were performed by using group, shake, time and their interactions as fixed effects. Since baseline differences can be expected between subjects and baselines values may influence the magnitude of response [26], baseline values were included as covariate in the model. The scope of this manuscript was to compare postprandial responses. The interaction group x shake does not contain a response and was therefore not determined. Studentized residuals >2.5, obtained from the mixed model, were considered outliers. A least significant difference (LSD) posthoc analysis was performed if significant differences were detected by linear mixed model. A value of P<0.05 was considered significant. All correlations were performed by Pearson's correlations (PASW statistics 18.0.3). Postprandial TG values of one person were identified as outlier for the correlation analysis and excluded from the correlation analysis.

RESULTS

A total of 18 lean and 18 obese individuals entered and completed the study. BMI, fasting plasma TG and insulin concentrations were greater in obese compared to lean subjects (**Table 3.2**).

Hemodynamics and pulse wave analysis

HF shake consumption was associated with a decrease in heart rate in lean subjects, whereas this postprandial decrease was not observed in obese subjects (P<0.05) (**Table 3.3**). Compared to the SFA and n3 PUFA shakes, the MUFA shake resulted in a less pronounced postprandial decrease in HR and a more pronounced postprandial decrease in BP (P<0.05). It also caused a more pronounced decrease in AIX compared to the n3 PUFA shake (P<0.05) (Table 3.3).

Table 3.2 Baseline characteristics of the lean and obese subjects

	Lean	Obese
Age (y)	61.8 ± 5.9	62.6 ± 3.2
BMI (kg/m^2)	23.8 ± 0.8	32.4 ± 3.0*
Body fat (%)	21.9 ± 5.7	38.2 ± 5.2*
VAT (cm^2)	102 ± 40	231 ± 75*
SAT (cm^2)	163 ± 52	350 ± 99*
VAT/SAT ratio	0.64 ± 0.20	0.68 ± 0.24
Waist circumference (cm)	89.3 ± 5.9	115.9 ± 10.0*
Plasma TG (mmol/L)	1.5 ± 0.5	2.1 ± 0.9*
Plasma FFA (mmol/L)	0.51 ± 0.21	0.51 ± 0.13
Plasma Insulin (mU/L)	6.1 ± 2.8	13.4 ± 6.6*
Plasma Glucose (mmol/L)	5.2 ± 0.4	5.5 ± 0.4
HOMA-IR	1.3 ± 0.7	3.4 ± 1.6*

Values are means ± SD, lean *n*=18 and obese *n*=18, * Different from lean subjects, *P*<0.05. TG, triglycerides; FFA, free fatty acids; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue. For VAT, SAT and VAT/SAT ratio; lean *n*=17 and obese *n*=18.

Plasma cytokines

Obese subjects had significantly higher fasting plasma concentrations of CRP, SAA and vWF compared to lean subjects (*P*<0.05) (**Table 3.4**). No differences in postprandial plasma cytokine response were observed between lean and obese. For both obese and lean subjects, shake consumption resulted in increased plasma IL8, sICAM1, sICAM3 and sVCAM1 concentrations and decreased plasma IL6, thrombomodulin and SAA concentrations with no differences in response between shakes (Table 3.4). SFA consumption was associated with higher plasma P-selectin concentrations 2 hours postprandially, compared to MUFA and n3 PUFA consumption (*P*<0.05) (Table 3.4).

Leukocyte relative cell count and cell surface expression

At baseline obese subjects had fewer monocytes relative to lymphocytes and neutrophils compared to lean subjects (*P*<0.05) (**Table 3.5**). Consumption of all shakes was associated with an increase in relative number of neutrophils at the expense of monocytes and lymphocytes. This relative increase in neutrophils was more pronounced in lean compared to obese subjects and less pronounced after SFA shake consumption compared to both other shakes (*P*<0.05) (Table 3.5).

Table 3.3 Baseline and postprandial changes in hemodynamics and arterial stiffness after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

		Shake									*P*-values					
		SFA			MUFA			n3 PUFA			Main effects				Interactions	
	Group	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	G	S	T	G x T	S x T
SBP	Lean	119 ± 17	-2 ± 7	0 ± 8^{b}	118 ± 14	-3 ± 8	-3 ± 7^{a}	118 ± 14	0 ± 8	1 ± 9^{ab}	NS	NS	0.045	NS	NS	NS
(mmHg)	Obese	126 ± 17	2 ± 8	5 ± 9^{b}	128 ± 15	-3 ± 8	0 ± 6^{a}	130 ± 17	-2 ± 9	-2 ± 8^{ab}						
DBP	Lean	72 ± 6	-2 ± 5^{b}	-1 ± 4^{b}	71 ± 7	-2 ± 6^{a}	-3 ± 6^{a}	72 ± 7	-1 ± 5^{b}	-1 ± 6^{b}	NS	NS	0.001	0.009	NS	NS
(mmHg)	Obese	74 ± 7	1 ± 3^{b}	2 ± 3^{b}	77 ± 8	-4 ± 4^{a}	-2 ± 4^{a}	77 ± 8	-1 ± 5^{b}	-1 ± 4^{b}						
Heart rate	Lean	59 ± 9	-4 ± 5^{b}	-5 ± 5^{b}	58 ± 9	-1 ± 5^{a}	-3 ± 5^{a}	61 ± 11	-5 ± 7^{b}	-5 ± 7^{b}	NS	0.006	<0.001	0.001	0.012	0.001
(BPM)	Obese	60 ± 7	-1 ± 4^{b}	-3 ± 4^{b}	60 ± 6	2 ± 4^{a}	0 ± 3^{a}	59 ± 7	-1 ± 4^{b}	-1 ± 5^{b}						
CSBP	Lean	112 ± 17	-3 ± 8^{b}	-2 ± 8^{b}	109 ± 15	-5 ± 8^{a}	-5 ± 7^{a}	109 ± 14	0 ± 8^{b}	0 ± 8^{ab}	NS	NS	0.017	0.025	NS	NS
(mmHg)	Obese	117 ± 16	0 ± 7^{b}	2 ± 9^{b}	119 ± 15	-5 ± 9^{a}	-1 ± 8^{a}	121 ± 16	-2 ± 10^{b}	-3 ± 9^{ab}						
AIX	Lean	18.6 ± 7.0	-1.6 ± 5.3^{b}	-3.0 ± 5.4^{ab}	17.8 ± 5.5	-3.3 ± 3.6^{a}	-5.4 ± 3.4^{a}	17.5 ± 6.3	-0.7 ± 4.5^{b}	-1.9 ± 4.9^{b}	NS	NS	<0.001	<0.001	NS	NS
(%)	Obese	21.4 ± 5.8	-3.3 ± 2.8^{b}	-4.2 ± 4.0^{ab}	21.5 ± 6.7	-4.8 ± 4.5^{a}	-4.2 ± 4.3^{a}	22.2 ± 5.6	-1.6 ± 3.6^{b}	-2.6 ± 5.3^{b}						

Values are means ± SD, lean *n*=18 and obese *n*=11. SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; CSBP, central/aortic systolic blood pressure; AIX augmentation index. Different letters indicate differences between shakes at a given time for lean and obese combined (*P*=0.05). *P*-values were calculated using linear mixed models for repeated measures. Baseline, baseline comparison between groups; G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; NS, not significant (*P*=0.05).

Table 3.4 Baseline and postprandial changes in plasma markers of cellular adherence, coagulation and systemic inflammation after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

		Shake									*P*-values					
		SFA			MUFA			n3 PUFA			Main effects				Interactions	
	Group	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	G	S	T	G x T	S x T
IL-6	Lean	1.62 ± 1.54	-0.11 ± 0.14	-0.12 ± 0.18	1.53 ± 1.51	-0.09 ± 0.17	-0.22 ± 0.38	1.59 ± 1.27	-0.04 ± 0.46	0.00 ± 0.63	NS	NS	NS	0.009	NS	NS
(pg/mL)	Obese	1.76 ± 0.89	0.01 ± 0.42	-0.11 ± 0.44	1.87 ± 0.93	-0.31 ± 0.47	-0.09 ± 0.29	2.28 ± 1.59	-0.09 ± 0.34	-0.03 ± 0.28						
IL-8	Lean	4.74 ± 0.98	0.59 ± 0.48	0.37 ± 0.64	4.66 ± 0.92	0.5 ± 0.76	0.28 ± 0.74	4.66 ± 1.04	0.17 ± 0.40	0.39 ± 0.86	NS	NS	NS	<0.001	NS	NS
(pg/mL)	Obese	4.66 ± 1.24	0.35 ± 0.42	0.23 ± 0.58	4.58 ± 1.30	0.38 ± 0.50	0.59 ± 0.61	4.92 ± 1.38	0.3 ± 0.66	0.56 ± 0.87						
E-selectin	Lean	15.7 ± 3.7	0.2 ± 1.0	-0.0 ± 1.2	15.8 ± 4.3	-0.1 ± 0.9	-0.5 ± 1.0	15.9 ± 4.9	-0.0 ± 1.2	-0.5 ± 1.7	NS	NS	NS	NS	NS	NS
(ng/mL)	Obese	18.1 ± 7.6	0.1 ± 0.9	-0.3 ± 0.8	18.1 ± 7.7	-0.1 ± 1.1	0.2 ± 0.8	17.7 ± 7.4	0.1 ± 0.9	-0.1 ± 1.3						
P-selectin	Lean	129 ± 23	16 ± 25[a]	7 ± 24	132 ± 19	1 ± 16[b]	0 ± 18	133 ± 25	-5 ± 11[b]	1 ± 18	NS	NS	0.037	NS	NS	NS
(ng/mL)	Obese	108 ± 26	9 ± 19[a]	7 ± 18	123 ± 37	2 ± 19[b]	3 ± 19	123 ± 37	1 ± 17[b]	1 ± 20						
sICAM3	Lean	1.58 ± 0.39	0.08 ± 0.12	0.07 ± 0.13	1.55 ± 0.35	0.06 ± 0.11	0.07 ± 0.10	1.57 ± 0.36	0.00 ± 0.13	0.05 ± 0.15	NS	NS	NS	<0.001	NS	NS
(ng/mL)	Obese	1.45 ± 0.19	0.05 ± 0.11	0.04 ± 0.10	1.45 ± 0.21	0.06 ± 0.12	0.07 ± 0.08	1.47 ± 0.22	0.06 ± 0.13	0.09 ± 0.15						
TM	Lean	3.47 ± 0.57	-0.01 ± 0.15	-0.12 ± 0.18	3.47 ± 0.57	0.00 ± 0.18	-0.13 ± 0.21	3.48 ± 0.51	-0.06 ± 0.18	-0.1 ± 0.24	NS	NS	NS	<0.001	NS	NS
(ng/mL)	Obese	3.37 ± 0.49	0.02 ± 0.15	-0.06 ± 0.17	3.39 ± 0.52	0.04 ± 0.20	-0.02 ± 0.12	3.36 ± 0.54	0.02 ± 0.23	-0.06 ± 0.25						

CRP	Lean	0.59 ± 0.47	0.03 ± 0.07	0.00 ± 0.09	0.80 ± 0.99	0.07 ± 0.28	0.06 ± 0.45	0.89 ± 0.90	-0.02 ± 0.13	-0.02 ± 0.11	0.011	NS	NS	NS	NS	NS
(µg/mL)	Obese	4.52± 6.20	-0.02 ± 287	0.04 ± 0.55	3.75± 3.24	0.20 ± 0.71	0.40 ± 1.30	2.09 ± 1.90	-0.12 ± 0.70	-0.19 ± 0.97						
SAA	Lean	0.78 ± 0.28	-0.05 ± 0.06	-0.07 ± 0.11	0.94 ± 0.53	-0.03 ± 0.09	-0.09 ± 0.14	1.47 ± 2.75	-0.21 ± 0.76	-0.26 ± 1.02	0.016	NS	NS	0.001	NS	NS
(µg/mL)	Obese	2.30 ± 2.28	-0.11 ± 0.17	-0.14 ± 0.22	2.29 ± 1.56	-0.10 ± 0.51	-0.16 ± 0.47	1.89 ± 1.53	-0.09 ± 0.17	-0.14 ± 0.26						
sICAM1	Lean	213 ± 37	-1 ± 13	1 ± 18	211 ± 44	6 ± 13	-0 ± 14	213 ± 43	4 ± 15	4 ± 18	NS	NS	NS	0.023	NS	NS
(ng/mL)	Obese	211 ± 45	7 ± 18	6 ± 19	214 ± 42	6 ± 13	3 ± 17	215 ± 49	4 ± 11	5 ± 13						
sVCAM1	Lean	319 ± 63	4 ± 24	11 ± 36	324 ± 73	3 ± 26	-3 ± 26	329 ± 82	6 ± 17	26 ± 77	NS	NS	NS	0.014	NS	NS
(ng/mL)	Obese	358 ± 69	11 ± 34	12 ± 39	352 ± 65	8 ± 26	7 ± 38	338 ± 42	19 ± 36	19 ± 36						
vWF	Lean	99 ± 30	2 ± 7	-1 ± 8	101 ± 26	5 ± 14	4 ± 16	101 ± 29	2 ± 14	10 ± 34	0.004	NS	NS	NS	NS	NS
(%)	Obese	130 ± 45	8 ± 20	6 ± 23	139 ± 37	1 ± 4	-4 ± 7	140 ± 48	-5 ± 19	-4 ± 20						

Values are means ± SD, lean n=18 and obese n=18. TM, thrombomodulin. Different letters indicate differences between shakes at a given time for lean and obese combined (P=0.05). P-values were calculated using linear mixed models for repeated measures. Baseline, baseline comparison between groups; G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; NS, not significant (P=0.05).

Table 3.5 Baseline and postprandial changes in in relative cell count and cell surface activation markers after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

		Shake						*P*-values					
		SFA		MUFA		n3 PUFA			Main effects			Interactions	
	Group	Baseline	Δ4h	Baseline	Δ4h	Baseline	Δ4h	Baseline	G	S	T	G x T	S x T
Relative cell count (%)*													
Neutrophils	Lean	65 ± 10	6 ± 6[b]	67 ± 10	10 ± 9[a]	67 ± 12	7 ± 7[a]	NS	NS	0.029	<0.001	0.038	0.031
	Obese	72 ± 10	2 ± 7[b]	70 ± 9	6 ± 7[a]	71 ± 12	4 ± 3[a]						
Monocytes	Lean	7 ± 2	-1 ± 1	6 ± 2	-2 ± 2	6 ± 3	-2 ± 2	0.019	NS	NS	<0.001	<0.001	NS
	Obese	4 ± 2	0 ± 1	5 ± 2	-1 ± 1	5 ± 2	-1 ± 1						
Lymphocytes	Lean	28 ± 9	-4 ± 5[c]	27 ± 9	-8 ± 7[a]	27 ± 11	-6 ± 6[b]	NS	NS	0.004	<0.001	0.013	0.003
	Obese	23 ± 9	-1 ± 6[c]	25 ± 8	-5 ± 5[a]	24 ± 11	-4 ± 3[b]						
CD11a (MFI)													
Neutrophils	Lean	4.02 ± 0.41	0.05 ± 0.25	3.96 ± 0.32	0.03 ± 0.18	4.00 ± 0.36	0.09 ± 0.18	NS	NS	NS	0.009	NS	NS
	Obese	3.78 ± 0.49	0.08 ± 0.19	3.82 ± 0.42	0.05 ± 0.17	3.80 ± 0.45	0.02 ± 0.24						
Monocytes	Lean	9.02 ± 0.92	0.22 ± 0.69	9.08 ± 1.13	0.16 ± 0.73	9.10 ± 1.55	0.56 ± 0.64	NS	NS	NS	<0.001	NS	NS
	Obese	8.81 ± 1.03	0.19 ± 0.93	8.77 ± 1.07	0.40 ± 0.46	8.87 ± 1.25	0.08 ± 0.50						
Lymphocytes	Lean	10.6 ± 1.3	-0.7 ± 0.9	10.3 ± 1.4	-0.7 ± 0.6	10.7 ± 1.7	-0.7 ± 0.9	NS	NS	NS	<0.001	<0.001	NS
	Obese	10.0 ± 2.6	0.2 ± 0.8	10.3 ± 3.0	-0.2 ± 0.8	10.2 ± 2.7	-0.1 ± 0.9						

CD11b (MFI)													
Neutrophils	Lean	3.99 ± 1.65	1.63 ± 1.78	3.38 ± 1.89	1.71 ± 1.23	4.45 ± 2.39	1.13 ± 1.37	NS	NS	NS	<0.001	NS	NS
	Obese	3.69 ± 1.96	1.60 ± 1.54	3.67 ± 1.61	1.14 ± 1.66	3.93 ± 2.27	0.75 ± 0.84						
Monocytes	Lean	3.07 ± 0.77	0.63 ± 0.95	3.20 ± 1.52	0.61 ± 1.05	3.36 ±1.43	0.76 ± 1.21	NS	NS	NS	<0.001	NS	NS
	Obese	2.91 ± 1.23	0.48 ± 1.35	2.74 ± 0.78	0.62 ± 0.94	3.09 ± 1.48	0.06 ± 0.55						
Lymphocytes	Lean	0.41 ± 0.13	-0.07 ± 0.08	0.38 ± 0.10	-0.06 ± 0.08	0.44 ± 0.18	-0.09 ± 0.10	NS	0.011	NS	<0.001	<0.001	NS
	Obese	0.38 ± 0.13	0.02 ± 0.09	0.40 ± 0.11	-0.03 ± 0.09	0.40 ± 0.12	-0.02 ± 0.10						
CD62l (MFI)													
Neutrophils	Lean	5.49 ± 1.32	0.12 ± 0.69	5.38 ± 1.65	-0.11 ± 0.96	5.04 ± 1.15	0.54 ± 1.25	NS	NS	NS	0.002	NS	NS
	Obese	5.26 ± 1.58	0.56 ± 1.08	5.32 ± 1.08	0.46 ± 1.07	5.49 ± 1.38	0.43 ± 1.34						
Monocytes	Lean	2.95 ± 0.91	0.11 ± 0.53	2.78 ± 0.86	0.14 ± 0.62	2.60 ± 0.65	0.42 ± 0.78	NS	NS	NS	<0.001	NS	NS
	Obese	2.58 ± 0.78	0.26 ± 0.47	2.52 ± 0.67	0.34 ± 0.58	2.79 ± 0.99	0.17 ± 0.57						
Lymphocytes	Lean	2.39 ± 0.64	0.16 ± 0.41	2.34 ± 0.90	0.15 ± 0.35	2.10 ± 0.62	0.44 ± 0.78	NS	NS	NS	0.001	NS	NS
	Obese	2.67 ± 0.92	0.22 ± 0.69	2.60 ± 0.83	0.17 ± 0.58	2.69 ± 0.97	0.07 ± 0.59						

Values are means ± SD, lean *n*=18 and obese *n*=18. MFI, mean fluorescent intensity (arbitrary units). Different letters indicate differences between shakes at a given time for lean and obese combined (*P*=0.05). *P*-values were calculated using linear mixed models for repeated measures. Baseline, baseline comparison between groups; G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; NS, not significant (*P*=0.05). * In proportion to the other two populations and expressed as percentage.

Table 3.6 Baseline and postprandial changes in metabolic parameters after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

		Shake									*P*-values					
		SFA			MUFA			n3 PUFA			Main effects				Interactions	
	Group	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	G	S	T	G x T	S x T
TG	Lean	1.53±0.49	0.64±0.40^{b}	0.48±0.48^{b}	1.37±0.41	0.91±0.47^{a}	1.41±1.00^{a}	1.50±0.50	0.33±0.30^{c}	0.78±0.58^{b}	0.021	NS	<0.001	<0.001	0.046	<0.001
(mmol/L)	Obese	2.07±0.99	0.58±0.40^{b}	0.78±0.61^{b}	2.13±1.06	1.14±0.68^{a}	2.28±1.18^{a}	1.98±0.78	0.30±0.26^{c}	0.77±0.45^{b}						
FFA	Lean	0.51±0.21	-0.21±0.19^{b}	-0.04±0.22	0.49±0.20	-0.10±0.18^{a}	0.03±0.23	0.55±0.23	-0.25±0.21^{b}	-0.03±0.17	NS	NS	<0.001	0.002	0.042	<0.001
(mmol/L)	Obese	0.48±0.10	-0.18±0.12^{b}	0.11±0.11	0.50±0.15	-0.09±0.16^{a}	0.08±0.16	0.54±0.15	-0.21±0.11^{b}	0.06±0.16						
Insulin	Lean	6.52±2.55	3.08±3.77^{b}	-1.82±2.08^{b}	5.80±3.38	2.73±3.88^{b}	1.00±3.98^{a}	6.02±2.55	-0.02±3.77^{a}	-1.64±2.56^{b}	<0.001	0.022	<0.001	0.003	0.032	0.001
(mU/L)	Obese	13.52±7.40	6.59±9.60^{b}	-1.67±3.33^{b}	12.81±5.24	4.39±6.62^{b}	1.79±6.07^{a}	13.91±7.25	2.33±5.87^{a}	-2.04±3.09^{b}						
Glucose	Lean	5.19±0.39	-0.30±0.35^{b}	-0.40±0.21^{b}	5.17±0.35	-0.29±0.31^{b}	-0.08±0.28^{a}	5.29±0.53	-0.42±0.39^{a}	-0.33±0.25^{b}	0.015	NS	<0.001	0.002	0.001	0.001
(mmol/L)	Obese	5.50±0.41	-0.16±0.48^{b}	-0.43±0.39^{b}	5.53±0.43	-0.18±0.32^{b}	-0.31±0.28^{a}	5.54±0.28	-0.43±0.38^{a}	-0.52±0.33^{b}						

Values are means ± SD, lean *n*=18 and obese *n*=18. Different letters indicate differences between shakes at a given time for lean and obese combined (*P*=0.05). *P*-values were calculated using linear mixed models for repeated measures. Baseline, baseline comparison between groups; G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; NS, not significant (*P*=0.05); TG, triglycerides; FFA, free fatty acids.

No significant differences in baseline CD11a, CD11b and CD62l cell surface expression were observed between lean and obese subjects. Lymphocyte CD11a and CD11b expression decreased significantly after shake consumption in lean subjects, but did not change in obese (P<0.05). Expressions of all other markers were postprandially increased with no difference in response between lean and obese or between shakes (Table 3.5).

Plasma metabolic markers and serum fatty acid composition

MUFA consumption was associated with a more pronounced increase in TG if compared with the SFA and n3PUFA consumption (P<0.05) (**Table 3.6**). The increase in plasma TG concentration was greater in the obese than in the lean men (P<0.05). The percentage of palmitic acid was 10% higher after the SFA challenge, the percentage of oleic acid was 60% higher after the MUFA challenge and the percentage of DHA was 950% higher after the n3 PUFA challenge, reflecting the composition of the shakes (**Supplemental Table S3.1**). Outcomes are based on single pools for each group at each given time point for each shake. Statistical tests were therefore not possible.

Correlations with postprandial TG

High fat shake consumption affected the augmentation index and increased plasma markers involved in cellular adherence and leukocyte cell surface activation markers. To determine if elevated TRLs in the postprandial state may be the cause of these effects, we tested the associations between changes in plasma TG concentrations, between 4 hours postprandially and baseline, with changes in these markers. Changes in TG correlated positively with changes in sICAM3 (r=0.45, P<0.01) after SFA consumption and with changes in sICAM1 (r=0.39, P=0.02), sVCAM1 (r=0.46, P<0.01) and IL-8 (r=0.48, P<0.01) after n3PUFA consumption. For leukocyte cell surface activation markers, changes in TG correlated negatively with changes in neutrophil CD11a expression (r=-0.37, P=0.03) after SFA consumption and positively with changes in monocyte CD11a expression (r=0.36, P=0.03) after MUFA consumption.

DISCUSSION

We compared the postprandial impact of high-fat shakes differing in fatty acid composition between lean and obese subjects by comprehensive characterization of the vascular, leukocyte activation and inflammatory cytokine response. In both groups and after all shakes, aortic augmentation index (AIX) decreased, plasma sICAM1, sICAM3, sVCAM1 and IL-8 increased,

monocytes and neutrophils CD11a, CD11b and CD621 expression increased and lymphocytes CD62l expression increased. For several measures, obese subjects had blunted postprandial responses to a HF challenge when compared to lean subjects, including lymphocyte CD11a and CD11b expression and heart rate. In addition, differences in postprandial responses between different fatty acid shakes were observed, such as a more pronounced decrease in AIX and blood pressure after MUFA consumption.

All postprandial changes point towards an activated state of cellular adherence and hence a atherogenic milieu. Firstly, the increase in plasma soluble adhesion molecules sICAM1, sICAM3 and sVCAM1 can mediate cellular adherence of leukocytes to the endothelium [27]. Secondly, the increase in plasma IL-8 can trigger neutrophil activation and recruitment [28] and monocyte adhesion to the vascular endothelium [29]. Thirdly, the increase expression of CD11a and CD11b as part of LFA-1 and MAC-1 integrins respectively and CD62l (l-selectin), can increase leukocyte recruitment and binding to the ICAMs expressed on the endothelium [27]. Altogether, this demonstrates that consumption of a HF challenge initiates an activated state of cellular adherence in the circulation, which is the initial phase in the development of atherosclerosis [27]. Something which previous studies had only partly demonstrated and in separate observations [9-11,30,31]. The current study additional shows that this HF-shake induced affect, is largely independent of type of fat consumed or being lean or obese, despite the clear differences in postprandial TG response between these factors.

Therefore, the question remains whether elevated TRLs in the postprandial state might be responsible for this activated state of cellular adherence [6-8]. Positive correlations between changes in TG and some soluble adhesion molecules were almost only observed after n3 PUFA consumption, indicating that the change in TG is not solely responsible for the postprandial changes in soluble adhesion molecules. Furthermore, the observed correlations between changes in TG and changes in neutrophil and monocyte CD11a expression had an opposite direction and were not very strong. A previous study did report positive correlations between changes in TG levels and changes in monocytes CD11b expression [10]. However, this correlation was observed in 10 subjects after 6 hours, whereas our findings were based upon 35 subjects and after 4 hours. Based on the correlation analysis in the current study, we cannot substantiate a role of postprandial TG on the pro-inflammatory postprandial response alone. Perhaps other mechanisms, such as increased postprandial plasma endotoxins after high fat intake also contribute to this postprandial inflammatory response [32]. These endotoxins are transported through the gut wall during chylomicron uptake and are known to activate inflammation [33].

With respect to the differences between fatty acid type in the HF shake, MUFA consumption was associated with a less pronounced postprandial decrease in heart rate and a more pronounced postprandial decrease in BP and AIX compared to the other shakes. A decrease in AIX a few hours after meal intake is well described in literature and probably due to changes in arterial stiffness and wave reflections caused by neutrally or hormone mediated peripheral vasodilation [34]. To our knowledge we are the first to describe significant differences in postprandial AIX between intake of different fatty acids types. Berry *et al.* compared postprandial effects of 50g fat derived from shea butter blend (SFA) or high oleic sunflower oil (MUFA) on AIX in younger subjects (18–40y), but did not observe differences between meals [35]. The differences in findings with our study might be explained by our older population or by the meal composition, as the mixed meal provided by Berry *et al.* was higher in carbohydrates and lower in fat. Interestingly, MUFA consumption was besides associated with a more pronounced decrease in AIX, also associated with a more pronounced increase in TG concentrations. However, no correlations were observed between changes in postprandial TG concentrations and AIX after MUFA consumption. This more pronounced decrease in AIX after MUFA consumption may have been caused by the higher insulin and or glucose concentrations after MUFA intake compared to SFA or n3 PUFA intake [36,37]. Insulin can induce sympathetic nervous system stimulation, which subsequently causes a decrease in vascular resistance hence, a decrease in AIX may be expected after an increase in insulin concentrations [36]. Unfortunately, the design of the current study was not suitable to verify this hypothesis, as postprandial peaks of both glucose and insulin can be expected before 2 hours postprandially. The questions remains what the impact would be of a lower postprandial response on the long term. Whereas a lower AIX in the fasting state is known to be associated with lower risk of CVD [38], the impact of postprandial AIX changes on long term outcome measures such as CVD are unknown.

SFA shake consumption resulted in increased postprandial plasma P-selectin concentrations if compared to the other shakes. P-selectin is expressed both by platelets and endothelial cells and modulates adhesion of leukocytes and platelets to the endothelium [39]. The observed postprandial SFA-induced increase in P-selectin might therefore enhance P-selectin modulated leukocyte adherence to the endothelium in the postprandial state.

Differences between lean and obese in postprandial HF response were characterized by a blunted responses in obese on HR, leukocyte count and lymphocyte CD11a and CD11b expression. CD11a and CD11b are important for early adhesion of leukocytes to ICAMs located on the endothelium [40,41]. The decreased postprandial lymphocyte CD11a and CD11b expression in lean subjects might point towards less lymphocyte adherence and subsequently less activation of the endothelium in the postprandial state, whereas this was not observed in obese subjects.

Interestingly, this decrease was restricted to lymphocyte CD11a and CD11b expression, as all other leukocyte adhesion markers were postprandially increased.

The current study allowed us to compare high fat meal responses of different fatty acids in lean and obese men. We investigated the postprandial vascular and inflammatory response before and 2 and 4h after milkshake consumption. As a consequence, we may have missed changes that occurred at earlier or later time points. Compared to lean individuals, obese individuals had higher insulin and TG levels at baseline and after shake consumption, indicating a reduced metabolic capacity. Nonetheless, obese subjects were not diabetic and not diagnosed with long-term medical conditions. A strong point of the study design is the match for milkshake volume, caloric content and macronutrients which enables us to draw conclusions about the effects of different fatty acid types. The amount of fat (95g; 88% of energy) used in this study is relatively high and does not reflect a common meal. However, the aim of the current study was to determine response capacity towards a high lipid load only differing in fatty acid type, which can be more easily achieved by these high amounts of fat. The fatty acids used in our study are commonly consumed and therefore expected to be well tolerated. As we did not include a shake with a normal fat content, we cannot rule out if the postprandial changes observed after all shakes are due to the high fat content per see or whether they also can be elicited by a common meal or by changes of circadian rhythm. A difference in habitual diet and or other lifestyle factors between lean and obese individuals may have influenced the postprandial response [42]. In order to minimize the effect of consumption of the meal from the previous day, subjects consumed a standardized low fat evening meal prior to each study day. In addition, all volunteers were not vegetarian and did not consume fish more than four times/wk and did not used fish oil supplements. A factor that may have contributed to the differences in TG response between the fatty acids may be the structure of the fat, which is able to modulate the intestinal absorption. The liquid structured high oleic acid sunflower oil may have been easier and better emulsified compared to the solid structured palm oil, leading to a more rapid lipid uptake after MUFA consumption [43]. This does however not explain the extended elevation in TG at 4 hours. Another explanation for the difference in TG response may be that the MUFA shake contained almost exclusively oleic acid (83% of total fat) while the other shakes contained a mixture of fatty acids. The consumption of high amounts of a single fatty acid might affect clearance capacity, resulting in higher circulating levels of TG for a longer period of time, which might have affected the observed differences in blood pressure, heart rate and augmentation index. Yet, no correlations between postprandial changes in TG concentrations and AIX were observed after MUFA consumption. Based on the more pronounced increase in TG and more pronounced decrease in AIX after MUFA intake, one might speculate that a

high fat MUFA shake is metabolic more challenging for the body compared to a shake high in SFA or n3 PUFA.

In summary, this study demonstrates that HF shake consumption initiate an activated state of cellular adherence and an atherogenic postprandial milieu as reflected by the increase in several plasma soluble adhesion markers and the increase in several leukocyte cell surface adhesion markers. Repeated exposure to such an atherogenic postprandial milieu may on the long run increase CVD risk. We additionally demonstrate that this high-fat induced response is largely independent of type of fatty acids consumed or being lean or obese, despite the clear differences in postprandial TG responses between groups and the different fatty acids shakes. Combined with a lack of clear correlations between changes in postprandial plasma and leukocyte adherence markers and changes in TG levels, these findings suggests that besides increased TG other mechanisms are involved in the high-fat consumption-induced activated state of cellular adherence. Fatty acid type did affected postprandial hemodynamics and arterial stiffness with a more pronounced decrease after MUFA consumption. Indicating that this fatty acid is the most potent fatty acid to provoke a physiological vascular response.

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SUPPLEMENT

Supplemental Table S3.1 Baseline and postprandial changes in metabolic parameters after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

	Shake					
	SFA		MUFA		n3 PUFA	
	Baseline	Δ4h	Baseline	Δ4h	Baseline	Δ4h
	% of total		% of total		% of total	
Palmitic acid						
Lean	27. 5	2.9	25.8	-11.5	27.1	-0.6
Obese	28.1	2.5	27.9	-11.2	29.1	0.3
Oleic acid						
Lean	35.8	2.5	38.1	22.8	37.4	-2.5
Obese	36.1	1.1	36.0	20.7	35.7	-0.6
DHA						
Lean	0.68	-0.09	0.44	-0.12	0.32	4.56
Obese	0.66	-0.09	0.63	-0.16	0.55	2.86

Pooled samples for lean (*n*=18) and obese (*n*=18) men at baseline and 4 hours after shake consumption measured in serum. Only values for palmitic acid, oleic acid and DHA are displayed.

Chapter 4

Postprandial fatty acid-specific changes in circulating oxylipins in lean and obese men after a high fat challenge

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ABSTRACT

Background: : Circulating oxylipins could have an effect on peripheral tissues and seem to play an important role in endothelial function. These circulating plasma oxylipins are esterified in triglyceride-rich lipoproteins (TRLs). The magnitude of the postprandial TRLs response after a high fat meal can be affected by BMI and fatty acid type. We therefore aimed to characterize the impact of a high fat meal challenge differing in fatty acid composition, on the postprandial oxylipin response in lean and obese men.

Methods: In a double-blind randomized cross-over challenge study we characterized the postprandial oxylipin response after different high-fat challenges, either high in saturated (SFA), mono-unsaturated (MUFA) or n3 poly-unsaturated fatty acids (PUFA), in lean and obese men.

Results: Plasma oxylipin profiles were significantly altered at 2 and 4 h after shake consumption when compared to baseline. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-derived oxylipins increased after n3 PUFA shake consumption. MUFA shake consumption increased levels of cytochrome P450 (CYP450)-mediated oxylipins. SFA shake consumption led to an increase in linoleic acid (LA)-derived HODEs. No differences were observed between lean and obese subjects at baseline or after shake consumption.

Conclusions: We are the first demonstrating that circulating oxylipins profiles are acutely affected after a high fat meal challenge and these changes are strongly influenced by different dietary fatty acids. These circulating oxylipins may affect endothelial function. However, future research is needed to elucidate how these circulating oxylipins in the postprandial phase may affect the endothelium.

INTRODUCTION

Oxylipins including eicosanoids are a spectrum of bioactive and signaling compounds made by oxidation of essential fatty acids and are involved in pro- or anti-inflammatory processes [1]. Three families of enzymes, namely cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450), allow the biosynthesis of a wide range of oxylipins: prostanoids and thromboxanes synthesized by COX, leukotrienes and monohydroxyalcohols [HETEs, HODEs, HEPEs] produced by LOX and epoxides synthezised by CYP450 which are transferred to their diol form by soluble epoxide hydrolase (sEH) in a fast turnover rate [2-4]. Oxylipins are known for their local autocrine and paracrine inflammatory effects, but there is a growing body of evidence that oxylipins may also act as endocrine molecules [5]. These circulating oxylipins could have an effect on peripheral tissues and seem to play an important role in endothelial function [5]. The majority of these circulating plasma oxylipins are esterified in triglyceride-rich lipoproteins (TRLs) and can be released by lipoprotein lipase (LPL) anchored on endothelial cells [6,7]. A high fat meal causes an increase in plasma TRL and is known to affect endothelial function [8]. The magnitude of the postprandial TRLs response after a high fat meal has been shown to be affected by BMI and fatty acid type [9]. The question remains whether plasma oxylipins are also changed after high fat meals differing in fatty acid composition. It is also unclear, whether the acute oxylipin responses are affected by BMI. We therefore aimed to characterize the acute effect of different high fat meal challenges on circulating oxylipins in lean and obese men.

MATERIALS AND METHODS

In this manuscript, we included a subpopulation of a previously reported study [9], namely 18 lean subjects and 18 obese subjects. The six obese type 2 diabetic subjects were excluded from this study because of the lower number.

Subjects

In total, 18 lean (predefined BMI 18–25 kg/m^2) and 18 obese (BMI >29 kg/m^2) male subjects between 50–70 years old were recruited. All subjects were non-smoking, normoglycemic (WHO criteria) and not diagnosed with any long-term medical condition. A more detailed description of the study population has been published previously [9]. All subjects gave written informed consent and the study was approved by Medical Ethics Committee of Wageningen University. The study was conducted according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO).

Study design

The study was a double blind randomized cross-over intervention study in which each participant visited the research facility three times. On each study day, subjects consumed a milkshake with 95g of fat, which was either high in saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) or n3 poly-unsaturated fatty acids (PUFA). Blood was collected at baseline (t=0) and 2 and 4 h after milkshake consumption. Subjects were randomly assigned to a treatment order and a one-week washout period was the minimum between consecutive study days. On the day prior to each study day, subjects consumed a standardized low fat evening meal, were refrained from alcohol or strenuous exercise, and were not allowed to eat or drink anything after 08.00 pm, except water.

Shake composition

All three shakes were isocaloric and macronutrient composition differed only in fatty acid composition (**Supplemental Table S4.1**). The shakes contained low-fat yoghurt, low-fat milk, strawberry flavour, 7.5g of sugar and 95g of the test fat. The SFA shake contained 95g palm oil (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) and the MUFA shake contained 95g high-oleic acid sunflower oil (Aldoc BV, Schiedam, The Netherlands). The n3 PUFA shake contained 40g palm oil and 55g Marinol D-40 (Lipid Nutrition, Wormerveer, The Netherlands), of which 40% was docosahexanoic acid (DHA). Vitamin E (165mg Tocoblend L50, Vitablend, Wolvega, The Netherlands) was added to the SFA and MUFA shakes to match vitamin E present in Marinol D-40. All three shakes had a volume of 500mL. Nutritional values of the shakes were calculated using the Dutch Nutrient Databank (NEVO 2006).

Oxylipin profiling analysis

The oxylipin profiling platform is a target analysis and addresses over 100 target metabolites. Plasma oxylipin profiles were obtained by a method previously described by Strassburg *et al.* [10]. In short, compound extraction was performed with solid phase extraction using a hydrophilic-lipophilic balance (HLB) (Oasis, Waters) and samples were analyzed by liquid chromatography (Agilent 1260, San Jose, CA, USA) coupled to electrospray ionization on a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA), while chromatographic separation was achieved on an Ascentis Express C18 (2.1x150 mm, 2.7 μm particles) (Supelco). Peak determination and peak area integration was performed with Mass Hunter Quan (Agilent, San Jose, CA, USA, Version B.04.00). Peak areas of target metabolites were corrected by

appropriate internal standards (ISTD) and calculated response ratios were used throughout the analysis. Oxylipins which are usually addressed by this analytical platform, but were below the detection limits, were not used for further analysis. Batch to batch correction was performed based on a method previously described by van der Kloet *et al.* [11].

Statistical analysis

Statistical analysis was performed by linear mixed models for repeated measures (PASW statistics 18.0.3). Baseline comparisons between groups were performed using baseline values in the mixed model procedure with group as main effect and shake as repeated factor. Comparisons of postprandial responses were performed by using group, shake, time and their interactions as fixed effects. Since baseline differences can be expected between groups, baseline values were included as covariate in the model. Benjamini and Hochberg false discovery rate correction (q=5%) was applied to correct for false positives. A least significant difference (LSD) posthoc analysis was performed, if significant differences were detected by linear mixed model. A value of $P<0.05$ was considered significant. All correlations were performed by Pearson's correlations (PASW statistics 18.0.3). One subject was identified as an outlier and therefore removed from the analysis.

RESULTS

A total of 18 lean and 18 obese individuals completed the study. Baseline subject characteristics are displayed in **Table 4.1**.

Table 4.1 Baseline characteristics of the subjects

	Lean (*n*=18)	Obese (*n*=18)
Age (yrs)	61.8 ± 5.9	62.6 ± 3.2
BMI (kg/m^2)	23.8 ± 0.8	32.4 ± 3.0 *
TG (mmol/L)	1.5 ± 0.5	2.1 ± 0.9 *
FFA (mmol/L)	0.51 ± 0.21	0.51 ± 0.13
Insulin (mmol/L)	6.1 ± 2.8	13.4 ± 6.6 *
Glucose (mmol/L) (mmol/L)	5.2 ± 0.4	5.5 ± 0.4

Data are presented as mean ± SD. TG, triglycerides; FFA, free fatty acids.* Significantly different from lean subjects ($P<0.05$).

Plasma metabolic markers and TG fatty acid composition

Baseline and postprandial concentrations of glucose, triglycerides (TG), free fatty acids (FFA) and insulin are shown in **Supplemental Table S4.2**. In addition, changes in fatty acid composition of the TG fraction in pooled serum samples 4 h after the high-fat challenges are shown in Supplemental Table S4.3. At baseline, obese subjects had significantly higher TG and insulin levels when compared to lean subjects. All three high-fat challenges increased TG and FFA concentrations at 2 and 4 h. The increase in TG was higher after the MUFA challenge ($P<0.001$) in both lean and obese subjects when compared with SFA and n3 PUFA shake consumption. Obese subjects showed a significantly higher increase in TG ($P=0.046$) after the MUFA shake when compared to lean subjects. Furthermore, increases in specific FFA in de TG fraction were observed that reflected the composition of the shakes (**Supplemental Table S4.3**). The percentage of palmitic acid was 1.1-fold higher after the SFA challenge, the percentage of oleic acid was 1.6-fold higher after the MUFA challenge and the percentage of DHA was 9.5-fold higher after the n3 PUFA challenge.

Oxylipin profiling

A total of 36 oxylipins were detected, which were derived from 6 different precursor FA (**Supplemental Table S4.4**). These included oxylipins produced by either n6 PUFAs, e.g. AA and LA, or n3 PUFAs, e.g. EPA and DHA. The detected oxylipins including the statistical outcomes of the linear mixed model are shown in **Table 4.2**. No significant differences in oxylipin levels between lean and obese subjects were observed at baseline or after high fat shake consumption. We therefore do not discriminate between the two groups for the rest of the results section.

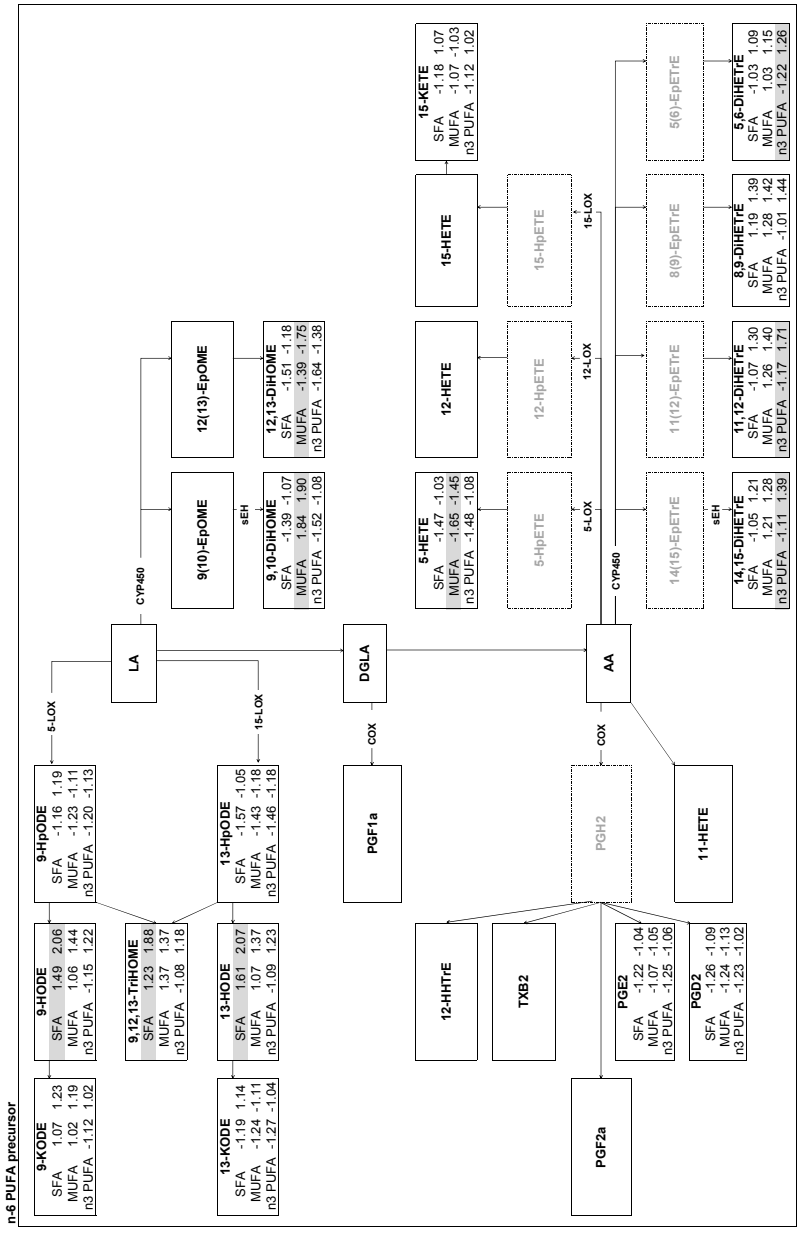
Postprandial oxylipin responses

The majority of the detected oxylipin compounds were affected by shake consumption, with the exception of the LA-derived epoxides 12(13)-EpOME and 9(10)-EpOME and the AA-derived compounds PGF2α, TXB2, 11-HETE, 12-HHTrE, 12-HETE and 15-HETE (Table 4.2). Fold changes of the postprandially changed oxylipins are visualized in a metabolic pathway scheme of enzymatic oxylipin formation (**Figure 4.1**). The COX products PGE2 and PGD2, the LOX product, 5-HETE and the CYP450 products 12,13-DiHOME decreased 2 h and 4 h after shake consumption. The LA-derived oxylipins 13-HODE, 9-HODE and 9,12,13-TriHOME from the LOX pathway increased after shake consumption. In addition, the AA-derived DiHETrEs, EPA-derived DiHETEs and DHA-derived 19,20-DiHDPA from the CYP450 enzymatic pathway were generally increased 4 h after shake consumption

Table 4.2 Detected oxylipins and the statistical outcomes of the linear mixed model

			P-values					
				Main effects			Interaction	
Precursor FA	Enzymatic pathway	Oxylipin	Baseline	G	S	T	G x T	S x T
LA	CYP450	12(13)-EpOME	-	-	-	-	-	-
LA	CYP450	12,13-DiHOME	-	-	-	<0.001	-	<0.05
LA	CYP450	9(10)-EpOME	-	-	-	-	-	-
LA	CYP450	9,10-DiHOME	-	-	<0.001	<0.001	-	<0.001
LA	CYP450	9,12,13-TriHOME	-	-	<0.001	<0.001	-	<0.05
LA	LOX	13-HODE	-	-	<0.001	<0.001	-	<0.001
LA	LOX	13-HpODE	-	-	-	<0.001	-	-
LA	LOX	13-KODE	-	-	-	<0.001	-	-
LA	LOX	9-HODE	-	-	<0.001	<0.001	-	<0.01
LA	LOX	9-HpODE	-	-	-	-	-	-
LA	LOX	9-KODE	-	-	-	-	-	-
DGLA	COX	PGF1a	-	-	-	-	-	-
AA	COX	PGD2	-	-	-	<0.001	-	-
AA	COX	PGE2	-	-	-	<0.001	-	-
AA	COX	PGF2a	-	-	<0.05	-	-	-
AA	COX	TXB2	-	-	-	-	-	-
AA	COX	11-HETE	-	-	-	-	-	-
AA	COX	12-HHTrE	-	-	-	-	-	-
AA	CYP450	14,15-DiHETrE	-	-	<0.01	<0.001	-	<0.01
AA	CYP450	11,12-DiHETrE	-	-	<0.001	<0.001	-	<0.001
AA	CYP450	8,9-DiHETrE	-	-	<0.05	<0.001	-	-
AA	CYP450	5,6-DiHETrE	-	-	-	<0.001	-	<0.01
AA	LOX	12-HETE	-	-	-	-	-	-
AA	LOX	15-HETE	-	-	-	-	-	-
AA	LOX	15-KETE	-	-	-	<0.05	-	-
AA	LOX	5-HETE	-	-	<0.05	<0.001	-	-
ALA	LOX	9-HOTrE	-	-	<0.001	<0.01	-	<0.001
EPA	COX	TXB3	-	-	<0.001	<0.001	-	<0.001
EPA	CYP450	17,18-DiHETE	-	-	<0.001	<0.001	-	<0.001
EPA	CYP450	14,15-DiHETE	-	-	<0.001	<0.001	-	<0.001
EPA	LOX	12-HEPE	-	-	<0.001	<0.001	-	<0.01
EPA	LOX	15-HEPE	-	-	<0.001	<0.01	-	<0.01
EPA	LOX	5-HEPE	-	-	<0.001	<0.001	-	<0.001
DHA	CYP450	19(20)-EpDPE	-	-	<0.001	<0.001	-	<0.001
DHA	CYP450	19,20-DiHDPA	-	-	<0.001	<0.001	-	<0.001
DHA	LOX	17-HDoHE	-	-	<0.001	<0.001	-	<0.001

AA, arachidonic acid; DHA, docosahexaenoic acid; DGLA, dihomo-gamma-linolenic acid; EPA, eicosapentaenoic aicd; LA, linoleic acid. *P*-values were calculated using linear mixed models for repeated measures with a false discovery rate (FDR) correction for multiple testing. Baseline, baseline comparison between groups; G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; -, not significant (*P*=0.05).


n-6 PUFA precursor
LA
DGLA
AA
CYP460
5-LOX
15-LOX
COX
sEH
12-LOX
9-KODE
SFA 1.07 1.23
MUFA 1.02 1.19
n3 PUFA -1.12 1.02
9-HODE
SFA 1.49 2.06
MUFA 1.06 1.44
n3 PUFA -1.15 1.22
9-HpODE
SFA -1.16 1.19
MUFA -1.23 -1.11
n3 PUFA -1.20 -1.13
9,12,13-TriHOME
SFA 1.23 1.88
MUFA 1.37 1.37
n3 PUFA -1.08 1.18
13-KODE
SFA -1.19 1.14
MUFA -1.24 -1.11
n3 PUFA -1.27 -1.04
13-HODE
SFA 1.61 2.07
MUFA 1.07 1.37
n3 PUFA -1.09 1.23
13-HpODE
SFA -1.57 -1.05
MUFA -1.43 -1.18
n3 PUFA -1.46 -1.18
PGF1a
PGH2
PGF2a
PGE2
SFA -1.22 -1.04
MUFA -1.07 -1.05
n3 PUFA -1.25 -1.06
PGD2
SFA -1.26 -1.09
MUFA -1.24 -1.13
n3 PUFA -1.23 -1.02
TXB2
12-HHTrE
11-HETE
9(10)-EpOME
9,10-DIHOME
SFA -1.39 -1.07
MUFA 1.84 1.90
n3 PUFA -1.52 -1.08
12(13)-EpOME
12,13-DiHOME
SFA -1.51 -1.18
MUFA -1.39 -1.75
n3 PUFA -1.64 -1.38
5-HETE
SFA -1.47 -1.03
MUFA -1.65 -1.45
n3 PUFA -1.48 -1.08
5-HpETE
12-HETE
12-HpETE
15-HETE
15-HpETE
15-KETE
SFA -1.18 1.07
MUFA -1.07 -1.03
n3 PUFA -1.12 1.02
14(15)-EpETrE
11(12)-EpETrE
8(9)-EpETrE
5(6)-EpETrE
14,15-DiHETrE
SFA -1.05 1.21
MUFA 1.21 1.28
n3 PUFA -1.11 1.39
11,12-DiHETrE
SFA -1.07 1.30
MUFA 1.26 1.40
n3 PUFA -1.17 1.71
8,9-DiHETrE
SFA 1.19 1.39
MUFA 1.28 1.42
n3 PUFA -1.01 1.44
5,6-DiHETrE
SFA -1.03 1.09
MUFA 1.03 1.15
n3 PUFA -1.22 1.26

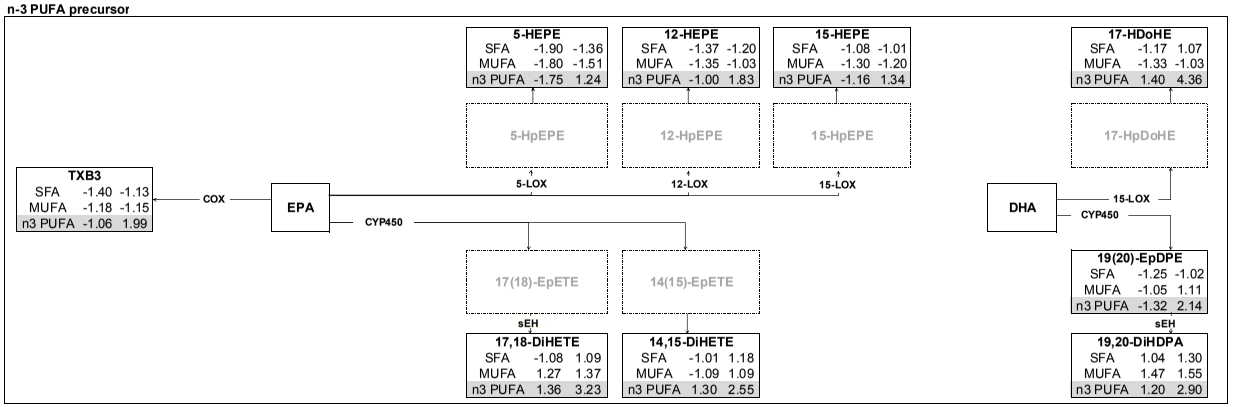


Figure 4.1 Changes in oxylipin profiles after high saturated fatty acid (SFA), mono-unsaturated fatty acid (MUFA) or n3 polyunsaturated fatty acid (PUFA) consumption. Data are presented in a metabolic pathway scheme of enzymatic oxylipin formation. Precursor fatty acids (small box); AA, arachidonic acid; DHA, docosahexaenoic acid; DGLA, dihomo-gamma-linolenic acid; EPA, eicosapentaenoic aicd; LA, linoleic acid. Names on the arrows represent enzymatic pathways: lox; lipoxygenase, cox; cyclooxygenase, CYP450; cytochrome P450, sEH; soluble epoxide hydrolase. Each large black box represents an oxylipin. Boxes with dotted lines represent oxylipins not detected in this study. Boxes with continues lines and without values were detected, but did not changed significantly over time. Boxes with continues lines and with values were detected and changed significantly over time ($P<0.05$ for time effect); values in the boxes represent fold changes after 2h (1st column) or 4h (2nd column) compared to baseline. Highlighted shakes and fold changes in gray indicate the deviating shake for significant shake-time effects ($P<0.05$).

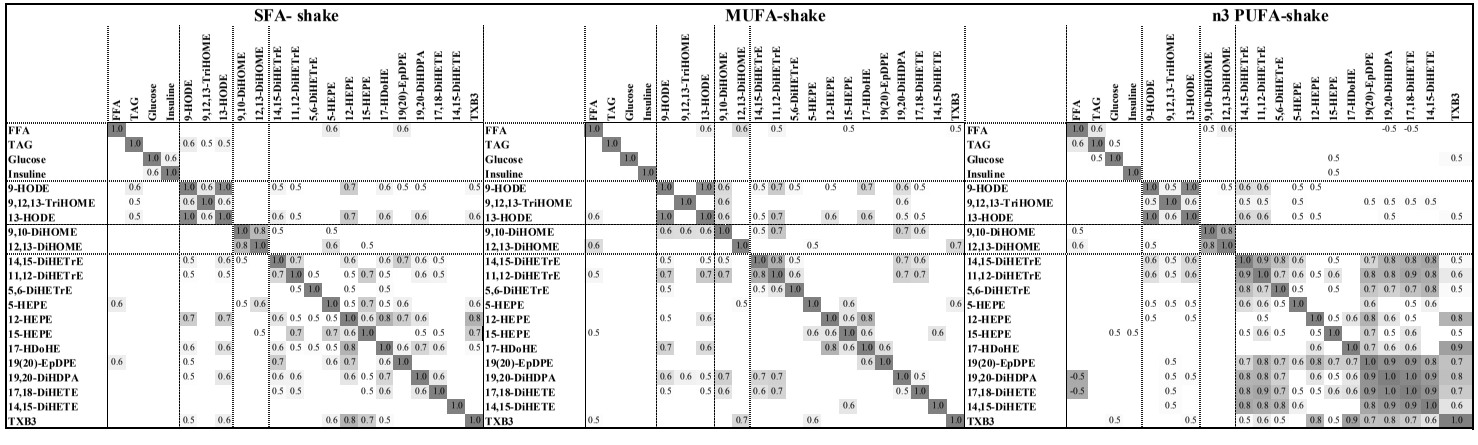


Figure 4.2 Correlation matrix of changes in selected oxylipins (with fatty acid specific responses) and metabolic parameters 4 hours after high fat saturated fatty acid (SFA), mono-unsaturated fatty acid (MUFA) or n3 polyunsaturated fatty acid (PUFA) consumption. Correlations were performed on delta values (T4-T0). Values represent significant (P<0.05) correlation coefficients ≥0.5. Correlation coefficients are displayed on a gray-scale, ranging from 0.5 or -0.5 (white) to 1 or -1(gray).

Differences between shakes

Differences in oxylipin responses between the three different shakes are also visualized in the metabolic pathway scheme (**Figure 4.1**). After SFA shake consumption the increase in the LA-derived alcohols 13-HODE, 9-HODE and the triol 9,12,13-TriHOME was more pronounced when compared to the other shakes. LA-derived diol 9,10-DiHOME increased after MUFA shake consumption, but decreased after SFA and PUFA shake consumption. MUFA consumption also caused more decreased levels of the LA-derived diol 12,13-DiHOME and the AA-derived alcohol 5-HETE when compared to the other shakes. After the n3 PUFA challenge the concentrations of all detected EPA- and DHA-derived metabolites involved in the CYP450 pathway were more increased when compared to the other shakes. In addition, the EPA-derived HEPEs produced by LOX enzymes and the AA-derived TBX3 produced by COX enzymes were only increased after n3 PUFA consumption. High fat n3 PUFA consumption also led to stronger increases in AA-derived DiHETrEs from the CYP450 enzymatic pathway.

Correlations of selected oxylipins

To determine associations between postprandial changes in individual oxylipins, we correlated delta values of each oxylipin per shake (**Figure 4.2**). For this correlation analyzes we used delta values of 4 h versus baseline and selected only the oxylipins that were significantly changed between the shakes. Changes in LA-derived 13-HODE, 9-HODE and 9,12,13-TriHOME strongly correlated with each other, especially after SFA and n3 PUFA consumption. Changes in EPA- and DHA-derived metabolites and AA-derived DiHETrEs correlated with each other after n3 PUFA consumption. These results demonstrate that these oxylipins all change mostly in dependence on their precursor oxylipins. Changes in plasma TG correlated positively with the changes in 13-HODE, 9-HODE and 9,12,13-TriHOME after SFA consumption.

DISCUSSION

Oxylipins are involved in a vast number of inflammatory processes and evidence of the importance of circulating oxylipins in endothelial function is growing. We are the first demonstrating that a high fat meal acutely alters circulating oxylipin profiles and that these changes are highly affected by the dietary fatty acid composition. A high fat n3 PUFA enriched shake increased EPA- and DHA-derived oxylipins. High fat SFA shake consumption predominantly increased LA-derived oxylipins from the LOX pathway. A MUFA enriched

shake increased LA-derived oxylipins from the CYP450 pathway. No differences in oxylipin response were observed between lean and obese subjects.

We previously found that high-fat consumption initiates an activated state of cellular adherence and an atherogenic milieu [12]. Oxylipins are important modulators of the inflammatory response and changes in circulating oxylipins after a high fat challenge test, may contribute to this pro-atherogenic response [5]. Overall, high fat meal consumption increased LA-derived oxylipins from the LOX pathway and EPA, DHA and AA-derived oxylipins from the CYP450 pathway. High fat meal consumption did not increase COX-related oxylipins, which are typical pro-inflammatory lipid mediators [3]. Many postprandially changed oxylipin tended to decrease 2 h postprandially, followed by an increase 4 h postprandially. The initial decrease in oxylipin after 2 hours might be due to a reduced lipolysis caused by the acute postprandial increase in insulin. The increase in insulin may reduce the release of fatty acids from the adipose tissue and TRLs and hence may reduce oxylipin formation [13,14]. After a decline in insulin, lipolysis is restored and fatty acids are again released from the tissues and TRLs into plasma, hence explaining the increase in oxylipins 4 h postprandially [13-15].

Fatty acid type highly affected the postprandial circulating oxylipin response. Correlations showed that these fatty acid specific-affected oxylipins all changed in a similar direction and magnitude. Although, this can be expected, these associations have never been shown in humans in a postprandial state. High SFA consumption postprandially increased LOX-mediated and LA-derived 9-HODE and 13-HODE. HODEs have been shown to increase inflammatory mediators, such as TNFα, ICAM and reactive oxygen species in vitro [5,7]. These changes after SFA consumption may point towards a pro-atherogenic environment for the endothelium. MUFA consumption increased 9,10-DiHOME and decreased 12,13-DiHOME. These LA-derived oxylipins have been studied in vitro, but both adverse and beneficial effects have been reported [16]. It is therefore unclear how postprandial changes in these oxylipins after MUFA consumption may affect vascular health. Most observed differences in circulating oxylipin response between fatty acids were observed after n3 PUFA consumption. High fat n3 PUFA consumption led to stronger increases in CYP450-mediated and AA-derived DiHETrEs, which are hydrolysis products of epoxyeicosatrienoic acids (EETs). EETs were not detected in the current study, possibly due to a fast turnover rate to their corresponding DiHETrEs. EETs have anti-inflammatory properties, but these characteristics seem to diminish after hydrolysis to their corresponding DiHETrEs [2]. The question therefore remains if, how and in which way our observed effects on DiHETrEs can affect the endothelium. High fat n3 PUFA consumption also increased oxylipins from EPA and DHA precursors. The biological effects of these EPA/DHA-derived oxylipins are poorly described in literature. It has been suggested that EPA- and

DHA-derived oxylipins have a lower pro-inflammatory potency compared to AA-derived oxylipins [17]. Shifting the balance from AA-derived oxylipins towards EPA- and DHA-derived oxylipins due to enzymatic competition might be beneficial to lower inflammation on the long run [17-19]. More studies are needed to disentangle acute and chronic effects. Nonetheless, these bioactive oxidized lipids are liberated from TRLs in close proximity to the endothelium. Repeated exposure of these elevated oxylipins in the postprandial phase might therefore affect endothelial function on the long run.

The current prevailing hypothesis is that local oxylipins are not stored, but synthesized de novo from membrane bound FA [3]. The source of circulating oxylipins in plasma and how they are regulated postprandially is still largely unknown [5]. Interestingly, we observed that precursor fatty acids of several changed oxylipins reflected the FA composition of the shakes. For example, the n3 PUFA shake contained high amounts of EPA and DHA, hence consumption of this shake increased many EPA- and DHA- derived oxylipins. Moreover, the SFA shake contained the highest amounts of LA due to the palm oil naturally comprising of around 9% of LA, followed by the MUFA shake containing high oleic sunflower oil with approximately 3.6% less LA [20]. The concentration of LA was lower in the n3 PUFA shake as well, because a part of the SFA was substituted by n3 PUFA. As a possible consequence of the different LA concentrations present in the shakes, the consumption of the SFA shake increased LA-derived oxylipins 9-HODE, 9,12,13-TriHOME and 13-HODE, while these increases were less pronounced after MUFA and n3 PUFA consumption. These findings suggest that the dietary exogenous fatty acids may act directly as precursor molecules for the biosynthesis of these circulating oxylipins. However, not all observed changes reflected the fatty acids composition of the shake. For example, AA was not present in our intervention shakes, whereas many AA-derived were increased postprandially. Dietary fatty acids may also postprandially affect circulating oxylipin profiles indirectly. It is known that dietary fatty acids are potent signaling molecules [21]. Fatty acids may activate distinct enzymatic or inflammatory pathways, thereby affecting the synthesis of oxylipin from membrane bound fatty acids. Future postprandial challenge studies with ^{13}C-labeled fatty acids are needed to determine if the consumed fatty acids affect the biosynthesis of these circulating oxylipins indirectly by activating distinct enzymatic pathways and/or can act directly as precursor molecules.

In conclusion, we are the first demonstrating that circulating oxylipins profiles are affected after consumption of a high fat meal challenge and that these changes are strongly influenced by the fatty acid composition of the meal challenge. It is difficult to evaluate how these acute postprandial changes affect health, but our findings may suggest that SFA consumption creates a more pro-atherogenic environment, whereas n3 PUFA consumption seems to create a less

harmful environment for the endothelium. This current study urges future research to elucidate the formation of these postprandial circulating oxylipins and their mechanism of action and potential role in cardiovascular disease risk.

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Conflict of interest

None.

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SUPPLEMENT

Supplemental Table S4.1 Nutritional values of the three intervention high fat shakes

Nutritional value	SFA	MUFA	n3 PUFA
Energy (kcal)	990	990	990
Protein (g)	10	10	10
Carbohydrates (g)	19	19	19
Fat (g)	95	95	95
Saturated fatty acids (g)	51	8	32
Monounsaturated fatty acids (g)	37	79	25
Polyunsaturated fatty acids (g)	6	8	38
ALA (g)	0	0	0
EPA (g)	0	0	3
DHA (g)	0	0	23
Vitamin E (mg)	165	165	165

Calculated using the Dutch Food Composition table (NEVO 2006). ALA, alpha-linolenic acid (18:3).

Supplemental Table S4.2 Baseline and postprandial changes in metabolic parameters after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

		Shake									*P*-values					
		SFA			MUFA			n3 PUFA				Main effects			Interactions	
	Group	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	G	S	T	G x T	S x T
TG	Lean	1.53±0.49	0.64±0.40^{b}	0.48±0.48^{b}	1.37±0.41	0.91±0.47^{a}	1.41±1.00^{a}	1.50±0.50	0.33±0.30^{c}	0.78±0.58^{b}	0.021	NS	<0.001	<0.001	0.046	<0.001
(mmol/L)	Obese	2.07±0.99	0.58±0.40^{b}	0.78±0.61^{b}	2.13±1.06	1.14±0.68^{a}	2.28±1.18^{a}	1.98±0.78	0.30±0.26^{c}	0.77±0.45^{b}						
FFA	Lean	0.51±0.21	-0.21±0.19^{b}	-0.04±0.22	0.49±0.20	-0.10±0.18^{a}	0.03±0.23	0.55±0.23	-0.25±0.21^{b}	-0.03±0.17	NS	NS	<0.001	0.002	0.042	<0.001
(mmol/L)	Obese	0.48±0.10	-0.18±0.12^{b}	0.11±0.11	0.50±0.15	-0.09±0.16^{a}	0.08±0.16	0.54±0.15	-0.21±0.11^{b}	0.06±0.16						
Insulin	Lean	6.52±2.55	3.08±3.77^{b}	-1.82±2.08^{b}	5.80±3.38	2.73±3.88^{b}	1.00±3.98^{a}	6.02±2.55	-0.02±3.77^{a}	-1.64±2.56^{b}	<0.001	0.022	<0.001	0.003	0.032	0.001
(mU/L)	Obese	13.52±7.40	6.59±9.60^{b}	-1.67±3.33^{b}	12.81±5.24	4.39±6.62^{b}	1.79±6.07^{a}	13.91±7.25	2.33±5.87^{a}	-2.04±3.09^{b}						
Glucose	Lean	5.19±0.39	-0.30±0.35^{b}	-0.40±0.21^{b}	5.17±0.35	-0.29±0.31^{b}	-0.08±0.28^{a}	5.29±0.53	-0.42±0.39^{a}	-0.33±0.25^{b}	0.015	NS	<0.001	0.002	0.001	0.001
(mmol/L)	Obese	5.50±0.41	-0.16±0.48^{b}	-0.43±0.39^{b}	5.53±0.43	-0.18±0.32^{b}	-0.31±0.28^{a}	5.54±0.28	-0.43±0.38^{a}	-0.52±0.33^{b}						

Values are means ± SD, lean *n*=18 and obese *n*=18. Different letters indicate differences between shakes at a given time for lean and obese combined (*P*=0.05). *P*-values were calculated using linear mixed models for repeated measures. Baseline, baseline comparison between groups; G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; NS, not significant (*P*=0.05); TG, triglycerides; FFA, free fatty acids.

Supplemental Table S4.3 Baseline and postprandial changes in triglyceride fatty acid composition after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

	Shake					
	SFA		MUFA		n3 PUFA	
	Baseline	Δ4h	Baseline	Δ4h	Baseline	Δ4h
	% of total		% of total		% of total	
Palmitic acid						
Lean	27. 5	2.9	25.8	-11.5	27.1	-0.6
Obese	28.1	2.5	27.9	-11.2	29.1	0.3
Oleic acid						
Lean	35.8	2.5	38.1	22.8	37.4	-2.5
Obese	36.1	1.1	36.0	20.7	35.7	-0.6
DHA						
Lean	0.68	-0.09	0.44	-0.12	0.32	4.56
Obese	0.66	-0.09	0.63	-0.16	0.55	2.86

Pooled samples for lean (*n*=18) and obese (*n*=18) men at baseline and 4 hours after shake consumption measured in serum. Only values for palmitic acid, oleic acid and DHA are displayed.

Supplemental Table S4.4 Baseline and postprandial changes in oxylipins after high fat SFA, MUFA and n3 PUFA shake consumption in lean and obese men

		SFA			MUFA			PUFA		
Oxylipin	Group	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h
12(13)-EpOME	Lean	0.04±0.006	-0.013±0.007	-0.004±0.006	0.027±0.002	0±0.005	-0.003±0.004	0.04±0.007	-0.011±0.008	-0.013±0.008
	Obese	0.04±0.007	-0.009±0.007	-0.004±0.006	0.044±0.007	0.001±0.011	-0.015±0.007	0.032±0.003	-0.006±0.004	0.003±0.004
12,13-DiHOME	Lean	0.522±0.075	-0.224±0.066	-0.118±0.08	0.464±0.108	-0.221±0.09	-0.269±0.109	0.584±0.084	-0.24±0.074	-0.222±0.092
	Obese	0.416±0.046	-0.104±0.047	-0.023±0.05	0.393±0.063	-0.041±0.065	-0.126±0.061	0.396±0.037	-0.11±0.035	-0.049±0.037
9(10)-EpOME	Lean	0.082±0.012	0.007±0.022	0.03±0.022	0.066±0.01	-0.011±0.01	0.006±0.017	0.071±0.009	-0.018±0.01	-0.018±0.01
	Obese	0.095±0.024	-0.011±0.028	0.001±0.031	0.111±0.033	0.012±0.032	-0.023±0.028	0.064±0.007	-0.006±0.012	0.001±0.013
9,10-DiHOME	Lean	0.608±0.088	-0.247±0.064	-0.07±0.082	0.687±0.12	0.554±0.137	0.484±0.087	0.569±0.078	-0.198±0.061	-0.107±0.081
	Obese	0.488±0.109	-0.063±0.052	0.002±0.084	0.477±0.087	0.337±0.113	0.46±0.143	0.428±0.047	-0.075±0.042	0.035±0.047
9,12,13-TriHOME	Lean	0.252±0.032	0.141±0.032	0.255±0.067	0.196±0.012	0.072±0.019	0.078±0.019	0.358±0.095	-0.026±0.079	0.032±0.078
	Obese	0.334±0.07	-0.008±0.064	0.263±0.158	0.227±0.019	0.082±0.031	0.075±0.022	0.233±0.024	0.011±0.029	0.1±0.039
13-HODE	Lean	0.757±0.072	0.667±0.088	0.932±0.127	0.732±0.099	0.097±0.108	0.286±0.127	1.04±0.124	-0.002±0.169	0.219±0.165
	Obese	0.81±0.136	0.29±0.154	0.747±0.253	0.897±0.122	0.013±0.152	0.322±0.135	0.829±0.087	-0.105±0.102	0.293±0.171
13-HpODE	Lean	0.115±0.025	-0.053±0.021	-0.023±0.022	0.094±0.014	-0.032±0.014	-0.02±0.012	0.108±0.018	-0.034±0.016	-0.043±0.019
	Obese	0.11±0.017	-0.029±0.021	0.016±0.021	0.118±0.026	-0.038±0.025	-0.012±0.029	0.109±0.015	-0.024±0.018	0.003±0.011
13-KODE	Lean	0.191±0.024	-0.033±0.02	0.019±0.019	0.195±0.023	-0.026±0.019	-0.035±0.027	0.219±0.03	-0.047±0.03	-0.05±0.034
	Obese	0.204±0.027	-0.035±0.029	0.038±0.033	0.219±0.032	-0.055±0.034	-0.008±0.036	0.195±0.021	-0.02±0.026	0.042±0.019
9-HODE	Lean	0.825±0.084	0.665±0.163	1.006±0.176	0.753±0.121	0.15±0.132	0.41±0.158	1.157±0.148	-0.105±0.234	0.233±0.216
	Obese	0.908±0.15	0.189±0.165	0.838±0.308	1.064±0.183	-0.032±0.243	0.392±0.223	0.938±0.098	-0.19±0.116	0.339±0.2
9-HpODE	Lean	0.074±0.014	-0.01±0.013	0.001±0.012	0.083±0.013	-0.018±0.014	-0.017±0.014	0.087±0.016	-0.015±0.008	-0.029±0.016
	Obese	0.08±0.014	-0.011±0.014	0.028±0.022	0.092±0.013	-0.015±0.014	0±0.016	0.08±0.01	-0.001±0.012	0.011±0.008
9-KODE	Lean	0.031±0.004	0.013±0.004	0.019±0.004	0.033±0.003	0.002±0.004	0.007±0.004	0.04±0.005	-0.007±0.006	-0.006±0.006
	Obese	0.043±0.009	-0.007±0.009	-0.001±0.012	0.035±0.005	0±0.006	0.006±0.005	0.03±0.002	0.001±0.004	0.008±0.004

Supplemental Table S4.4 continues on next page.

Supplemental Table S4.4 *Continued*

		SFA			MUFA			PUFA		
Oxylipin	Group	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h
PGF1a	Lean	0.025±0.004	-0.005±0.004	-0.002±0.005	0.024±0.002	0.001±0.002	-0.001±0.003	0.027±0.002	-0.003±0.002	-0.001±0.004
	Obese	0.023±0.001	0.001±0.002	0.001±0.003	0.025±0.002	-0.002±0.002	0.001±0.002	0.026±0.002	-0.001±0.003	0±0.003
PGD2	Lean	0.07±0.007	-0.018±0.006	-0.003±0.007	0.06±0.007	-0.011±0.009	-0.008±0.005	0.075±0.008	-0.014±0.009	-0.006±0.01
	Obese	0.065±0.007	-0.012±0.007	-0.008±0.006	0.063±0.005	-0.013±0.005	-0.007±0.003	0.063±0.005	-0.013±0.004	0.002±0.007
PGE2	Lean	0.147±0.015	-0.028±0.009	-0.005±0.011	0.119±0.011	-0.008±0.014	-0.007±0.01	0.154±0.016	-0.031±0.016	-0.019±0.018
	Obese	0.112±0.011	-0.02±0.013	-0.006±0.009	0.111±0.01	-0.008±0.009	-0.004±0.006	0.118±0.009	-0.024±0.009	0.002±0.011
PGF2a	Lean	0.095±0.007	0.009±0.008	0.002±0.009	0.096±0.011	0.005±0.009	0.005±0.007	0.101±0.012	-0.015±0.011	-0.015±0.009
	Obese	0.108±0.009	0.002±0.006	0.004±0.01	0.112±0.009	0.002±0.008	0.01±0.009	0.104±0.013	0.002±0.009	-0.004±0.009
TXB2	Lean	0.918±0.1	-0.095±0.076	0.068±0.098	0.898±0.112	0±0.124	0.003±0.123	1.075±0.154	0.024±0.207	0.034±0.206
	Obese	0.742±0.09	-0.016±0.113	0.126±0.126	0.952±0.145	-0.116±0.156	-0.013±0.181	0.783±0.104	-0.074±0.079	0.068±0.106
11-HETE	Lean	0.854±0.098	-0.11±0.131	0.005±0.114	0.713±0.106	0.063±0.17	0.24±0.157	0.902±0.108	0.045±0.262	0.093±0.235
	Obese	0.608±0.076	-0.069±0.104	0.108±0.118	0.996±0.215	-0.276±0.237	-0.011±0.273	0.637±0.074	-0.026±0.083	0.1±0.114
12-HHTrE	Lean	0.86±0.103	-0.015±0.157	0.051±0.117	0.783±0.143	0.115±0.199	0.193±0.202	1.002±0.157	0.109±0.274	0.057±0.31
	Obese	0.669±0.074	0.019±0.11	0.172±0.135	0.989±0.196	-0.153±0.26	0.003±0.263	0.692±0.106	0.005±0.085	0.024±0.12
14,15-DiHETrE	Lean	0.036±0.002	-0.002±0.002	0.008±0.003	0.039±0.003	0.011±0.004	0.015±0.005	0.046±0.004	0±0.008	0.023±0.007
	Obese	0.04±0.002	-0.002±0.002	0.008±0.003	0.039±0.003	0.006±0.004	0.009±0.004	0.05±0.006	-0.011±0.007	0.016±0.01
11,12-DiHETrE	Lean	0.014±0.001	0±0.002	0.006±0.002	0.016±0.002	0.006±0.002	0.007±0.003	0.019±0.003	0±0.004	0.018±0.005
	Obese	0.015±0.001	-0.002±0.001	0.004±0.002	0.015±0.002	0.003±0.002	0.006±0.003	0.02±0.004	-0.007±0.005	0.012±0.007
8,9-DiHETrE	Lean	0.006±0.001	0.002±0.001	0.003±0.001	0.007±0.001	0.002±0.001	0.004±0.002	0.01±0.001	0.001±0.003	0.005±0.002
	Obese	0.007±0.001	0.001±0.001	0.003±0.001	0.008±0.001	0.002±0.001	0.002±0.001	0.011±0.002	-0.003±0.002	0.004±0.003
5,6-DiHETrE	Lean	0.007±0.001	0±0.001	0.002±0.001	0.009±0.002	0.002±0.001	0.002±0.001	0.011±0.001	-0.001±0.002	0.004±0.002
	Obese	0.01±0.001	-0.001±0.001	0±0.001	0.01±0.001	-0.001±0.001	0.001±0.001	0.013±0.002	-0.003±0.002	0.003±0.002
12-HETE	Lean	3.135±0.542	-0.321±0.461	-0.08±0.435	2.791±0.69	-0.145±0.839	0.37±0.763	3.427±0.601	0.246±0.893	-0.153±0.926
	Obese	2.051±0.287	0.06±0.43	0.573±0.518	3.435±0.783	-0.485±1.104	0.415±1.001	2.182±0.331	-0.067±0.302	0.051±0.359

15-HETE	Lean	0.139±0.021	-0.01±0.01	0.023±0.018	0.109±0.013	0.01±0.016	0.001±0.01	0.188±0.026	-0.028±0.019	-0.038±0.016
	Obese	0.112±0.014	-0.005±0.016	0.002±0.014	0.138±0.029	-0.034±0.03	-0.018±0.03	0.125±0.014	-0.014±0.01	-0.002±0.013
15-KETE	Lean	0.046±0.005	-0.004±0.004	0.002±0.007	0.045±0.005	-0.005±0.005	-0.002±0.006	0.057±0.006	-0.005±0.007	-0.005±0.006
	Obese	0.043±0.007	-0.01±0.008	0.002±0.007	0.044±0.006	-0.007±0.005	-0.002±0.006	0.048±0.005	-0.006±0.004	0.01±0.007
5-HETE	Lean	0.034±0.004	-0.013±0.004	-0.009±0.005	0.026±0.002	-0.009±0.004	-0.006±0.003	0.032±0.003	-0.011±0.004	0±0.004
	Obese	0.03±0.003	-0.009±0.004	0.004±0.003	0.038±0.009	-0.019±0.011	-0.016±0.011	0.038±0.004	-0.009±0.003	-0.003±0.005
9-HOTrE	Lean	0.047±0.008	0.008±0.005	0.025±0.007	0.055±0.018	-0.031±0.017	-0.036±0.018	0.067±0.009	-0.024±0.009	-0.015±0.01
	Obese	0.047±0.007	-0.002±0.007	0.025±0.013	0.04±0.006	-0.004±0.007	-0.007±0.006	0.048±0.006	-0.014±0.006	0.002±0.007
TXB3	Lean	0.069±0.009	-0.025±0.007	-0.01±0.007	0.061±0.01	-0.015±0.008	-0.016±0.009	0.087±0.017	0±0.02	0.099±0.03
	Obese	0.054±0.008	-0.01±0.006	-0.004±0.008	0.051±0.008	-0.004±0.006	0.001±0.005	0.067±0.01	-0.015±0.01	0.051±0.023
17,18-DiHETE	Lean	0.018±0.002	0±0.002	0.006±0.002	0.018±0.002	0.009±0.002	0.008±0.002	0.031±0.006	0.034±0.017	0.117±0.025
	Obese	0.02±0.002	-0.004±0.002	-0.002±0.003	0.02±0.003	0.002±0.003	0.006±0.003	0.046±0.014	-0.012±0.018	0.056±0.029
14,15-DiHETE	Lean	0.006±0.001	0.002±0.001	0.004±0.002	0.008±0.001	-0.001±0.001	0.002±0.002	0.009±0.002	0.009±0.003	0.024±0.005
	Obese	0.009±0.002	-0.002±0.002	-0.001±0.002	0.008±0.001	0±0.001	-0.001±0.001	0.014±0.003	-0.003±0.004	0.012±0.007
12-HEPE	Lean	0.862±0.158	-0.284±0.099	-0.17±0.111	0.688±0.114	-0.13±0.114	-0.088±0.095	1.189±0.328	-0.025±0.261	1.123±0.483
	Obese	0.771±0.183	-0.16±0.181	-0.101±0.165	0.799±0.138	-0.191±0.121	0.035±0.116	0.782±0.112	-0.083±0.081	0.474±0.28
15-HEPE	Lean	0.018±0.002	-0.003±0.002	-0.003±0.004	0.012±0.002	-0.003±0.002	-0.002±0.002	0.023±0.005	-0.005±0.005	0.009±0.005
	Obese	0.015±0.002	0±0.003	0.003±0.003	0.017±0.003	-0.003±0.003	-0.004±0.002	0.018±0.002	-0.002±0.002	0.009±0.006
5-HEPE	Lean	0.029±0.004	-0.014±0.004	-0.013±0.004	0.019±0.003	-0.012±0.003	-0.009±0.004	0.03±0.006	-0.014±0.006	0.008±0.004
	Obese	0.028±0.004	-0.013±0.004	-0.003±0.003	0.03±0.005	-0.011±0.004	-0.011±0.004	0.033±0.003	-0.013±0.004	0.008±0.005
19(20)-EpDPE	Lean	0.059±0.018	-0.004±0.005	-0.002±0.008	0.038±0.009	-0.007±0.011	-0.005±0.01	0.075±0.027	0.012±0.006	0.264±0.065
	Obese	0.035±0.004	0.006±0.009	0.003±0.011	0.033±0.004	-0.002±0.004	0.01±0.006	0.136±0.048	-0.047±0.048	0.114±0.071
19,20-DiHDPA	Lean	0.089±0.012	0.012±0.007	0.033±0.009	0.086±0.01	0.06±0.011	0.059±0.008	0.144±0.031	0.102±0.059	0.444±0.095
	Obese	0.097±0.009	-0.005±0.008	0.023±0.01	0.093±0.012	0.024±0.012	0.036±0.014	0.199±0.055	-0.053±0.068	0.246±0.119
17-HDoHE	Lean	0.864±0.187	-0.048±0.09	0.148±0.154	0.862±0.251	-0.156±0.167	-0.057±0.135	1.851±0.712	0.716±0.484	5.682±1.598
	Obese	1.08±0.314	-0.263±0.288	-0.048±0.224	0.947±0.283	-0.236±0.227	-0.004±0.175	0.889±0.17	0.182±0.101	3.355±1.033

Response ratios were calculated by peak area of oxylipin compound divided by peak area of internal standard. Values are mean ± SEM.

Chapter 5

Distinct changes in peripheral blood mononuclear cell gene expression profiles between lean and obese subjects after a high-fat challenge differing in fatty acid type

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In preparation.

ABSTRACT

Background: Atherosclerosis usually affects middle-aged adults, but the process often starts in early life. A promising strategy to detect early disturbances in vascular health is to impose subjects to a metabolic challenge, such as a high fat load. The ability to respond to such a challenge may reflect the flexibility of cells and organs and can be used to detect early perturbations in health status. We aimed to detect small disturbances in health status by comparing whole genome expression changes in peripheral blood mononuclear cells (PBMCs) between human subjects with different disease risk profiles after a high mono-unsaturated fatty acids (MUFA) or high saturated fatty acid (SFA) challenge.

Methods: In a double-blind cross-over study, 17 lean and 15 obese middle-aged men received two high-fat milkshakes containing 95g fat, either high in SFAs or MUFAs. PBMC gene expression profiles were determined before and 4 hours after shake consumption and induced gene expression changes were compared between obese and lean subjects and between the different fatty acids.

Results: Pro-thrombotic genes were up-regulated in obese relative to lean subjects after SFA intake. After MUFA intake, genes involved in posttranslational protein modifications were up-regulated and various G-protein coupled receptors were down-regulated in obese relative to lean subjects. In both lean and obese subjects, the SFA shake decreased the expression of genes involved in the biosynthesis and uptake of cholesterol and increased the expression of genes involved in cholesterol efflux. The MUFA shake increased the expression of inflammatory genes and of peroxisome proliferator-activated receptor α (PPARα) target genes involved in the β-oxidation.

Conclusions: Subject with different metabolic risk phenotypes have distinct changes in PBMC gene expression profiles after a high-fat challenge test with the most deviating effect for MUFA. The gene expression changes after SFA intake may suggest an intracellular situation of excess cholesterol after SFA intake. The up-regulation in expression of β-oxidation genes after MUFA intake may suggest an increased flow of fatty acids towards the β-oxidation. Both SFA and MUFA-regulated pathways are important in cellular lipid homeostasis. Dysregulation of these pathways may lead to increased lipid accumulation, an essential process in the formation of lipid-loaded macrophages.

INTRODUCTION

Cardiovascular diseases (CVD) are still one of the leading causes of deaths worldwide and the underlying pathological condition of many CVD is atherosclerosis. Atherosclerosis is a slowly progressing condition characterized by a thickening of the artery wall as a result of lipid accumulation and leukocyte infiltration in the sub-endothelial space [1]. Endothelial dysfunction is regarded as a pivotal factor in the onset and progression of atherosclerosis [2]. Atherosclerosis often affects older or middle-aged adults, but the process often starts early in life. A valuable strategy to detect early disturbances in vascular health is to impose subjects to a metabolic challenge test. The ability to respond to such a challenge may reflect the metabolic flexibility of cells and organs and can therefore be a useful tool to detect early perturbations in health status, even before this can be measured under fasting conditions [3]. A typical pro-atherogenic meal challenge is a high fat load [4-6]. One of the current hypotheses is that the elevated concentrations of plasma triglyceride rich lipoproteins (TRLs) after a high fat meal activate endothelial cells and leukocytes and thereby affect endothelial function [20,34,35]. We recently observed that a challenge high in mono-unsaturated fatty acids (MUFA) elicited a more pronounced postprandial increase in triglyceride (TG) concentrations than a challenge high in saturated fatty acids (SFA), especially in obese individuals [7]. These findings suggest that MUFAs are more potent fatty acids to challenge the metabolic response capacity than SFAs and that obese individuals have perturbed postprandial metabolic responses.

A major limitation for early detection of atherosclerosis is the limited accessibility of endothelial cells, especially in a pre-clinical setting. Cells that are available and easy to collect are circulating peripheral blood mononuclear cells (PBMCs). PBMCs are a mixture of T-cells, B-cells and monocytes, which can interact with the endothelium and play a role in the onset and progression of atherosclerosis. For example, monocytes are able to differentiate to macrophages and foam cells that are involved in the formation of fatty streaks during atherosclerosis development [22]. PBMCs are therefore a valuable source of material to study changes in metabolic and inflammatory processes. A powerful way to evaluate whole genome wide adaptive changes in gene transcription is by unbiased high-throughput transcriptome analysis. This allows the identification of affected stress related and metabolic compensatory pathways in PBMCs that may reflect the resilience capacity of these cells. Comparing postprandial changes in PBMC gene expression profiles after intake of SFAs and MUFAs may increase our understanding on how these fatty acids may exert their acute effects on PBMCs. Furthermore, challenging lean and obese subjects with a high-fat shake enriched with SFAs or MUFAs may allow us to detect small disturbances in cellular response capacity related to health status. We therefore aimed to characterize PBMC whole genome expression changes in response to a high MUFA or high SFA challenge in lean and obese subjects.

MATERIALS AND METHODS

Subjects

We included 18 lean (predefined BMI 18–25 kg/m^2) and 18 obese (predefined BMI >30 kg/m^2) male subjects between 50–70 years old from a subpopulation of a previously reported study [7]. Six obese type 2 diabetic subjects from the original study were excluded because of the lower number. All lean and obese subjects were non-smoking, normoglycemic (WHO criteria) and not diagnosed with any long-term medical condition. All subjects gave written informed consent and the study was approved by the local Medical Ethics Committee. The study was registered at clinicaltrials.gov as NCT00977262.

Study design

The study was a double-blind randomized cross-over intervention study in which each participant visited the university two times. On each study day, the subjects consumed milkshake with 95g of fat, which was high in either SFA or MUFA. Blood samples were collected before and 4 hours after a milkshake consumption. Subjects were randomly assigned to a treatment order and a one-week washout period was the minimum between consecutive study days. On the day prior to each study day, subjects consumed a standardized low fat evening meal, were refrained from alcohol or strenuous exercise and were not allowed to eat or drink anything except water after 08.00 pm. This study was conducted according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO).

Intervention shakes

Both shakes had a volume of 500ml, were isocaloric and macronutrient composition differed only in fatty acid composition (**Supplemental Table S5.1**). The SFA shake contained 95g palm oil and the MUFA shake contained 95g high-oleic acid sunflower oil. Vitamin E (165mg) was added to the shakes to match vitamin E present in the n3 polyunsaturated fatty acid (PUFA) shake, that was used in the original study design. A more detailed description of the intervention shakes is previously reported [7].

PBMC and RNA isolation

PBMCs were isolated before and 4 hours after milkshake consumption by BD Vacutainer Cell Preparation Tubes. RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantified (Nanodrop ND 1000, Nanodrop technologies, Wilmington, Delaware USA) and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK).

Micro-array processing

PBMC samples from 17 lean and 15 obese subjects yielded enough RNA of sufficient quality at all collection points to perform microarray analysis. Microarray analysis was performed for each individual at baseline and 4 hours after SFA and MUFA consumption, resulting in a total of 128 microarrays.

Total RNA was labeled using a one-cycle cDNA labeling kit (MessageAmp™ II-Biotin Enhanced Kit; Ambion Inc, Nieuwekerk a/d IJssel, Netherlands) and hybridized to GeneChip® Human Gene 1.1 ST Array targeting 19,738 unique gene identifiers (Affymetrix Inc. Santa Clara, CA). Sample labelling, hybridization to chips and image scanning were performed according to the manufacturers' instructions.

Microarray analysis

Microarrays were analysed by using MADMAX pipeline for statistical analysis of microarray data (https://madmax.bioinformatics.nl/). Microarrays were analysed using the reorganized oligonucleotide probes, which combines all individual probes for a gene [8]. Expression values were calculated using the robust multichip average (RMA) method and normalized by quantile normalization [9,10]. Genes with normalized signals >20 on >8 arrays were defined as expressed and selected for further analysis. At baseline, the expression of genes between lean and obese was defined as different when the *P*-value <0.05 in a t-test with Bayesian correction (Limma) [11]. Gene expression was defined as postprandially changed between T0 and T4 when the *P*-value was <0.05 in a paired t-test with Bayesian correction (Limma). Differences in gene expression changes between groups or shakes were calculated from the individual signal to log ratios (SLR). Between lean and obese, changes after shake consumption were defined as different when the *P*-value <0.05 in a t-test with Bayesian correction (Limma). Changes after SFA and MUFA shake consumption were defined as different when the *P*-value <0.05 in a paired t-test with Bayesian correction (Limma). In the latter comparison we combined lean

and obese subjects to end up with a total of 32 participants to increase power. Data were further analysed with gene set enrichment analysis (GSEA) [12]. Gene sets with a false discovery rate (FDR) *Q*-value <0.25 were defined as significantly regulated. As a large number of gene sets was changed with FDR *Q*-value <0.25 we used a FDR *Q*-value <0.05 in Cytoscape (Cytoscape 2.8.3) to visualize clusters of postprandially changed gene sets.

Plasma metabolic markers and TG fatty acid composition

Plasma TG, glucose, insulin and free fatty acids (FFA) were assessed during fasting condition and 2 and 4h after milkshake consumption and have been published [7].

RESULTS

Subject characteristics and metabolic response

Baseline characteristics of the 17 lean and 15 obese subjects of which microarrays were performed are displayed in **Table 5.1**. Baseline and postprandial concentrations of glucose, TG, FFA and insulin are shown in **Supplemental Table S5.2**. Obese subjects had higher fasting TG and insulin concentrations than lean subjects. MUFA consumption was associated with a more pronounced increase in TG compared with the SFA consumption as has been published [7].

Differences between lean and obese subjects

Figure 5.1 is a flow chart of the number of genes of which the expression was significantly different between lean and obese subjects at baseline and of the number of genes that changed differently in expression in obese relative to the lean subjects after a high-fat SFA or MUFA challenge. At baseline 294 genes were significant differently expressed between lean and obese

Table 5.1 Baseline characteristics of the subjects

	Lean (*n*=17)	Obese (*n*=15)
Age (yrs)	62 ± 6	63 ± 4
Height (cm)	177 ± 8	179 ± 6
Weight (kg)	75 ± 7	105 ± 13*
BMI (kg/m^2)	23.8 ± 0.9	32.5 ± 3.3*

Data are presented as mean ± SD. * Significantly different from lean subjects (*P*<0.05).

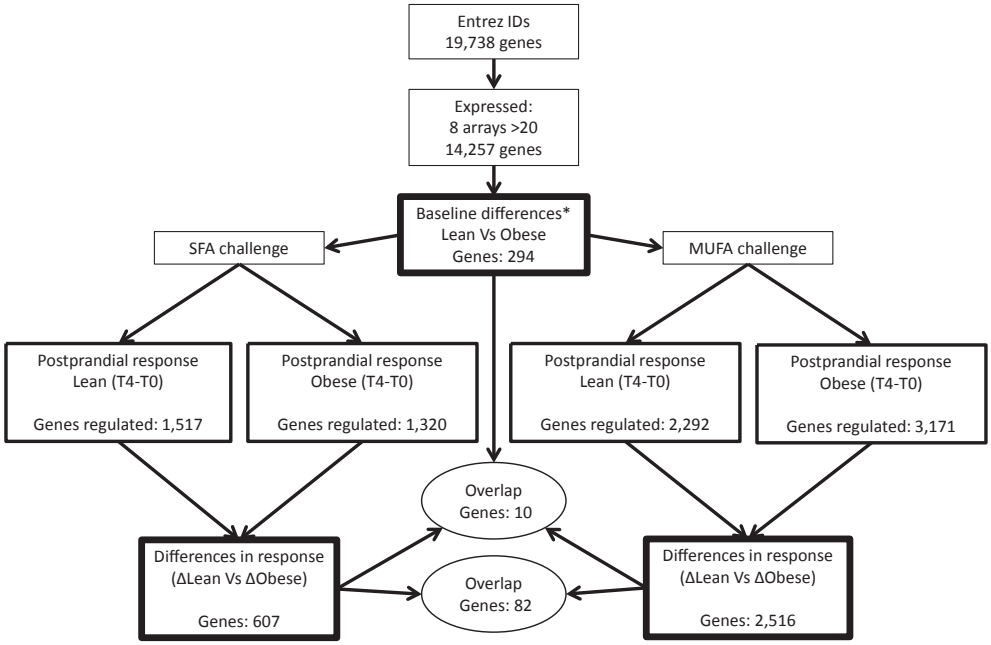


Figure 5.1 Flow diagram showing the number of genes of which the expression was significantly different between lean (n=17) and obese (n=15) subjects at baseline, the number of genes that changed in expression after a high-fat SFA or MUFA challenge and of the number of genes that changed differently in expression in obese relative to the lean subjects after a high-fat SFA or MUFA challenge. A change was significant if P<0.05. * Baseline differences between lean and obese subject were calculated from the mean signal intensities of the two baseline recordings.

subjects. The SFA challenge changed the expression of 1,320 genes in obese and 1,517 genes in lean and the expression of 607 genes changed significant differently between lean and obese (delta obese T4-T0 vs. delta lean T4-T0). The MUFA challenge changed the expression of 3,171 genes in obese and 2292 genes in lean and the expression of 2,516 genes changed significantly different between lean and obese (delta obese T4-T0 vs. delta lean T4-T0). A total of 10 genes was significant differently expressed between lean and obese at baseline and changed significantly different in expression in obese relative to the lean subjects after both challenges i.e. *TLE2*, *RNF5P1*, *VAV3*, *PLEK2*, *KIAA1522*, *SORCS3*, *IKBKE*, *ZNF740*, *CSGALNACT2* and *TRAV12*-2. A total of 82 genes changed significantly different in expression in obese relative to the lean subjects after both the SFA and MUFA shake.

At baseline, GSEA identified 15 gene sets that were lower expressed in obese compared to lean subjects (FDR Q<0.25). All gene sets were located in one cluster that largely included the same

genes. These genes predominantly belonged to the human leukocyte antigen (HLA) class II and included *HLA-DQA2*, *HLA-DQB1* and *HLA-DRB5*.

The gene sets (FDR $Q< 0.05$) significant differently changed in response to SFA challenge in obese relative to the lean subjects (delta obese T4-T0 vs. delta lean T4-T0) are visualised in **Supplemental Figure S5.1**. Four gene sets that were up-regulated in obese and down-regulated in lean subjects after SFA intake belonged to a cluster involved in 'platelet activation'. Individual changes of significantly changed genes from these gene sets are visualized in **Figure 5.2** and included chemokine's released from activated platelets (*PF4*, *PPBP*), platelet integrins (*ITGA2B*, *ITGB3*), P-selectin (*SELP*) and genes involved in platelet aggregation and thrombus formation (*PDE3A*, *CD9*, *GP1BA*, *GP6* and *GP9*).

The gene sets (FDR $Q< 0.05$) significant differently changed in response to the MUFA challenge in obese relative to the lean subjects are visualised in **Supplemental Figure S5.2**. A cluster with genes involved in posttranslational protein modification was up-regulated in obese and down-regulated in lean subjects. A cluster of G protein coupled receptors (GPCR) was down-regulated in obese subjects and up-regulated in lean subjects. Individual expression changes of genes significantly changed within these clusters are visualized in a heatmap (**Figure 5.3**) and included genes involved in protein glycolysation (*RFT1*, *PMM2*, *B4GALT6*, *ALG8*, *ALG2* and *ST3GAL4*), glycosylphosphatidyl-inositol (GPI) anchor synthesis (*PIGN*, *PIGF*, *SEC31A*, *SEC24C*, *SEC24B* and *PGAP3*) and various G protein coupled receptors.

Differences between a SFA and MUFA challenge

To identify difference in gene expression changes between the SFA and the MUFA challenge, we combined data of lean and obese subjects (in total n=32). **Figure 5.4** lists a flow diagram with the number of genes that changed significantly in expression after a SFA or MUFA challenge. SFA consumption changed the expression of 2,122 genes and MUFA consumption changed the expression of 2,270 genes. Expression of 1,489 genes changed significantly different between the MUFA shake and the SFA shake.

Gene sets identified with GSEA with a FDR $Q<0.05$ are visualised in **Supplemental Figure S5.3**. The most highly ranked significantly down-regulated gene sets after SFA consumption relative to MUFA, included 'cholesterol biosynthesis' and 'biosynthesis of unsaturated fatty acids'. Many genes from these gene sets are target genes of the transcription factor SREBP2 and SREBP1a. The gene *SREBF2* itself was also more down-regulated after SFA compared to MUFA intake ($P<0.001$). Furthermore, SFA intake increased the expression of genes involved in the efflux of cellular cholesterol i.e. *ABCA1* and *ABCG1* and decreased the expression of the

Cluster platelet function			Lean subjects			Obese subjects			Lean vs. Obese
Genes	Entrez ID	Description	Individual SLR	Mean SLR	*P*-values	Individual SLR	Mean SLR	*P*-values	*P*-values
MPL	4352	myeloproliferative leukemia virus oncogene		-0.13	0.19		0.31	0.00	0.00
EGF	1950	epidermal growth factor		-0.19	0.11		0.33	0.01	0.00
KIF3C	3797	kinesin family member 3C		-0.16	0.00		0.04	0.43	0.00
P2RX1	5023	purinergic receptor P2X, ligand-gated ion channel, 1		0.04	0.42		0.22	0.00	0.00
CAPZB	832	capping protein (actin filament) muscle Z-line, beta		-0.09	0.00		0.00	0.87	0.01
F2R	2149	coagulation factor II (thrombin) receptor		-0.15	0.01		0.11	0.08	0.01
THBD	7056	thrombomodulin		-0.43	0.00		-0.23	0.00	0.01
CLU	1191	clusterin		-0.04	0.67		0.28	0.01	0.01
GP9	2815	glycoprotein IX (platelet)		-0.04	0.62		0.25	0.00	0.01
PDGFA	5154	platelet-derived growth factor α polypeptide		-0.08	0.46		0.29	0.01	0.01
SPARC	6678	secreted protein, acidic, cysteine-rich (osteonectin)		-0.08	0.49		0.31	0.01	0.01
TUBB1	81027	tubulin, beta 1		-0.08	0.43		0.25	0.02	0.01
GNG11	2791	guanine nucleotide binding protein (G protein), gamma 11		-0.07	0.56		0.32	0.01	0.01
GUCY1A3	2982	guanylate cyclase 1, soluble, α3		-0.17	0.01		0.06	0.41	0.01
MGLL	11343	monoglyceride lipase		0.01	0.93		0.23	0.00	0.02
SELP	6403	selectin P (granule membrane protein 140kDa, antigen CD62)		-0.08	0.51		0.28	0.03	0.02
GUCY1B3	2983	guanylate cyclase 1, soluble, beta 3		-0.09	0.29		0.19	0.04	0.02
ITGA2B	3674	integrin, alpha 2b		-0.08	0.53		0.32	0.03	0.02
CD9	928	CD9 molecule		-0.15	0.11		0.16	0.12	0.02
FCER1G	2207	Fc fragment of IgE, high affinity I, receptor for /gamma polypept		-0.04	0.32		0.08	0.04	0.02
F13A1	2162	coagulation factor XIII, A1 polypeptide		-0.11	0.09		0.09	0.18	0.02
PDE3A	5139	phosphodiesterase 3A, cGMP-inhibited		-0.08	0.37		0.20	0.04	0.02
EHD3	30845	EH-domain containing 3		-0.07	0.31		0.14	0.06	0.02
VAV3	10451	vav 3 guanine nucleotide exchange factor		-0.10	0.07		0.09	0.13	0.02
ESAM	90952	endothelial cell adhesion molecule		-0.10	0.37		0.24	0.05	0.02
PPBP	5473	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)		-0.03	0.74		0.24	0.01	0.02
GP6	51206	glycoprotein VI (platelet)		-0.04	0.67		0.22	0.02	0.02
VCL	7414	vinculin		-0.06	0.20		0.09	0.09	0.03
PF4	5196	platelet factor 4		-0.10	0.37		0.23	0.05	0.03
GNGT2	2793	guanine nucleotide binding protein, gamma transd.act.polypept 2		-0.19	0.00		0.04	0.56	0.03
ITGB3	3690	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)		-0.03	0.77		0.32	0.01	0.03
MMRN1	22915	multimerin 1		-0.04	0.72		0.27	0.03	0.03
GP1BA	2811	glycoprotein Ib (platelet), α polypeptide		-0.01	0.94		0.35	0.01	0.03
PTGIR	5739	prostaglandin I2 (prostacyclin) receptor (IP)		-0.23	0.00		-0.06	0.34	0.05
PIK3R5	23533	phosphoinositide-3-kinase, regulatory subunit 5		-0.13	0.00		-0.04	0.18	0.05

Figure 5.2 Expression heatmap of significantly changed genes in obese relative to lean subjects after the SFA challenge involved in platelet activation. Expression changes are indicated as individual signal-log-ratios (SLR) of t=4h versus t=0h for lean (*n*=15) and obese (*n*=18) subjects. Subjects are ranked based on participant number. Down-regulation or up-regulation of gene expression is presented on a colour scale ranging from green (down-regulated, SLR ≤ -0.25) to red (up-regulated, SLR ≥ 0.25).

scavenger low-density lipoprotein receptor (*LDLR*) involved in influx of cholesterol. **Figure 5.5** shows a heatmap of gene expression changes per person of significantly changed genes after SFA of the above described pathways.

One of the most highly ranked gene set up-regulated after the MUFA challenge relative to the SFA challenge was the gene set 'PPAR targets'. **Figure 5.6** shows a heatmap of the significantly changed peroxisome proliferator-activated receptor (PPAR) target genes from this gene set. We included additional PPARα target genes in the heatmap which were shown to be regulated in PBMCs after *ex vivo* stimulation by the PPARα ligand WY14,643 [13] and were significantly

Cluster GCRP			Lean subjects			Obese subjects			Lean vs. Obese
Genes	Entrez ID	Description	Individual SLR	Mean SLR	*P*-values	Individual SLR	Mean SLR	*P*-values	*P*-values
GALR3	8484	galanin receptor 3		0.14	0.09		-0.25	0.00	0.00
GPR25	2848	G protein-coupled receptor 25		0.18	0.00		-0.09	0.17	0.00
GCGR	2642	glucagon receptor		0.10	0.09		-0.14	0.02	0.00
GPR62	118442	G protein-coupled receptor 62		0.05	0.49		-0.25	0.00	0.00
CXCL1	2919	chemokine (C-X-C motif) ligand 1		0.20	0.00		-0.05	0.42	0.00
GNG4	2786	guanine nucleotide binding protein (G protein), gamma 4		0.00	0.94		-0.26	0.00	0.00
GPR3	2827	G protein-coupled receptor 3		0.11	0.23		-0.26	0.01	0.01
AVP	551	arginine vasopressin		0.13	0.11		-0.18	0.03	0.01
ADCY6	112	adenylate cyclase 6		0.07	0.20		-0.14	0.01	0.01
AVPR1A	552	arginine vasopressin receptor 1A		0.18	0.01		-0.05	0.48	0.01
GNG7	2788	guanine nucleotide binding protein (G protein), gamma 7		0.11	0.02		-0.04	0.40	0.01
WNT1	7471	wingless-type MMTV integration site family, member 1		0.10	0.09		-0.11	0.08	0.01
FFAR1	2864	free fatty acid receptor 1		0.19	0.02		-0.12	0.17	0.01
MTNR1B	4544	melatonin receptor 1B		0.07	0.29		-0.19	0.01	0.01
WNT10B	7480	wingless-type MMTV integration site family, member 10B		0.08	0.18		-0.13	0.04	0.01
CCL17	6361	chemokine (C-C motif) ligand 17		0.04	0.44		-0.14	0.01	0.01
WNT16	51384	wingless-type MMTV integration site family, member 16		0.09	0.07		-0.09	0.07	0.01
ADRA1D	146	adrenergic, alpha-1D-, receptor		0.09	0.13		-0.11	0.08	0.01
BDKRB2	624	bradykinin receptor B2		0.13	0.05		-0.12	0.09	0.01
TUBB3	10381	tubulin, beta 3		0.08	0.40		-0.22	0.02	0.01
DRD2	1813	dopamine receptor D2		0.04	0.53		-0.19	0.00	0.01
PTH2	113091	parathyroid hormone 2		0.04	0.60		-0.21	0.00	0.02
S1PR2	9294	sphingosine-1-phosphate receptor 2		0.03	0.67		-0.20	0.01	0.02
OXTR	5021	oxytocin receptor		0.11	0.04		-0.06	0.31	0.02
ADCY5	111	adenylate cyclase 5		0.06	0.31		-0.17	0.01	0.02
TAS1R3	83756	taste receptor, type 1, member 3		0.10	0.13		-0.13	0.06	0.02
ADRB1	153	adrenergic, beta-1-, receptor		0.16	0.05		-0.12	0.16	0.02
EDN3	1908	endothelin 3		0.03	0.58		-0.15	0.01	0.02
WNT10A	80326	wingless-type MMTV integration site family, member 10A		0.07	0.26		-0.14	0.03	0.02
GRM5	2915	glutamate receptor, metabotropic 5		0.04	0.40		-0.13	0.02	0.02
CHRM1	1128	cholinergic receptor, muscarinic 1		0.09	0.20		-0.13	0.07	0.02
FZD10	11211	frizzled family receptor 10		0.05	0.37		-0.14	0.03	0.03
OPN4	94233	opsin 4		0.05	0.45		-0.15	0.02	0.03
NPY	4852	neuropeptide Y		0.11	0.04		-0.06	0.29	0.03
RLN3	117579	relaxin 3		0.01	0.92		-0.26	0.01	0.03
HRH3	11255	histamine receptor H3		0.04	0.59		-0.17	0.03	0.04
NPW	283869	neuropeptide W		0.06	0.41		-0.16	0.05	0.04
FZD1	8321	frizzled family receptor 1		0.17	0.01		-0.01	0.91	0.04
PDE4C	5143	phosphodiesterase 4C, cAMP-specific		0.03	0.67		-0.16	0.02	0.04
UTS2R	2837	urotensin 2 receptor		0.11	0.16		-0.11	0.16	0.04
GAL	51083	galanin prepropeptide		0.03	0.68		-0.18	0.01	0.04
WNT3A	89780	wingless-type MMTV integration site family, member 3A		0.06	0.29		-0.11	0.09	0.04
TBXA2R	6915	thromboxane A2 receptor		0.18	0.03		-0.07	0.44	0.04
GPR37L1	9283	G protein-coupled receptor 37 like 1		0.03	0.70		-0.19	0.02	0.05
KISS1R	84634	KISS1 receptor		0.06	0.34		-0.12	0.09	0.05
CNR2	1269	cannabinoid receptor 2 (macrophage)		0.02	0.73		-0.15	0.02	0.05
HTR5A	3361	5-hydroxytryptamine (serotonin) receptor 5A		0.03	0.65		-0.18	0.02	0.05
GNG8	94235	guanine nucleotide binding protein (G protein), gamma 8		0.07	0.29		-0.11	0.13	0.05
Cluster protein modification			**Lean subjects**			**Obese subjects**			**Lean vs. Obese**
Genes	**Entrez ID**	**Description**	**Individual SLR**	**Mean SLR**	***P*-values**	**Individual SLR**	**Mean SLR**	**P value**	***P*-values**
SEC24B	10427	SEC24 family, member B (S. cerevisiae)		-0.05	0.06		0.07	0.01	0.00
SEC24C	9632	SEC24 family, member C (S. cerevisiae)		-0.08	0.01		0.08	0.02	0.00
SEC31A	22872	SEC31 homolog A (S. cerevisiae)		-0.04	0.04		0.03	0.24	0.03
UAP1	6675	UDP-N-acteylglucosamine pyrophosphorylase 1		-0.16	0.00		0.04	0.46	0.00
RFT1	91869	RFT1 homolog (S. cerevisiae)		-0.08	0.07		0.06	0.16	0.02
B4GALT6	9331	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypept 6		-0.13	0.12		0.27	0.00	0.00
PIGF	5281	phosphatidylinositol glycan anchor biosynthesis, class F		-0.13	0.25		0.31	0.01	0.00
B4GALT4	8702	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypept 4		-0.19	0.01		0.11	0.14	0.01
GANAB	23193	glucosidase, alpha/ neutral AB		-0.05	0.04		0.05	0.04	0.01
PIGN	23556	phosphatidylinositol glycan anchor biosynthesis, class N		-0.09	0.13		0.15	0.03	0.01
ALG13	79868	asparagine-linked glycosylation 13 homolog (S. cerevisiae)		-0.07	0.10		0.10	0.02	0.01
MGAT2	4247	mannosyl-glycoprotein beta-1,2-N-acetylglucosaminyltransferase		-0.06	0.23		0.14	0.01	0.01
ST8SIA6	338596	ST8 alpha-N-acetyl-neuraminide α-2,8-sialyltransferase 6		-0.19	0.03		0.13	0.14	0.01
PMM2	5373	phosphomannomutase 2		-0.06	0.17		0.11	0.03	0.02
EIF5A	1984	eukaryotic translation initiation factor 5A		0.00	0.95		0.22	0.00	0.02
EDEM3	80267	ER degradation enhancer, mannosidase α-like 3		-0.05	0.15		0.06	0.15	0.02
MAN1A2	10905	mannosidase, alpha, class 1A, member 2		-0.06	0.11		0.06	0.09	0.02
ALG8	79053	asparagine-linked glycosylation 8, α-1,3-glucosyltransf hom.		-0.09	0.05		0.06	0.22	0.03
ALG2	85365	asparagine-linked glycosylation 2, α-1,3-mannosyltransf hom.		-0.07	0.09		0.06	0.19	0.03
GAS6	2621	growth arrest-specific 6		-0.29	0.00		-0.10	0.12	0.03
ST3GAL4	6484	ST3 beta-galactoside α-2,3-sialyltransferase 4		-0.14	0.01		0.02	0.72	0.03
B4GALT1	2683	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransf, polypep1		-0.06	0.05		0.03	0.32	0.03
PGAP3	93210	post-GPI attachment to proteins 3		-0.04	0.32		0.09	0.03	0.02

Figure 5.3 Expression heatmap of significantly changed genes in obese relative to lean subjects after the MUFA challenge involved in G protein coupled receptors (GPCR) and posttranslational protein modification. Expression changes are indicated as individual signal-log-ratios (SLR) of t=4h versus t=0h for lean (*n*=15) and obese (*n*=18) subjects. Subjects are ranked based on participant number. Down-regulation or up-regulation of gene expression is presented on a colour scale ranging from green (down-regulated, SLR ≤ -0.25) to red (up-regulated, SLR ≥ 0.25).

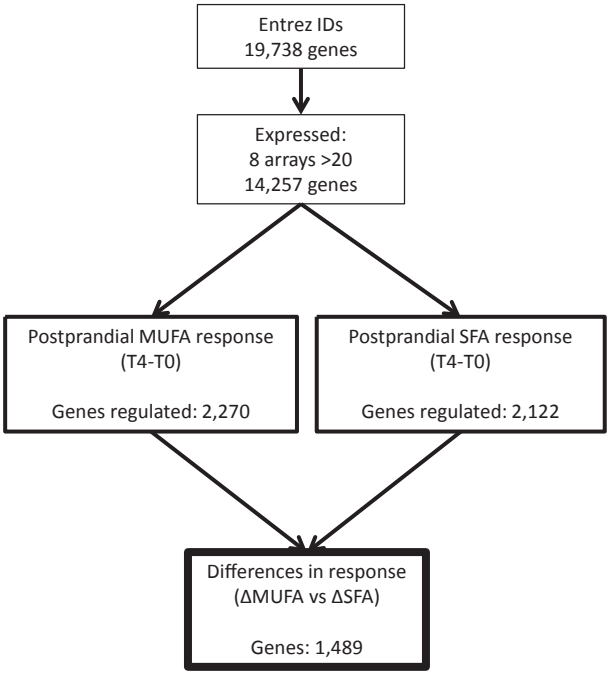
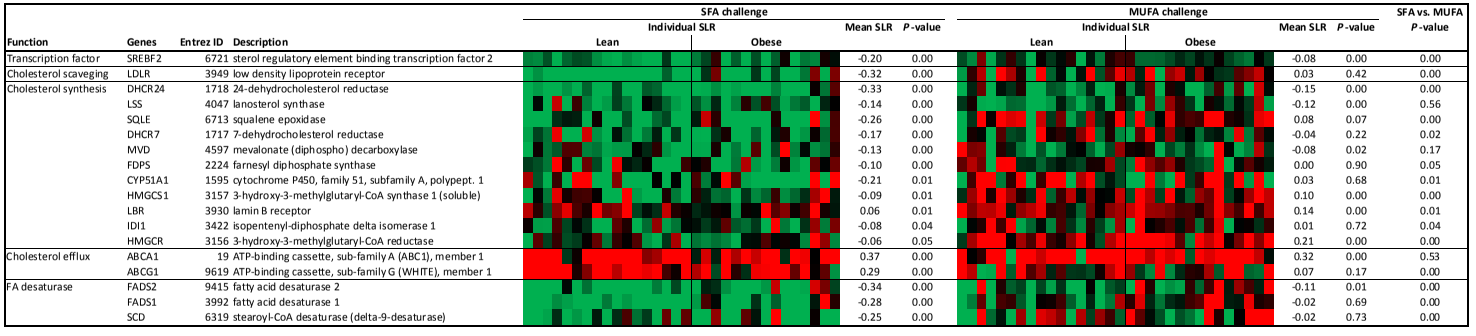


Figure 5.4 Flow diagram showing the number of genes of which the expression was changed after a high fat SFA or MUFA challenge and of the number of genes that changed in expression significant differently between the MUFA shake and the SFA shake in the whole study population (n=32). A change was significant if P<0.05.

changed by the MUFA challenge. Many PPAR target genes up-regulated after MUFA intake were involved in β-oxidation (Figure 5.6). Moreover, several inflammatory gene sets were also more pronouncedly up-regulated after MUFA consumption relative to SFA consumption, such as toll-like receptor signalling and IL8-CXCR1/2 pathways (Figure 5.6).

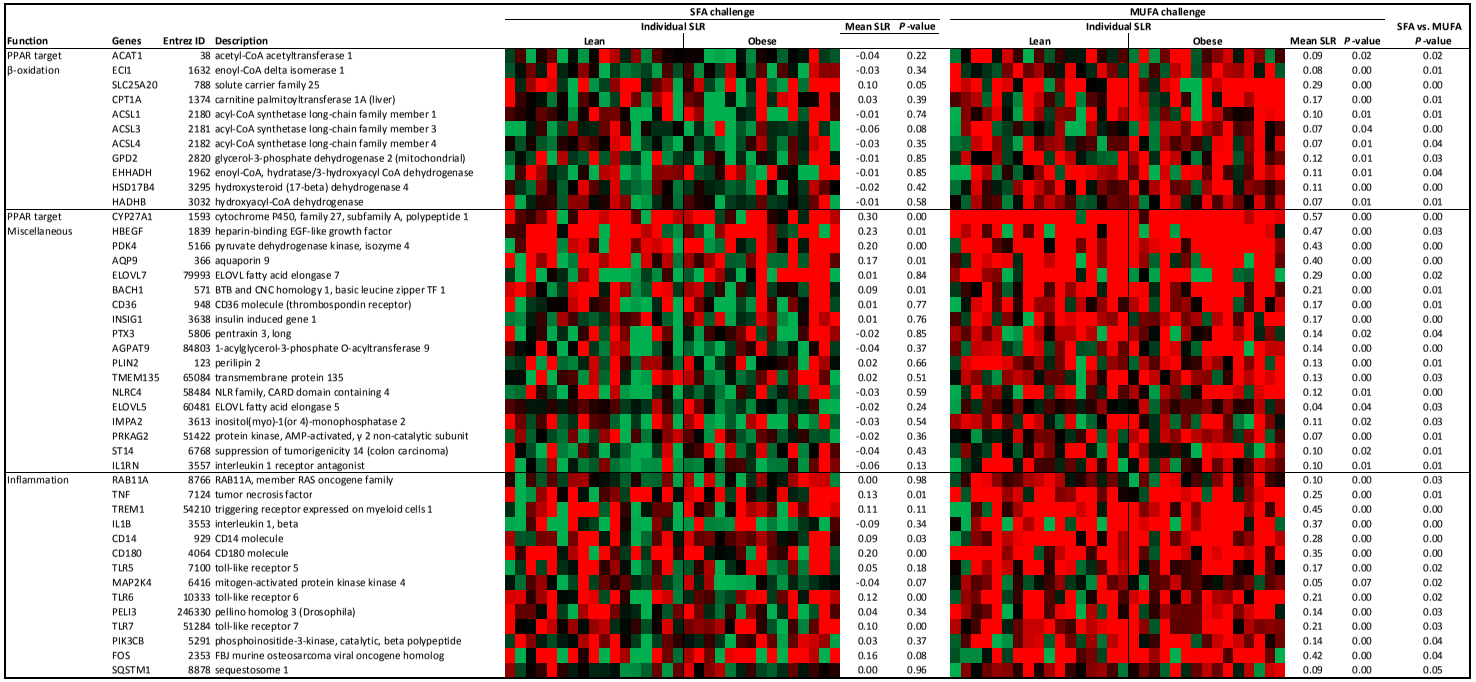
DISCUSSION

In the current study we showed that changes in PBMC gene expression profiles after a high fat challenge test are different between lean and obese subjects, with the most pronounced effect for MUFA. In both lean and obese subjects, the SFA shake decreased expression of genes involved in cholesterol biosynthesis and uptake and increased expression of genes involved in cholesterol efflux. The MUFA shake increased the expression of inflammatory genes and of PPARα target genes involved in β-oxidation.



				SFA challenge			MUFA challenge			SFA vs. MUFA
				Individual SLR	Mean SLR	*P*-value	Individual SLR	Mean SLR	*P*-value	*P*-value
Function	Genes	Entrez ID	Description	Lean Obese			Lean Obese			
Transcription factor	SREBF2	6721	sterol regulatory element binding transcription factor 2		-0.20	0.00		-0.08	0.00	0.00
Cholesterol scaveging	LDLR	3949	low density lipoprotein receptor		-0.32	0.00		0.03	0.42	0.00
Cholesterol synthesis	DHCR24	1718	24-dehydrocholesterol reductase		-0.33	0.00		-0.15	0.00	0.00
	LSS	4047	lanosterol synthase		-0.14	0.00		-0.12	0.00	0.56
	SQLE	6713	squalene epoxidase		-0.26	0.00		0.08	0.07	0.00
	DHCR7	1717	7-dehydrocholesterol reductase		-0.17	0.00		-0.04	0.22	0.02
	MVD	4597	mevalonate (diphospho) decarboxylase		-0.13	0.00		-0.08	0.02	0.17
	FDPS	2224	farnesyl diphosphate synthase		-0.10	0.00		0.00	0.90	0.05
	CYP51A1	1595	cytochrome P450, family 51, subfamily A, polypept. 1		-0.21	0.01		0.03	0.68	0.01
	HMGCS1	3157	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)		-0.09	0.01		0.10	0.00	0.00
	LBR	3930	lamin B receptor		0.06	0.01		0.14	0.00	0.01
	IDI1	3422	isopentenyl-diphosphate delta isomerase 1		-0.08	0.04		0.01	0.72	0.04
	HMGCR	3156	3-hydroxy-3-methylglutaryl-CoA reductase		-0.06	0.05		0.21	0.00	0.00
Cholesterol efflux	ABCA1	19	ATP-binding cassette, sub-family A (ABC1), member 1		0.37	0.00		0.32	0.00	0.53
	ABCG1	9619	ATP-binding cassette, sub-family G (WHITE), member 1		0.29	0.00		0.07	0.17	0.00
FA desaturase	FADS2	9415	fatty acid desaturase 2		-0.34	0.00		-0.11	0.01	0.00
	FADS1	3992	fatty acid desaturase 1		-0.28	0.00		-0.02	0.69	0.00
	SCD	6319	stearoyl-CoA desaturase (delta-9-desaturase)		-0.25	0.00		-0.02	0.73	0.00

Figure 5.5 Expression heatmap of significantly changed genes after SFA intake involved in cholesterol synthesis, cholesterol influx and efflux and of mono- and polyunsaturated fatty acid synthesis. Expression changes are indicated as individual signal-log-ratios (SLR) of t=4h versus t=0h for all subjects (*n*=32) after SFA and MUFA consumption. Subjects are split for group and ranked within these groups based on participant number. Down-regulation or up-regulation of gene expression is presented on a colour scale ranging from green (down-regulated, SLR ≤ -0.25) to red (up-regulated, SLR ≥ 0.25).



Function	Genes	Entrez ID	Description	SFA challenge: Individual SLR Lean	SFA challenge: Individual SLR Obese	SFA challenge: Mean SLR	SFA challenge: *P*-value	MUFA challenge: Individual SLR Lean	MUFA challenge: Individual SLR Obese	MUFA challenge: Mean SLR	MUFA challenge: *P*-value	SFA vs. MUFA *P*-value
PPAR target	ACAT1	38	acetyl-CoA acetyltransferase 1			-0.04	0.22			0.09	0.02	0.02
β-oxidation	ECI1	1632	enoyl-CoA delta isomerase 1			-0.03	0.34			0.08	0.00	0.01
	SLC25A20	788	solute carrier family 25			0.10	0.05			0.29	0.00	0.00
	CPT1A	1374	carnitine palmitoyltransferase 1A (liver)			0.03	0.39			0.17	0.00	0.01
	ACSL1	2180	acyl-CoA synthetase long-chain family member 1			-0.01	0.74			0.10	0.01	0.01
	ACSL3	2181	acyl-CoA synthetase long-chain family member 3			-0.06	0.08			0.07	0.04	0.00
	ACSL4	2182	acyl-CoA synthetase long-chain family member 4			-0.03	0.35			0.07	0.01	0.04
	GPD2	2820	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)			-0.01	0.85			0.12	0.01	0.03
	EHHADH	1962	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase			-0.01	0.85			0.11	0.01	0.04
	HSD17B4	3295	hydroxysteroid (17-beta) dehydrogenase 4			-0.02	0.42			0.11	0.00	0.00
	HADHB	3032	hydroxyacyl-CoA dehydrogenase			-0.01	0.58			0.07	0.01	0.01
PPAR target	CYP27A1	1593	cytochrome P450, family 27, subfamily A, polypeptide 1			0.30	0.00			0.57	0.00	0.00
Miscellaneous	HBEGF	1839	heparin-binding EGF-like growth factor			0.23	0.01			0.47	0.00	0.03
	PDK4	5166	pyruvate dehydrogenase kinase, isozyme 4			0.20	0.00			0.43	0.00	0.00
	AQP9	366	aquaporin 9			0.17	0.01			0.40	0.00	0.00
	ELOVL7	79993	ELOVL fatty acid elongase 7			0.01	0.84			0.29	0.00	0.02
	BACH1	571	BTB and CNC homology 1, basic leucine zipper TF 1			0.09	0.01			0.21	0.00	0.01
	CD36	948	CD36 molecule (thrombospondin receptor)			0.01	0.77			0.17	0.00	0.01
	INSIG1	3638	insulin induced gene 1			0.01	0.76			0.17	0.00	0.00
	PTX3	5806	pentraxin 3, long			-0.02	0.85			0.14	0.02	0.04
	AGPAT9	84803	1-acylglycerol-3-phosphate O-acyltransferase 9			-0.04	0.37			0.14	0.00	0.00
	PLIN2	123	perilipin 2			0.02	0.66			0.13	0.00	0.01
	TMEM135	65084	transmembrane protein 135			0.02	0.51			0.13	0.00	0.03
	NLRC4	58484	NLR family, CARD domain containing 4			-0.03	0.59			0.12	0.01	0.00
	ELOVL5	60481	ELOVL fatty acid elongase 5			-0.02	0.24			0.04	0.04	0.03
	IMPA2	3613	inositol(myo)-1(or 4)-monophosphatase 2			-0.03	0.54			0.11	0.02	0.03
	PRKAG2	51422	protein kinase, AMP-activated, γ 2 non-catalytic subunit			-0.02	0.36			0.07	0.00	0.01
	ST14	6768	suppression of tumorigenicity 14 (colon carcinoma)			-0.04	0.43			0.10	0.02	0.01
	IL1RN	3557	interleukin 1 receptor antagonist			-0.06	0.13			0.10	0.01	0.01
Inflammation	RAB11A	8766	RAB11A, member RAS oncogene family			0.00	0.98			0.10	0.00	0.03
	TNF	7124	tumor necrosis factor			0.13	0.01			0.25	0.00	0.01
	TREM1	54210	triggering receptor expressed on myeloid cells 1			0.11	0.11			0.45	0.00	0.00
	IL1B	3553	interleukin 1, beta			-0.09	0.34			0.37	0.00	0.00
	CD14	929	CD14 molecule			0.09	0.03			0.28	0.00	0.00
	CD180	4064	CD180 molecule			0.20	0.00			0.35	0.00	0.00
	TLR5	7100	toll-like receptor 5			0.05	0.18			0.17	0.00	0.02
	MAP2K4	6416	mitogen-activated protein kinase kinase 4			-0.04	0.07			0.05	0.07	0.02
	TLR6	10333	toll-like receptor 6			0.12	0.00			0.21	0.00	0.02
	PELI3	246330	pellino homolog 3 (Drosophila)			0.04	0.34			0.14	0.00	0.03
	TLR7	51284	toll-like receptor 7			0.10	0.00			0.21	0.00	0.03
	PIK3CB	5291	phosphoinositide-3-kinase, catalytic, beta polypeptide			0.03	0.37			0.14	0.00	0.04
	FOS	2353	FBJ murine osteosarcoma viral oncogene homolog			0.16	0.08			0.42	0.00	0.04
	SQSTM1	8878	sequestosome 1			0.00	0.96			0.09	0.00	0.05

Figure 5.6 Expression heatmap of significantly changed PPAR target genes and inflammatory genes after MUFA consumption. Expression changes are indicated as individual signal-log-ratios (SLR) for all subjects (*n*=32) after SFA and MUFA consumption. Subjects are split for group and ranked within these groups based on participant number. Down-regulation or up-regulation of gene expression is presented on a colour scale ranging from green (down-regulated, SLR ≤ -0.25) to red (up-regulated, SLR ≥ 0.25).

Whereas before the challenge, under fasting conditions, 294 genes were significantly different expressed between lean and obese subjects, the challenge increased those differences to 607 genes after the SFA challenge and 2,516 genes after the MUFA challenge. Based on this increase we conclude that the application of a high fat challenge test magnified differences in PBMC gene expression profiles between lean and obese subject. In addition, based on the higher number of genes differentially changed in obese relative to lean subjects after the MUFA compared to the SFA challenge, we hypothesise that a high MUFA challenge is more potent in inducing a transcriptional response in PBMCs than a challenge high in SFAs, hence resulting in expression regulation of a higher number of genes.

The observed differences in PBMC gene expression between lean and obese subjects at baseline and after the high fat challenges point towards a difference in haemostasis and immune function between lean and obese subjects. Firstly, the lower expression of HLA class II genes at baseline in obese relative to lean subjects may be relevant in autoimmunity and adaptive immune responses [14,15]. Secondly, SFA elicited in obese subjects a pro-thrombotic gene expression profile relative to lean subjects. Thirdly, GPI-anchored protein synthesis genes were up-regulated and various G-protein coupled receptors were down-regulated in obese relative to lean subjects after MUFA intake. GPI-anchored proteins on leukocytes regulate integrin-mediated cell adhesion [16,17] and many GPCRs are known to mediate both inflammatory and anti-inflammatory processes [18]. Changes in gene expression profiles involved in haemostasis and immune function in PBMCs after a high fat challenge may be one of the first detectable hallmarks for small disturbances in health status. Yet, it must be noted that this study was explorative and to what extend these observed changes in gene expression profiles are early markers for disease development and whether these are predictive for atherogenic processes remains to be elucidated.

SFA intake resulted in the following gene expression changes; a decrease in cholesterol uptake, a decrease in cholesterol biosynthesis and an increase in cholesterol efflux. Transcription of genes involved in cholesterol biosynthesis and uptake is largely regulated via SREBP2 activation in a cholesterol-dependent manner. An excess of intracellular cholesterol causes a down-regulation of genes involved in cholesterol biosynthesis and uptake [19]. The observed gene expression changes may therefore suggest an intracellular situation of excess cholesterol after SFA intake. In monocytes, an subpopulation of the PBMCs, intracellular cholesterol homeostasis is of crucial importance for monocyte health. Excess intracellular cholesterol will be stored as cholesteryl esters in lipid droplets, ultimately leading to macrophage activation and foam cell formation [20]. Foam cells are involved in the formation of fatty streaks during atherosclerosis development [20]. The question remains why those pathways are only affected by SFA and not by MUFA.

One might expect that the PBMCs are exposed to a higher concentration of cholesterol but the SFA in our shake consisted of cholesterol-free palm oil. However, a previous postprandial study in humans showed that the postprandial increase in VLDL, three hours after high fat meal intake, was more pronounced after SFA than after MUFA consumption [21]. As VLDL has a higher cholesterol content than chylomicrons, PBMCs may have been exposed to higher concentrations of cholesterol.

SFA intake also down regulated SREBP1a target genes encoding for desaturase enzymes [22]. SREBP1a is activated via a cholesterol-dependent manner similar to SREBP2 [23]. The decrease in gene expression of desaturase enzymes may therefore be an additional effect of a possible excess of intracellular cholesterol after SFA intake as described above. Delta 5 and delta 6 desaturases, e.g. FADS1 and FADS2, are essential for the formation of long-chain polyunsaturated fatty acids from dietary linoleic acid (LA) and α-linolenic acid (ALA). It has been proposed that a defect in the activity of delta 5 and delta 6 desaturases may decrease the formation of poly unsaturated fatty acids from LA and ALA and in turn, decrease the formation of anti-inflammatory mediators, such as prostaglandin E1 (PGE_1), prostacyclin (PGI_2), PGI_3, lipoxins and resolvins [24]. The lowering in gene expression of desaturase enzymes after repeated SFA intake may therefore, decrease the formation of such anti-inflammatory mediators.

MUFA intake increased the expression of many PPARα target genes. Previous *in vitro* and animal experiments demonstrated that unsaturated long-chain FA are better ligands of PPARs than SFA [25,26]. To our knowledge we are the first to describe such a difference between different dietary fatty acid types *in vivo* in PBMCs of human subjects. Our previous work in human PBMCs found that only a few PPARα target genes changed in expression after consumption of a high fat shake and no differences in gene expression changes were found between shakes high in SFA or PUFAs [27]. In that study, we challenged 21 healthy young men with SFA or PUFA enriched shakes of 55g of fat. The reason why we did find clear differences between shakes in the current study may be due to the larger samples size, e.g. 32 middle-aged males, the higher amount of consumed fat and/or the type of fat. The finding that many PPARα target genes that changed in expression were involved in β-oxidation is coherent with previous findings in *ex vivo* PBMC experiments from our group, that showed that one of the main targets of PPARα activation in human PBMCs is β-oxidation [13]. By up-regulation of the β-oxidation, PBMCs may reduce the amount of fatty acids available for intracellular lipid accumulation an essential process in the formation of lipid-loaded macrophages [28]. Besides the identified changes in expression of PPARα target genes, MUFA intake also up-regulated the expression of many pro-inflammatory genes. This may be explained by the fact that MUFAs, due to the unsaturated double bond, are more susceptible for oxidation, thereby inducing a pro-inflammatory response.

Saturated fatty acids are far less sensitive for oxidative stress, hence explaining the much lower pro-inflammatory response after acute intake of these FAs. In addition, we previously showed that acute consumption of high n3 PUFAs causes an increased stress and inflammatory gene expression response in PBMCs compared to SFA [27]. Long term low intake of n3 PUFAs however elicited an anti-inflammatory gene expression response in PBMCs [29]. Interestingly, in the latter study, it was also observed that long term low intake of the control, high oleic acid sunflower oil, down-regulated the expression of several genes involved in inflammation and cell adhesion. Therefore, we hypothesise that repeated moderate exposure to stress-inducing fatty acids such as MUFAs, will activate the transcriptional response, hence increasing the cellular capacity to adapt. This eventually will improve the resilience capacity of cells, leading to positive health effects on the long run.

In the current study we challenged two populations of middle-aged men with a different risk phenotype for developing CVD. Besides the higher insulin and TG levels under fasting conditions, the obese subjects were non-diabetic and not diagnosed with a long term medical condition. But, despite the apparently small differences in health status between our lean and obese subjects, changes in PBMC gene expression profiles were explicit after a challenge test, especially after the MUFA challenge. Still, individual responses showed quit some between-subject variation within the lean and obese group. Interestingly, changes in gene expression patterns of some obese individuals mimicked a more 'lean type' response. One might speculate that these obese individuals may have more healthy metabolic risk profile compared to the other obese individuals. The use of such 'gene expression fingerprints' after the application of a high fat challenge test may better reflect the resilience capacity of a person than BMI alone and may improve individual obese-related disease risk prediction in the future. By combining lean and obese subjects, we were able to examine the difference between high fat SFA in MUFA challenge on the PBMC gene expression response in 32 individuals in a cross-over design. We matched shake volume, caloric content and macronutrients which enabled us to draw conclusions about the effects of different fatty acid types. We did not discuss gene expression changes that were affected by MUFA or SFA, but were not differently affected between shakes. This study therefore presents an underestimation of the interpretation of the total transcriptional effects of SFA and MUFA challenge. However, those gene expression changes might also be induced by change in time as circadian effect on gene expression have been described [30]. The amount of 95g of fat (88 energy-%) used in this study is relatively high and does not reflect a common meal with more balanced macronutrient compositions. Yet, the high fat shake was used as a tool to determine response capacity towards a high lipid load only differing in fatty acid type. This can be more easily achieved by these high amounts of fat. By applying this challenge test

and comparing the induced gene expression changes after SFA and MUFA intake we were able to determine short term direct effects of fatty acids on transcriptional regulation. Still, these findings are temporarily and specific for the postprandial situation. Future studies are needed to elucidate how repeated moderate exposure to these fatty acids will lead to longer-term systemic whole body adaptations and improve the resilience capacity of cells and organs on the long run.

In conclusion, this study demonstrates the strengths of combining high-throughput transcriptomic tools with a high fat metabolic challenge test, to detect small disturbances in health status between subject with small differences in metabolic risk phenotype. Especially a high MUFA challenge magnified differences between lean and obese subjects. The observed gene expression changes after SFA intake suggest an intracellular situation of excess cholesterol after SFA intake, whereas the up-regulation in β-oxidation genes after MUFA intake may point towards a reduced amount of fatty acids available for intracellular lipid accumulation. These findings increase our understanding on how a SFA or MUFA challenge exert their distinct effects on stress-related and metabolic compensatory processes.

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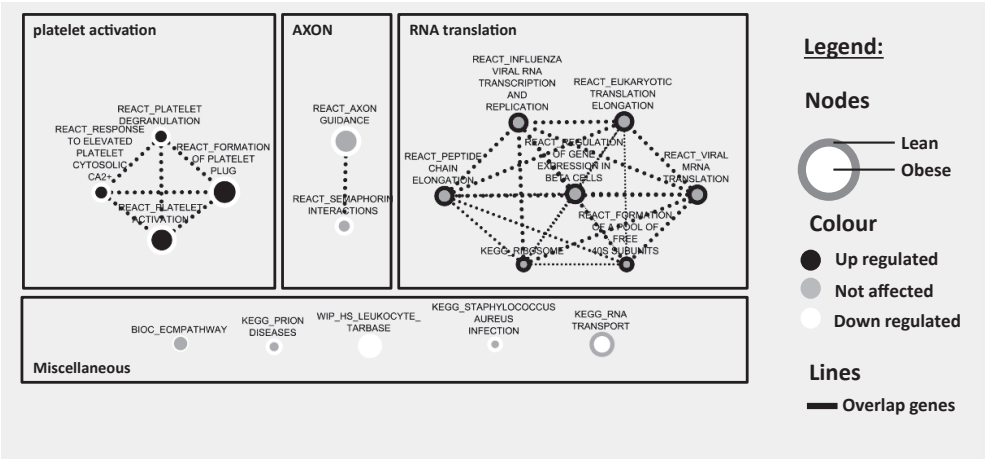
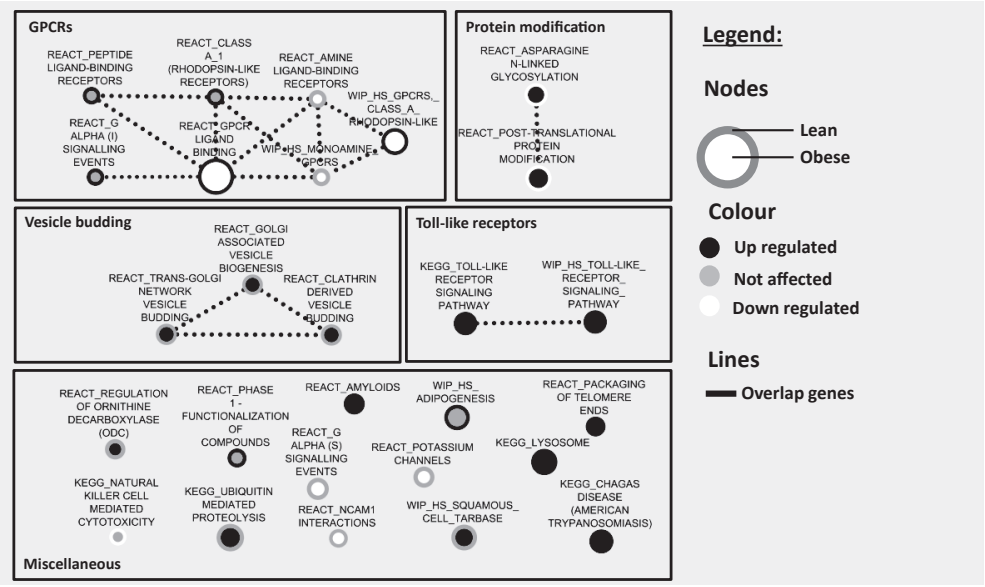
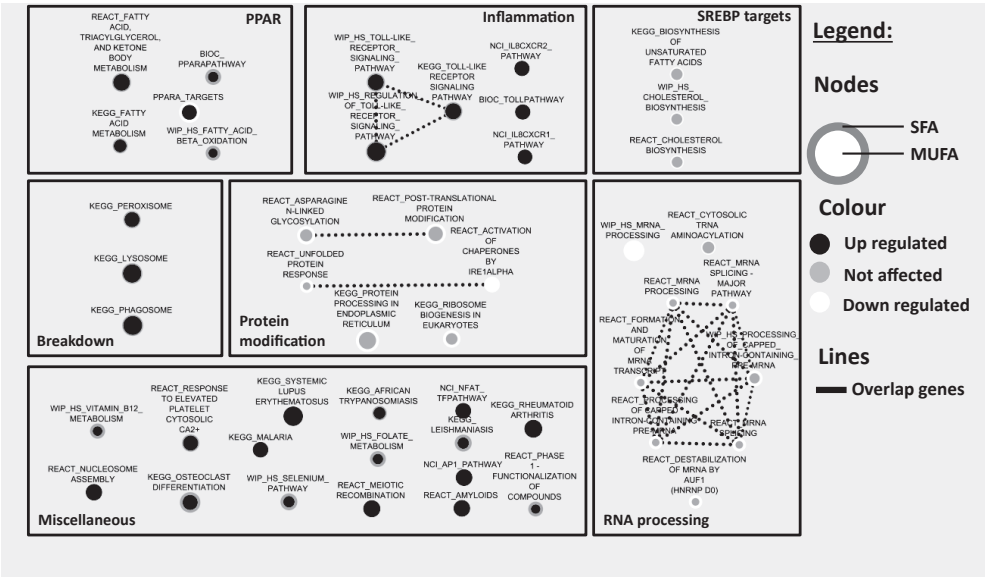
SUPPLEMENT



Supplemental Figure S5.1 Enrichment map of gene sets (FDR $Q<0.05$) significant differently changed in response to SFA challenge in obese ($n=15$) relative to the lean ($n=17$) subjects. Nodes represent gene sets, whereas dotted lines indicate overlapping genes between the gene sets (overlap cut-off: 0.9). Grey-scales in the nodes indicate if the gene set was up-regulated (black), down-regulated (white) or not affected (grey) in lean (inner node) or obese (outer node) subjects. Clusters of related gene sets were assigned a label on the basis of gene sets present in the cluster. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes database; BIOC, BioCarta Pathway Diagrams; REACT, Reactome knowledgebase; WIP_HS, WikiPathways Homo Sapiens; NCI, Nature Pathway Interaction database.



Supplemental Figure S5.2 Enrichment map of gene sets (FDR $Q<0.05$) significant differently changed in response to MUFA challenge in obese ($n=15$) relative to the lean ($n=17$) subjects. Nodes represent gene sets, whereas dotted lines indicate overlapping genes between the gene sets (overlap cut-off: 0.9). Grey-scales in the nodes indicate if the gene set was up-regulated (black), down-regulated (white) or not affected (grey) in lean (inner node) or obese (outer node) subjects. Clusters of related gene sets were assigned a label on the basis of gene sets present in the cluster. Abbreviations: GPCRs, G-protein coupled receptors; KEGG, Kyoto Encyclopedia of Genes and Genomes database; BIOC, BioCarta Pathway Diagrams; REACT, Reactome knowledgebase; WIP_HS, WikiPathways Homo Sapiens; NCI, Nature Pathway Interaction database.



Supplemental Figure S5.3 Enrichment map of gene sets (FDR $Q<0.05$) that changed significant differently between the MUFA shake and the SFA shake in the combined data of lean and obese subject (in total $n=32$). Nodes represent gene sets, whereas dotted lines indicate overlapping genes between the gene sets (overlap cut-off: 0.9). Grey-scales in the nodes indicate if the gene set was up-regulated (black), down-regulated (white) or not affected (grey) after MUFA (inner node) or SFA (outer node) intake. Clusters of related gene sets were assigned a label on the basis of gene sets present in the cluster. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes database; BIOC, BioCarta Pathway Diagrams; REACT, Reactome knowledgebase; WIP_HS, WikiPathways Homo Sapiens; NCI, Nature Pathway Interaction database.

Supplemental Table S5.1 Nutritional values of the intervention high fat shakes

Nutritional value	SFA	MUFA
Energy (kcal)	990	990
Protein (g)	10	10
Carbohydrates (g)	19	19
Fat (g)	95	95
Saturated fatty acids (g)	51	8
Monounsaturated fatty acids (g)	37	79
Polyunsaturated fatty acids (g)	6	8
ALA (g)	0	0
EPA (g)	0	0
DHA (g)	0	0
Vitamin E (mg)	165	165

Calculated using the Dutch Food Composition table (NEVO-table) using VBS KOMEET v.4 software. SFA = Saturated Fatty Acid; MUFA = Mono-Unsaturated Fatty Acid; ALA = alpha-linolenic acid (C18:3); EPA = eicosapentaenoic acid (C20:5); DHA = docosahexaenoic acid (C22:6).

Supplemental Table S5.2 Baseline and postprandial changes in metabolic parameters in plasma after high fat shake consumption

		Shake						*P*-value				
		SFA			MUFA			Main effects			Interaction effects	
		Baseline	Δ2h	Δ4h	Baseline	Δ2h	Δ4h	G	S	T	G x T	S x T
TG (mmol/L)	Lean	1.50 ± 0.50	0.64 ± 0.41	0.49 ± 0.49	1.40 ± 0.40	0.91 ± 0.49	1.41 ± 1.03	0.894	<0.001	<0.001	0.064	<0.001
	Obese	2.10 ± 1.10	0.55 ± 0.43	0.76 ± 0.65	2.20 ± 1.10	1.11 ± 0.71	2.28 ± 1.29					
FFA (mmol/L)	Lean	0.51 ± 0.22	-0.21 ± 0.20	-0.03 ± 0.23	0.50 ± 0.20	-0.11 ± 0.19	0.03 ± 0.24	0.171	<0.001	0.001	0.050	0.001
	Obese	0.49 ± 0.09	-0.19 ± 0.12	0.12 ± 0.11	0.49 ± 0.12	-0.07 ± 0.11	0.08 ± 0.15					
Insulin (mmol/L)	Lean	6.42 ± 2.59	3.08 ± 3.88	-1.67 ± 2.04	5.88 ± 3.47	2.77 ± 4.00	0.97 ± 4.10	0.010	<0.001	0.488	0.191	0.028
	Obese	12.33 ± 5.38	6.43 ± 9.48	-1.29 ± 3.49	11.79 ± 4.44	4.29 ± 5.13	1.67 ± 5.96					
Glucose (mmol/L)	Lean	5.20 ± 0.40	-0.29 ± 0.36	-0.41 ± 0.21	5.20 ± 0.40	-0.28 ± 0.31	-0.09 ± 0.29	0.047	<0.001	0.029	0.105	0.008
	Obese	5.50 ± 0.40	-0.15 ± 0.53	-0.41 ± 0.40	5.50 ± 0.50	-0.20 ± 0.32	-0.31 ± 0.31					

Data are presented as mean ± SD. G, group effect; S, shake effect; T, time effect; G x T, group x time interaction; S x T, shake x time interaction; TG, triglycerides; FFA, free fatty acids. *P*<0.05 was considered significant.

Chapter 6

Dark chocolate improves vascular function and leukocyte adherence capacity, no additional effect of high flavanol content

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Submitted for publication.

ABSTRACT

Background: Recently, the European food and safety authority improved the health claim that cocoa flavanols consumption helps to maintain endothelium-dependent vasodilation. We aimed to elaborate on this claim by investigating the effect of chocolate consumption on flow mediated dilation (FMD) and other markers of vascular health. We additionally investigated if high flavanol chocolate (HFC) consumption is preferred above normal dark chocolate (NFC) and if a higher flavanol content affects taste and motivation to consume chocolate.

Methods: In a double-blind cross-over study, we investigated the effects of acute intake and of 4-week daily HFC and NFC consumption on FMD, augmentation index (AIX), leukocyte numbers, plasma cytokines and leukocyte cell surfaces molecules in healthy overweight middle-aged men. In addition, the responsiveness to a postprandial high-fat challenge test after a 4-week HFC or NFC background was investigated. We additionally constructed sensory profiles from the HFC and NFC and collected motivation scores to eat these chocolates during the intervention.

Results: 4-week daily intake of chocolate increased FMD, decreased AIX, decreased leukocyte cell count, decreased plasma soluble adhesion molecules concentrations and decreased leukocyte adherence marker expression, with no difference between HFC and NFC. Additional flavanols did affect taste and did negatively affect motivation to consume chocolate.

Conclusions: Chocolate consumption improved FMD and AIX and initiated a less activated state of cellular adherence. Higher flavanol concentrations did not have an additional beneficial effect on vascular health, but did affect taste negatively.

INTRODUCTION

Several human intervention studies demonstrated that flavanol-enriched chocolate/cocoa intake can improve flow mediated dilatation (FMD) [1,2]. The European Food and Safety Authority (EFSA) therefore approved a health claim: 'cocoa flavanols help maintain endothelium-dependent vasodilation, which contributes to normal blood flow' [3]. Despite a clear cause and effect relationship has been established between cocoa flavanol consumption and improvement of FMD, the underlining mechanisms remain largely unknown. The major bioactive flavanol considered responsible for this beneficial effect seem (–)-epicatechins [4]. Several *in vitro* experiments in endothelial cells demonstrated that (–)-epicatechins are able to increase NO bioavailability [5,6]. Other factors known to be important in endothelial health, such as inflammation, leukocyte adherence and coagulation, might be affected by cocoa flavanols as well, but are less extensively studied *in vivo* [1]. Recent findings also demonstrated that 100 g/d intake of flavanol-rich dark chocolate for 3 days, reduced the postprandial impairment of arterial function after a glucose tolerance test [7]. This raises the question whether cocoa flavanols consumption makes subjects also more resistant to atherogenic nutritional challenges such as a high-fat meal [8-10].

The scientific evidence for the EFSA health claim that cocoa flavanols help maintain endothelium-dependent vasodilation is based on intervention studies in which healthy subjects consumed chocolate or cocoa powder beverages with an increased flavanol content. Chocolate is the predominantly consumed cocoa derived product. From a public heath perspective, increasing chocolate flavanol content might therefore be of strategic importance to improve vascular health and thereby decrease cardio vascular disease (CVD) risk at the population level. However, the question remains to what extend the increased flavanol content of chocolate will enhance the beneficial effect on vascular function and if normal dark chocolate also elicit these effects. Furthermore, flavanols have been described as astringent and bitter, increasing flavanol content of chocolate might therefore affect taste perception, hence affecting the taste [11] and motivation to consume such chocolates on a daily basis for a number of weeks [12-14].

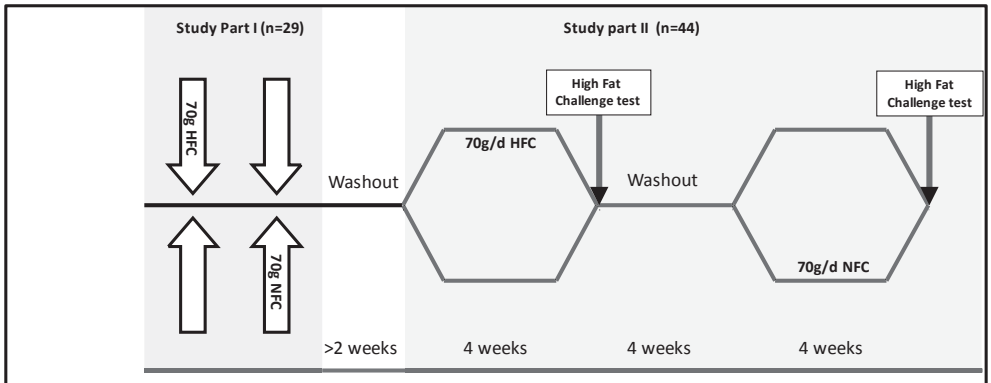
In the current study we aimed to investigate how chocolate consumption affects vascular health and if additional flavanols are necessary to elicits these effects on vascular function. We investigated the effect of acute and prolonged flavanol-enriched dark chocolate or regular dark chocolate consumption on FMD and several other markers related to vascular and endothelial function such as heamodynamics, augmentation index, leukocyte count, leukocyte cell surfaces activation markers and plasma cytokines involved in cellular adherence, coagulation and inflammation. We additively investigated if prolonged daily intake of high flavanol chocolate will alter vascular resilience capacity to a high fat challenge test. Our additional aim was to

investigate if increased flavanol content in chocolate affects the sensory profile of chocolate, the appreciation and the motivation to consume these chocolates for a longer period.

METHODS

Subjects

Healthy overweight (predefined BMI 25–32 kg/m^2) male subjects between 45–70 years old were recruited. All subjects were non-smoking, normoglycemic (WHO criteria) and not diagnosed with any long-term medical condition or high blood pressure (systolic>160 mmHg and/or diastolic>100 mmHg). Furthermore, subjects were not allowed to use medication or to take food supplements known to interfere with glucose homeostasis, blood pressure (BP), coagulation or inflammation. All subjects gave written informed consent and the study was approved by the Medical Ethics Committee of Wageningen University.



	Visit 1	Visit 2	Before 4-weeks	After 4-weeks	Before 4-weeks	After 4-weeks
Fasting						
T= 0h	a,b,c,d,f,h	a,b,c,d,f,h	a,b,c,d,e,h	a,b,c,d,e,g,h	a,b,c,d,e,h	a,b,c,d,e,g,h
Postprandial						
T = 1,5h				b		b
T = 2h	a,b,c,d,f,h	a,b,c,d,f,h				
T = 3h				a,b,c,d,e		a,b,c,d,e

Figure 6.1 Study design; Part I; cross-over acute intervention study. Samples were collected at baseline and 2 hours postprandially after chocolate intake. Part II; cross-over 4-week intervention study. Fasting samples were collected before and after the intervention. A high fat postprandial challenge test was implemented at the end of both intervention periods. Additional samples were collected 1.5 hours and 3 hours postprandially. Determined study outcomes: a = vascular measures; b = plasma metabolic markers; c = plasma cytokines; d = differentiated blood cell count; e = leukocyte cell surface markers; f = plasma flavanols; g = urinary flavanols, h = plasma ALAT/ASAT.

Study design

The study consisted of two study parts (**Figure 6.1**). Part I comprised an acute intervention study in which the postprandial effects of either high flavanol chocolate (HFC) or normal flavanol chocolate (NFC) consumption on vascular health was investigated. In part II, the effects of daily intake of HFC or NFC for 4 weeks on vascular health were investigated. In addition, the responsiveness to a postprandial high fat challenge test after a 4 week chocolate background of either HFC or NFC was investigated. Randomization was performed for both study parts by an independent research assistant using a computer-generated table. For study part II we constructed 25 blocks with a size of 2. Researchers as well as participants were blinded to randomization until after data analysis. Sample size was calculated with assumption that within-patient standard deviation of the FMD measurement was 2.13. Effect size between treatments were estimated 2.1 units FMD% for study part I and 1.6 units FMD% for study part II [15,16]. This study was performed at the Wageningen University from January until July 2011, according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO).

Part I: Acute effects of chocolate consumption

Part I comprised a double blind randomized cross-over acute intervention study, in which 29 participants visited the university two times. On each study day, subjects consumed 70g HFC or NFC. Vascular measurements, blood count, plasma cytokines, plasma (–)-epicatechins, plasma metabolic markers and plasma liver function markers were determined at baseline and 2 hours after chocolate consumption. This timeframe was chosen, because effects of flavanol consumption on vascular function are most pronounced two hours after consumption [17]. A minimum of one-week washout period was established between the two study days. All participants of part I also entered part II.

Part II: Long term effects of 4 week chocolate consumption

An additional 15 volunteers were recruited to start up with a total of 44 participants for study part II. Part II comprised a double blind randomized cross-over 4-week intervention study in which participants consumed 70g of chocolate daily in two 4-week periods, randomly assigned to either HFC or NFC, with a 4-week washout period between periods. Chocolates were dispensed in bars of 35g; one bar was consumed in the afternoon and one in the evening. Fasting vascular measurements, blood count, plasma cytokines, leukocyte activation markers, plasma metabolic markers and plasma liver function markers were determined before and after both intervention periods. Flavanol concentrations in 24h-urine samples were determined at the end of each intervention period as a marker of compliance.

After fasting measures at the end of each intervention period, participants received a high-fat (HF) challenge test containing 95g of fat, high in MUFA. Additional postprandial measures of vascular function, blood count, leukocyte activation markers, plasma cytokines and plasma metabolic markers were determined three hours after shake consumption. An additional blood sample was taken 1.5 hours after shake consumption, in order to characterize the plasma insulin, glucose, triglycerides (TG) and free fatty acid (FFA) response in better detail.

Chocolates were distributed weekly and extra bars were distributed. Participants were asked to bring back empty packages and left-over chocolates, and to keep a diary to record at what time bars were eaten. A personal dietary consult was planned before the intervention to prevent weight gain. During this consult, participants were advised to refrain from certain energy dense food products from their normal diet. If weight gain or loss >1kg was recorded during their weekly visit, an additional consult was scheduled to correct. Participants were not allowed to consume other chocolates or to consume more than two cups of tea, two glasses of red wine and to eat one apple per day, which are important sources of dietary flavanols. On the day prior to each testing day, subjects consumed a standardized low-fat evening meal, were refrained from alcohol or strenuous exercise and were not allowed to eat or drink anything except water after 08.00pm.

Chocolate flavanol content

HFC and NFC were provided by Barry Callebaut® and had both a cocoa mass of 58.0%. According to the manufacturer, the HFC (ACTICOA® chocolate) contained 2.2% flavanols of which, based on estimation from previous analysis, 10.5% were assumed to be (–)-epicatechins. The NFC was commonly produced chocolate with a similar macronutrient composition, caloric content, and caffeine and theobromine concentrations. According to the manufacturer, NFC contained 0.2% flavanols of which, based on estimation, 14% were (–)-epicatechins. A dose of 70g HFC or NFC would therefore represent respectively 1,540mg and 140mg flavanols of which 162mg and 27mg were (–)-epicatechin respectively. To ensure that these concentrations were valid for our produced chocolates, we asked the manufacturer to reanalyses our samples. Flavanol concentrations deviated from the original product sheet as 70g of HFC or NFC comprised a flavanol content of respectively 1,610mg and 490mg which is estimated to represents 169mg and 96mg of (–)-epicatechin respectively. As these concentrations substantially deviated from the original product sheet, we decided to analyse flavanol content independently from the manufacturer, based on a method previously described [18,19]. This analysis showed that 70g HFC or NFC comprised 1,078mg and 259mg flavanols of which 349mg

and 97mg (–)-epicatechin respectively. Intended and self-analysed flavanol concentrations of the chocolates are summarized in **Table 6.1**.

Table 6.1 Chocolate flavanol composition

	Expected values from manufacturer		Re-analysed values from manufacturer		Self-analysed values	
	NFC	HFC	NFC	HFC	NFC	HFC
Total flavanols (%)	0.2	2.2	0.7	2.3	0.4	1.5
Total flavanols (mg)	140	1,540	490	1,610	259	1,078
Catechin (mg)	9*	54*	32*	56*	22	45
(–)-Epicatechin (mg)	27*	162*	96*	169*	119	394

All values are calculated for a dose of 70g chocolate. NFC, normal flavanol chocolate; HFC, high flavanol chocolate. * based on estimation by the manufacturer.

High fat challenge test

The high-fat challenge test consisted of a milkshake containing 95g of fat, high in mono-unsaturated fatty acids (MUFA). Previous work demonstrated that vascular responses after a high-fat MUFA challenge were more pronounced if compared to challenges high in saturated fatty acids (SFA) or n3 polyunsaturated fatty acids (PUFA) [34]. The shake contained low-fat yoghurt, low-fat milk, strawberry flavour, 7.5g of sugar and 95g of high-oleic acid sunflower oil (Aldoc BV, Schiedam, The Netherlands). Nutritional values of the shake were calculated based on the food composition table (NEVO 2007) and presented in **Supplemental Table S6.1**.

Measures of vascular function

All vascular measurements were performed after 10 min of rest. Brachial systolic BP (SBP), diastolic BP (DBP) and heart rate (HR) were assessed automatically (DINAMAP® PRO 100) for 10 minutes with a 3 min interval. Central systolic blood pressure (CSBP) and the heart rate corrected augmentation index (AIX), a measure of wave reflection and arterial stiffness [20], were assessed by pulse wave analysis of the radial artery (SphygmoCor®CP System, ATcor Medical) as described previously [34]. FMD was assessed (Picus, ART.LAB v2.1, Esaote benelux bv.) as previously described [21]. FMD vessel recordings were analysed and judged by a blinded technician. One subject in study part one and 4 subjects in part II were removed from the analysis due to bad recordings.

Blood markers of inflammation and vascular heath

Plasma cytokine concentrations were analysed on preformatted arrays (Meso Scale Diagnostics, LLC) on a SECTOR Imager 2400 reader (Meso Scale Diagnostics, LLC) and plasma vWF concentrations were measured by a custom ELISA as previously described [22]. A differentiated leukocyte cell count was performed by a hospital laboratory (ZGV, Ede, the Netherlands).

Expression of leukocyte cell surface markers was determined by flow cytometry (FACSCanto™II, Becton-Dickinson). Markers were selected based on the involvement in activation and adherence to the endothelium. In short, whole blood was stained with fluorescent labelled monoclonal antibodies (MAbs) (Becton-Dickinson, The Netherlands); FITC conjugated CD66b, APC-cy7 conjugated CD11b, APC conjugated CD62l, PE conjugated CD11c and PerCP-cy5.5 conjugated CD45. Cell populations were identified by scatter properties and CD45 expression (FACSDiva™ Software v6.1.2). Non-specific binding was ruled out by using isotype matched MAbs. Expression of cell surface markers is expressed as Mean Florescence Intensity (MFI) in arbitrary units (AU).

Markers of compliance, metabolism and liver function

Plasma (–)-epicatechins and 24-hour urinary flavanol concentrations were determined by HPLC as previously described [23]. Plasma triglycerides (TG), glucose, insulin and free fatty acids (FFA) concentrations and plasma concentrations of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were analysed by a hospital laboratory (SHO, Velp, the Netherlands).

Taste and motivation to eat chocolate

During the intervention subjects filled out questionnaires about the motivation to consume chocolate on days 1, 7, 14, 21, and 27. Questions were asked about sensory boredom; desire to eat something sweet, desire to eat something savoury, and desire to eat chocolate, and questions on the liking and wanting of the chocolate; i.e. pleasantness and desire finish. Questions on sensory boredom were answered before consumption, questions on wanting and liking of the chocolate after consumption of one bite. All questions were answered on a 9-point scale.

In a sensory panel test separate from the main study, 31 untrained subjects (aged 25±7y; BMI 22.1±2.8 kg/m^2) evaluated the analytical sensory properties of the HFC and NFC on a 9-point scale. Samples were offered in pieces of 5–7g at room temperature (±20°C) in a randomized order. Between samples, subjects cleaned their palate and tongue with crackers and water. The

20 attributes in the sensory test were generated by means of a panel of 6 independent subjects that had experience with sensory testing. Next to the intervention chocolates, two commercially available chocolates with a comparable cocoa content Verkade Puur (Koninklijke Verkade, Zaandam, The Netherlands) and Delicata Puur (Albert Heijn, Zaandam, The Netherlands), were used for attribute generation and descriptive analyses.

Statistical analysis

Statistical analysis was performed by linear mixed models for repeated measures (PASW statistics 18.0.3). Study outcomes of study part I were analysed by using 'treatment' [CFH of CLF], 'time' [T0 or T2 hours] and 'treatment*time' as fixed effects. Study outcomes of study part II were analysed using 'treatment' [CFH of CLF], 'time' [before or after 4-week intervention] and 'treatment*time' as fixed effects. Study outcomes of the high fat challenge test were analysed by using 'treatment' [CFH or CLF background], 'time' [T0 or T3 hours] and 'treatment*time' as fixed effects. Baseline was included in the model as covariate for all study parts.

RESULTS

Subject characteristics intervention study

Three participants dropped out or were excluded during study part II, one due to medical reasons not related to the study, one due to disliking of the chocolate and one due to failure to adhere to the treatment. Baseline characteristics of the 29 participants that finished part I and the 41 participants that finished part II are listed in **Supplemental Table S6.2**.

Part I: Acute chocolate ingestion

Table 6.2 lists mean baseline and postprandial changes after HFC and NFC ingestion on plasma (–)-epicatechin concentrations, vascular function, leukocyte count, plasma cytokines and metabolic parameters. Plasma (–)-epicatechin concentrations increased 2 hours after chocolate intake, with a 5 fold more pronounced increase after HFC compared to NFC ingestion.

Chocolate ingestion decreased AIX, with no difference in postprandial response between HFC or NFC. Other measures of vascular function, such as FMD, were not affected 2 hours after chocolate ingestion. Haematocrit and number of thrombocytes, lymphocytes, monocytes and neutrophils were increased 2 hours after chocolate ingestion. The increase in erythrocytes after chocolate ingestion was more pronounced after HFC consumption if compared to NFC

Table 6.2 Mean baseline and postprandial changes after HFC and NFC ingestion on markers of vascular health (study part I)

	Treatment	Time		*P*-value		
		Baseline	Δ2h	Treat-ment	Time	Interaction Time*Treatment
Plasma epicatechin	NFC	0.00 ± 0.00	0.13 ± 0.04	<0.001	<0.001	<0.001
	HFC	0.00 ± 0.00	0.67 ± 0.23			
Vascular function						
SBP (mmHg)	NFC	128 ± 13	2 ± 7	NS	NS	NS
	HFC	130 ± 12	0 ± 7			
DBP (mmHg)	NFC	80 ± 7	0 ± 3	NS	NS	NS
	HFC	79 ± 6	0 ± 3			
HR (BPM)	NFC	60 ± 8	0 ± 5	NS	NS	NS
	HFC	58 ± 7	2 ± 5			
AIX (%)	NFC	21 ± 5	-5 ± 5	NS	<0.001	NS
	HFC	21 ± 5	-5 ± 4			
FMD (%)	NFC	5.5 ± 2.2	0.9 ± 2.35	NS	NS	NS
	HFC	4.6 ± 2.4	-0.40 ± 3.76			
Leukocyte count						
Haematocrit (l/l)	NFC	0.42 ± 0.02	0.01 ± 0.01	NS	<0.001	NS
	HFC	0.42 ± 0.02	0.01 ± 0.01			
Erythrocytes (#/pl)	NFC	4.71 ± 0.37	0.11 ± 0.10	NS	<0.001	NS
	HFC	4.71 ± 0.38	0.13 ± 0.07			
Thrombocytes (#/nl)	NFC	201 ± 25	6 ± 6	NS	<0.001	NS
	HFC	201 ± 28	7 ± 7			
Leukocytes (#/nl)	NFC	5.1 ± 1.1	0.7 ± 0.6	NS	<0.001	NS
	HFC	5.0 ± 1.2	0.7 ± 0.7			
Lymphocytes (#/nl)	NFC	1.49 ± 0.34	0.16 ± 0.29	NS	<0.001	NS
	HFC	1.51 ± 0.42	0.15 ± 0.24			
Monocytes (#/nl)	NFC	0.50 ± 0.12	-0.02 ± 0.07	NS	NS	NS
	HFC	0.49 ± 0.15	0.01 ± 0.08			
Neutrophils (#/nl)	NFC	2.9 ± 0.9	0.6 ± 0.5	NS	<0.001	NS
	HFC	2.8 ± 0.9	0.6 ± 0.8			
Plasma cytokines						
CRP (µg/ml)	NFC	1.61 ± 1.32	0.10 ± 0.36	NS	NS	NS
	HFC	2.01 ± 2.30	0.02 ± 0.11			
SAA (µg/ml)	NFC	3.14 ± 8.43	-0.23 ± 1.32	NS	NS	NS
	HFC	2.88 ± 6.35	-0.06 ± 0.20			
sICAM1 (ng/ml)	NFC	213 ± 39	8 ± 11	0.013	0.015	0.007
	HFC	220 ± 43	0 ± 13			

Table 6.2 continues on next page

Table 6.2 *Continued*

	Treatment	Time		*P*-value		
		Baseline	Δ2h	Treat-ment	Time	Interaction Time*Treatment
sVCAM1 (ng/ml)	NFC	329 ± 57	3 ± 27	NS	NS	NS
	HFC	346 ± 67	2 ± 37			
E-selectin (ng/ml)	NFC	13.7 ± 4.3	-0.2 ± 2	NS	NS	NS
	HFC	13.9 ± 3.8	-0.1 ± 1.4			
P-selectin (ng/ml)	NFC	124 ± 28	-0.2 ± 16.5	NS	NS	NS
	HFC	126 ± 27	-0.8 ± 19.7			
sICAM3 (ng/ml)	NFC	2.0 ± 0.5	0 ± 0.1	NS	0.011	NS
	HFC	2.0 ± 0.5	0 ± 0.1			
Thrombomodulin (ng/ml)	NFC	3.5 ± 0.6	0 ± 0.2	NS	NS	NS
	HFC	3.5 ± 0.7	0 ± 0.2			
IL-1β (pg/ml)	NFC	0.91 ± 0.68	0.06 ± 0.14	NS	0.001	NS
	HFC	0.89 ± 0.69	0.07 ± 0.11			
IL-6 (pg/ml)	NFC	1.5 ± 0.6	-0.1 ± 0.4	NS	0.006	NS
	HFC	1.5 ± 0.7	-0.1 ± 0.3			
IL-8 (pg/ml)	NFC	4.7 ± 1.3	0.1 ± 0.6	NS	NS	NS
	HFC	4.8 ± 1.6	0.2 ± 0.8			
TNFα (pg/ml)	NFC	10.3 ± 4.0	0 ± 0.8	NS	NS	NS
	HFC	10.4 ± 3.9	0 ± 0.9			
vWF (%)	NFC	130 ± 40	1.6 ± 8.7	NS	0.010	NS
	HFC	134 ± 46	6.8 ± 14.8			
Plasma metabolic parameters						
Glucose (mmol/l)	NFC	5.7 ± 0.5	0.2 ± 0.6	NS	0.001	NS
	HFC	5.8 ± 0.5	0.3 ± 0.6			
TG (mmol/l)	NFC	1.3 ± 0.5	0.2 ± 0.2	NS	<0.001	NS
	HFC	1.2 ± 0.4	0.2 ± 0.1			
FFA (mmol/l)	NFC	0.44 ± 0.12	-0.06 ± 0.12	NS	<0.001	NS
	HFC	0.46 ± 0.10	-0.06 ± 0.12			
Insulin (mmol/l)	NFC	7.1 ± 3.7	8.3 ± 6.4	0.046	<0.001	0.042
	HFC	6.4 ± 2.3	11.8 ± 7.7			
Other						
ALAT (U/l)	NFC	21 ± 7	2 ± 2	NS	<0.001	NS
	HFC	21 ± 8	1 ± 1			
ASAT (U/l)	NFC	33 ± 5	2 ± 2	0.375	<0.001	0.351
	HFC	34 ± 5	2 ± 3			

Values are mean ±SD, *n*=29. NFC, normal flavanol chocolate; HFC, high flavanol chocolate; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; AIX, heart rate corrected augmentation index; FMD, flow mediated dilation; NS, not significant; TG, triglycerides; FFA, free fatty acids.

consumption. Plasma sICAM1 concentrations were increased after HFC ingestion only. Plasma sICAM3, IL-1β and vWF concentrations were increased and plasma IL-6 concentration was decreased 2 hours after chocolate intake, with no difference between HFC or NFC. Chocolate ingestion increased plasma glucose and TG and decreased plasma FFA. Insulin was increased 2 hours after ingestion of both chocolates, with a more pronounced increase after HFC compared to NFC.

Part II: 4-week chocolate intervention

Participants did not gain or lose weight during the intervention period (P=0.565). Compliance to the study protocol was good, as reflected by the consumed bars and urinary flavanol concentrations. In total 4,930 out of 4,952 bars were consumed during the intervention, 99.3% of the HFC bars and 99.7% of the NFC bars. At the end of the intervention, urinary flavanol concentration were 1.34 ± 0.99μg/ml after the NFC period and 8.75 ± 7.79μg/ml after the HFC period (P<0.001).

Table 6.3 lists the effects of 4-week daily consumption of HFC and NFC on vascular measurements, leukocyte count, plasma cytokines, leukocyte activation markers and markers of liver function in the fasting state, including the postprandial outcomes after the HF challenge test. 4-weeks chocolate consumption increased fasting FMD by 1% point and decreased fasting AIX by 1% point on average, with no difference in response between HFC and NFC. Both FMD and AIX values returned to baseline after washout period (**Figure 6.2**). 4-week chocolate consumption additionally decreased number of leukocytes and decreased plasma sICAM1 and sICAM3 concentrations in the fating state, with no difference between HFC and NFC. Plasma TNFα concentrations were decreased after HFC and increased after NFC intervention. 4-week chocolate consumption also lowered expression of several cell surface molecules on leukocytes; on lymphocytes, CD62L and CD11b expression decreased by 4.8% and 5.0% on average. On monocytes, CD62l expression decreased by 17.9% on average. On neutrophils, CD66b and CD11c expression decreased by 3.9% and 10.2% on average. No significant differences on leukocyte cell surface molecule expression were observed between the HFC and NFC intervention. Chocolate consumption did not affect fasting TG, insulin, ALAT or ASAT. A minor but significant increase of 0.1mmol/l in fasting plasma glucose and 0.3mmol/l in plasma FFA was observed after 4-week chocolate intervention, with no difference in response between HFC and NFC (**Table 6.4**).

Table 6.3 Mean baseline and changes in fasting vascular health outcomes before and after 4-weeks HFC and NFC consumption (study part II), including postprandial outcomes after the high-fat challenge test at the end of each intervention

		Time			*P*-value					
					4 week intervention			High-fat challenge		
	Treat-ment	0 weeks/0h	Δ intervention 4 weeks/0h	Δ HF challenge 4 weeks/3h	Treat-ment	Time (w)	Interac-tion	Treatment background	Time (h)	Interac-tion
Vascular function										
SBP (mmHg)	NFC	128 ± 14	0 ± 9	-2 ± 6	NS	NS	NS	NS	NS	NS
	HFC	128 ± 15	-1 ± 8	-1 ± 7						
DBP (mmHg)	NFC	79 ± 7	0 ± 5	-2 ±4	NS	NS	NS	NS	<0.001	NS
	HFC	79 ± 6	-1 ± 4	-1 ± 4						
HR (BPM)	NFC	59 ± 8	1 ± 5	2 ± 4	NS	0.049	NS	NS	0.004	NS
	HFC	59 ± 6	1 ± 5	1 ± 6						
AIX (%)	NFC	21 ± 7	-1 ± 4	-4 ± 3	NS	0.024	NS	NS	<0.001	NS
	HFC	21 ± 6	-1 ± 3	-3 ± 4						
FMD (%)	NFC	4.9 ± 2.4	0.9 ± 3.6	-1.4 ± 3.8	NS	0.010	NS	NS	<0.001	NS
	HFC	4.7 ± 2.7	1.2 ± 3.7	-1.8 ± 2.7						
Leukocyte count										
Haematocrit (l/l)	NFC	0.43 ± 0.02	0.00 ± 0.01	0.01 ± 0.01	NS	NS	NS	NS	<0.001	NS
	HFC	0.43 ± 0.02	0.00 ± 0.02	0.01 ± 0.01						
Erythrocytes (#/pl)	NFC	4.80 ± 0.36	-0.01 ± 0.17	0.17 ± 0.12	NS	NS	NS	NS	<0.001	NS
	HFC	4.74 ± 0.33	0.05 ± 0.20	0.13 ± 0.08						
Thrombocytes (#/nl)	NFC	197 ± 28	-1 ± 14	11.98 ± 9.90	NS	NS	NS	NS	<0.001	NS
	HFC	195 ± 30	-1 ± 22	10.54 ± 7.24						

Table 6.3 continues on next page.

Table 6.3 *Continued*

		Time			*P*-value					
					4 week intervention			Hig-fat challenge		
	Treat-ment	0 weeks/0h	Δ intervention 4 weeks/0h	Δ HF challenge 4 weeks/3h	Treat-ment	Time (w)	Interac-tion	Treatment background	Time (h)	Interac-tion
Leukocytes (#/nl)	NFC	5.1 ± 1.4	-0.3 ± 0.8	0.73 ± 0.55	NS	0.023	NS	NS	<0.001	NS
	HFC	5.1 ± 1.5	-0.1 ± 0.9	0.77 ± 0.78						
Lymphocytes (#/nl)	NFC	1.48 ± 0.42	-0.05 ± 0.19	0.10 ± 0.22	NS	NS	NS	NS	<0.001	NS
	HFC	1.47 ± 0.44	-0.03 ± 0.20	0.12 ± 0.23						
Monocytes (#/nl)	NFC	0.49 ± 0.13	-0.03 ± 0.09	0.04 ± 0.08	NS	NS	NS	NS	<0.001	NS
	HFC	0.48 ± 0.13	-0.01 ± 0.09	0.03 ± 0.07						
Neutrophils (#/nl)	NFC	3.0 ± 1.1	-0.3 ± 0.7	0.10 ± 0.22	NS	NS	NS	NS	<0.001	NS
	HFC	2.9 ± 1.2	-0.0 ± 0.8	0.12 ± 0.23						
Plasma cytokines										
CRP (µg/ml)	NFC	2.51 ± 4.98	-0.44 ± 2.13	0.09 ± 0.32	NS	NS	NS	NS	NS	NS
	HFC	3.19 ± 5.49	0.37 ± 7.12	0.21 ± 1.05						
SAA (µg/ml)	NFC	2.10 ± 2.39	-0.10 ± 1.55	-0.09 ± 0.22	NS	NS	NS	NS	NS	NS
	HFC	3.12 ± 4.33	1.53 ± 8.91	-0.23 ± 1.46						
sICAM1 (ng/ml)	NFC	211 ± 64	-7 ± 14	6 ± 11	NS	0.001	NS	NS	<0.001	NS
	HFC	209 ± 62	-5 ± 17	7 ± 11						
sVCAM1 (ng/ml)	NFC	315 ± 46	-1 ± 29	11 ± 22	NS	NS	NS	NS	<0.001	NS
	HFC	316 ± 53	0 ± 34	11 ± 21						
E-selectin (ng/ml)	NFC	13.7 ± 4.8	0.1 ± 2.2	-0.1 ± 1.5	NS	NS	NS	NS	NS	NS
	HFC	13.8 ± 5.3	0.2 ± 1.7	-0.3 ± 1.3						
P-selectin (ng/ml)	NFC	123 ± 29	-3.6 ± 16.9	4.4 ± 14.6	NS	NS	NS	NS	0.020	NS
	HFC	124 ± 24	-2.7 ± 11.7	3.7 ± 16.9						

sICAM3 (ng/ml)	NFC	1.8 ± 0.4	-0.04 ± 0.14	0.04 ± 0.11	NS	0.023	NS	NS	<0.001	NS
	HFC	1.8 ± 0.4	-0.03 ± 0.12	0.05 ± 0.1						
TM (ng/ml)	NFC	3.3 ± 0.6	0.0 ± 0.2	0.0 ± 0.2	NS	NS	NS	NS	NS	NS
	HFC	3.4 ± 0.6	-0.1 ± 0.2	0.0 ± 0.1						
IL-1β (pg/ml)	NFC	0.65 ± 0.49	-0.01 ± 0.11	0.02 ± 0.09	NS	NS	NS	NS	NS	NS
	HFC	0.65 ± 0.47	-0.03 ± 0.10	0.03 ± 0.11						
IL-6 (pg/ml)	NFC	1.4 ± 0.8	-0.1 ± 0.6	-0.1 ± 0.2	NS	NS	NS	NS	0.036	NS
	HFC	1.4 ± 0.7	0.0 ± 1.1	-0.2 ± 0.9						
IL-8 (pg/ml)	NFC	4.6 ± 1.1	0.2 ± 0.6	0.7 ± 1.1	NS	NS	NS	NS	<0.001	NS
	HFC	4.7 ± 1.5	0.0 ± 1.1	0.6 ± 0.6						
TNFα (pg/ml)	NFC	10.0 ± 3.1	0.3 ± 1.0	0.1 ± 0.7	0.012	NS	0.012	NS	0.034	NS
	HFC	10.3 ± 3.3	-0.3 ± 1.1	0.2 ± 0.6						
vWF (%)	NFC	120 ± 39	1 ± 19	-3 ± 15	NS	NS	NS	NS	NS	NS
	HFC	124 ± 43	-1 ± 14	1 ± 14						
Leukocyte activation										
Lymphocyte										
CD11c (AU)	NFC	0.29 ± 0.17	-0.00 ± 0.01	0.03 ± 0.10	NS	NS	NS	NS	0.003	NS
	HFC	0.29 ± 0.16	-0.00 ± 0.01	0.03 ± 0.09						
CD62L (AU)	NFC	2.79 ± 0.83	-0.10 ± 0.64	0.09 ± 0.68	NS	0.047	NS	NS	NS	NS
	HFC	2.90 ± 0.92	-0.17 ± 0.57	0.03 ± 0.57						
CD11b (AU)	NFC	0.25 ± 0.09	-0.01 ± 0.05	0.03 ± 0.05	NS	0.012	NS	NS	<0.001	NS
	HFC	0.27 ± 0.09	-0.02 ± 0.05	0.03 ± 0.06						
Monocyte										
CD11c (AU)	NFC	2.83 ± 0.80	-0.09 ± 0.71	0.52 ± 1.63	NS	NS	NS	NS	0.013	NS
	HFC	3.06 ± 1.76	-0.30 ± 1.43	0.20 ± 0.61						

Table 6.3 continues on next page.

Table 6.3 *Continued*

		Time			*P*-value					
					4 week intervention			High-fat challenge		
	Treat-ment	0 weeks/0h	Δ intervention 4 weeks/0h	Δ HF challenge 4 weeks/3h	Treat-ment	Time (w)	Interac-tion	Treatment background	Time (h)	Interac-tion
CD62L (AU)	NFC	2.15 ± 0.87	-0.35 ± 0.74	-0.16 ± 0.53	NS	<0.001	NS	NS	0.025	NS
	HFC	2.23 ± 0.93	-0.43 ± 0.78	-0.07 ± 0.36						
CD11b (AU)	NFC	1.25 ± 0.30	-0.04 ± 0.23	0.25 ± 0.58	NS	NS	NS	NS	<0.001	NS
	HFC	1.36 ± 0.57	-0.15 ± 0.54	0.19 ± 0.24						
Neutrophil										
CD66b (AU)	NFC	2.63 ± 0.67	-0.06 ± 0.37	0.02 ± 0.53	NS	0.004	NS	NS	NS	NS
	HFC	2.72 ± 0.62	-0.14 ± 0.35	-0.01 ± 0.21						
CD11c (AU)	NFC	0.64 ± 0.17	-0.05 ± 0.15	0.05 ± 0.33	NS	0.017	NS	NS	NS	NS
	HFC	0.70 ± 0.34	-0.09 ± 0.32	-0.01 ± 0.11						
CD62L (AU)	NFC	3.85 ± 0.97	0.01 ± 1.03	0.33 ± 0.96	NS	NS	NS	NS	<0.005	NS
	HFC	4.08 ± 0.91	-0.23 ± 0.89	0.22 ± 0.77						
CD11b (AU)	NFC	1.04 ± 0.24	-0.02 ± 0.18	0.11 ± 0.48	NS	NS	NS	NS	0.016	NS
	HFC	1.15 ± 0.45	-0.14 ± 0.48	0.09 ± 0.16						
Markers of liver function										
ALAT (U/l)	NFC	21 ± 7	0 ± 3	-	NS	NS	NS	-	-	-
	HFC	20 ± 6	-1 ± 4	-						
ASAT (U/l)	NFC	33 ± 5	0 ±5	-	NS	NS	NS	-	-	-
	HFC	33 ± 6	1 ±5	-						

Values are mean ±SD, *n*=41. NFC, normal flavanol chocolate; HFC, high flavanol chocolate; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; AIX, heart rate corrected augmentation index; FMD, flow mediated dilation; AU, arbitrary units; TM, thrombomodulin; NS, not significant.

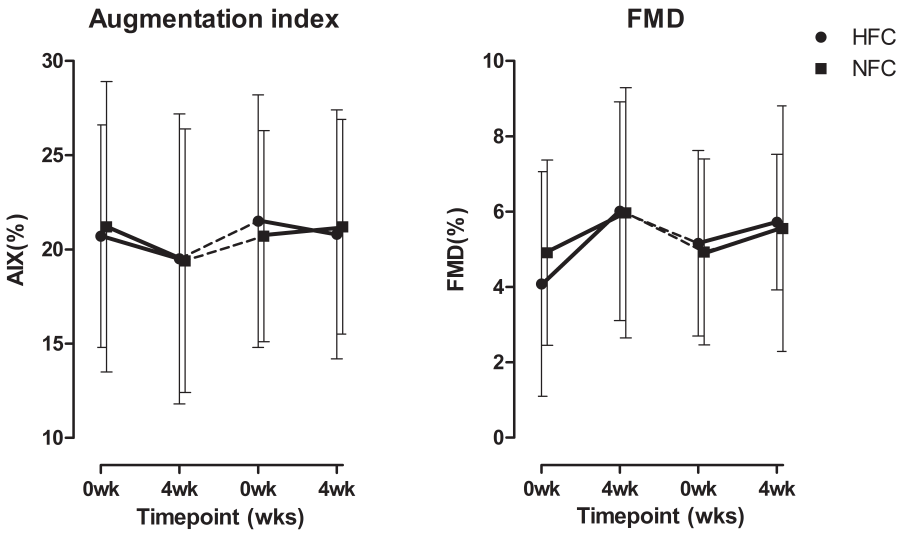


Figure 6.2 The effects of 4-week HFC or NFC consumption on AIX and FMD. Results are split for both intervention periods in order to visualize the effect of the washout period. Data are presented as mean ± SD.

High fat challenge test

Table 6.3 lists the effects of a 4-week HFC or NFC background on the postprandial response to a high-fat shake. High-fat shake consumption decreased FMD by 1.8% point, AIX by 4% point and diastolic blood pressure by 2mmHg on average, with no difference between a HFC or NFC background. High-fat shake consumption increased haematocrit and number of thrombocytes, erythrocytes, lymphocytes, monocytes and neutrophils. Plasma concentrations of sICAM1, sVCAM1, sICAM3, P-selectin, IL-8 and TNFα concentrations were increased, whereas plasma concentrations of IL-6 were decreased three hours after shake consumption. In addition, shake consumption increased lymphocyte CD11c and CD11b expression, monocyte CD11c and CD11b expression and neutrophil CD62l and CD11b expression, while monocyte CD62l expression was decreased. No differences between a HFC or NFC background were observed on blood cell count, plasma cytokines or leukocyte activation markers. No differences in response in plasma glucose, TG, insulin and FFA between a HFC or NFC background were observed (**Table 6.4**).

Table 6.4 Mean baseline and changes in fasting metabolic parameters before and after 4-weeks HFC and NFC consumption (study part II), including postprandial outcomes after the high-fat challenge test at the end of each intervention

		Time				*P*-value					
						4 week intervention			High-fat challenge		
Metabolic parameters	Treatment back-ground	0 weeks/0h	Δ intervention 4 weeks/0h	Δ High-fat challenge 1.5h	Δ High-fat challenge 3h	Treat-ment	Time (w)	Inter-action	Treatment background	Time (h)	Inter-action
Glucose (mmol/l)	NFC	5.6 ± 0.4	0.1 ± 0.3	-0.6 ± 0.6	-0.3 ± 0.4	NS	0.002	NS	NS	<0.001	NS
	HFC	5.5 ± 0.4	0.1 ± 0.3	-0.7 ± 0.5	-0.4 ± 0.3						
TG (mmol/l)	NFC	1.4 ± 0.6	-0.1 ± 0.4	0.4 ± 0.2	1.1 ± 0.5	NS	NS	NS	NS	<0.001	NS
	HFC	1.4 ± 0.7	0.0 ± 0.4	0.4 ± 0.2	1.0 ± 0.5						
FFA (mmol/l)	NFC	0.48 ± 0.11	0.02 ± 0.10	-0.21 ± 0.13	0.04 ± 0.12	NS	0.010	NS	NS	0.002	NS
	HFC	0.46 ± 0.11	0.03 ± 0.11	-0.22 ± 0.11	0.04 ± 0.13						
Insulin (mmol/l)	NFC	6.1 ± 2.7	0.7 ± 2.2	6.4 ± 7.7	3.8 ± 3.1	NS	NS	NS	NS	<0.001	NS
	HFC	6.9 ± 3.1	0.0 ± 2.2	8.4 ± 7.7	3.7 ± 3.0						

Values are mean ±SD, *n*=41. NFC, normal flavanol chocolate; HFC, high flavanol chocolate.; TG, triglycerides; FFA, free fatty acids.

Taste and motivation to eat chocolate

The sensory profile of the HFC and NFC are visualized in radar charts (**Supplemental Figure S6.1**). The HFC chocolate was less grainy, less hard, more soft, less creamy, less sweet, more astringent, more bitter and had a higher off-flavour compared to the NFC. The aftertaste of the HFC was described as more bitter, less sweet, higher after taste and higher dry mouth. These differences in taste did affect motivation scores of the participants during the intervention (**Supplemental Table S6.3**). The desire for chocolate score was on average 1.7 point lower if subjects received HFC compared to NFC. The chance that they would choose the chocolates themselves was on average 2.8 points lower for HFC compared to NFC. The wanting to finish the chocolate score was 2.5 point lower for the HFC group compared to the NFC group.

DISCUSSION

We extensively characterised the impact of chocolate consumption on vascular health and investigated if high flavanol dark chocolate in this respect is preferred above common dark chocolate. 4-week daily consumption of dark chocolate improved FMD and decreased AIX, leukocyte cell count, plasma concentrations of soluble adhesion molecules and protein expression of adherence marker on leukocytes. Chocolate with an increased flavanol content did not have an additional beneficial effect on markers of vascular health if compared to regular dark chocolate. However, higher flavanol content affected taste and had a negative effect on the motivation to eat chocolate.

The 1% point increase in FMD after 4-week daily consumption of dark chocolate is in line with the approved health claim by EFSA that cocoa flavanols have a positive effect on endothelium-dependent vasodilation [3]. A meta-analysis investigating the association between FMD on the relative risk of cardiovascular events, found that each 1% increase in FMD is associated with a relative risk of cardiovascular events of 0.87 (95%CI: 0.83, 0.91) [24]. These findings illustrate that our observed changes in FMD after chocolate intervention may contribute to a cardiovascular disease risk reduction. Next to the improvement in FMD, we are the first demonstrating that 4-week daily intake of dark chocolate additionally lowers AIX by 1% point. The AIX is a measure of wave reflection and arterial stiffness and a meta-analysis investigating the association between fasting AIX on the relative risk of cardiovascular events, found that each 10% increase of AIX is associated with a relative risk of cardiovascular events of 1.32 (95%CI 1.093–1.588) [25]. Based on the observed improvements in FMD and AIX, our results support the EFSA health claim that chocolate consumption is able to improve vascular function.

Interestingly, we did not observe an additional beneficial effect of the HFC on measures of vascular function.

Besides investigating the effect of chocolate consumption on vascular function, we additionally aimed to elucidate how chocolate is able to affect endothelial function. The observed changes in leukocyte cell count, plasma cytokines and leukocyte adherence markers after chocolate consumption, point towards a less activated state of cellular adherence and hence a less atherogenic milieu. First, 4-week daily consumption of dark chocolate lowered leukocyte cell count in the circulation. Leukocytes can transmigrate through the endothelial layer and therefore play a crucial role in the formation of atherosclerosis. In addition, elevated leukocyte cell count is a marker for systemic inflammation and is associated with an increased CVD risk [26,27]. Secondly, dark chocolate consumption decreased protein expression of lymphocyte CD62L and CD11b, monocyte CD62L and neutrophil CD66b and CD11c. These cell surface molecules are involved in leukocyte recruitment and adherence to the endothelium during the initial steps of atherosclerosis. CD11b is part of MAC-1 integrin and together with CD11c and CD62L they can bind to ICAMs and CD34 on endothelial cells, thereby activating the endothelium [28]. CD66b is a degranulation marker of neutrophils and up regulated after activation [29]. Thirdly, chocolate consumption decreased plasma concentrations of soluble adhesion molecules sICAM1 and sICAM3. Both sICAMs can be secreted by the endothelium and by leukocytes. Lower levels of these soluble adhesion molecules have been postulated to be associated with lower risk of cardiovascular events [28]. The combination of lower numbers of leukocytes, decreased leukocyte adhesion molecules expression and decreased plasma soluble adhesion molecules after 4-week dark chocolate consumption, point towards reduced leukocyte adherence and subsequently reduced activation of the endothelium, the initial state of atherosclerosis. Interestingly, this decrease in endothelial activation is coherent with our observed improvement in vascular function as reflected by the increase in FMD and decrease in AIX.

Previous reports investigating the impact of chocolate consumption on factors of cellular adherence are limited or evaluated only few measures. The impact of chocolate consumption on leukocyte cell count has not been reported before. One previous study investigated the effect of cocoa powder intake leukocyte adherence markers and observed that 4-week daily intake of cocoa did not affect CD11b [30]. The difference with our decrease in CD11b may be because they included patients at high risk of CVD and used cocoa powder instead of chocolate. In addition to CD11b, we also studied CD66b, CD11c and CD62L. Chocolate/cocoa intervention studies investigating soluble adherence molecules in plasma are more abundant but inconclusive. Most are performed with cocoa powder [30-32] or in patients at risk of CVD [30-33] whereas our observations are in overweight, but apparently healthy men.

We additionally investigated in the same study population how chocolate ingestion affects vascular health postprandially, and if a background of 4-week NFC or HFC consumption is able to improve the capacity to respond to a high fat MUFA challenge test. Acute chocolate intake decreased AIX and plasma IL-6 and increased in plasma sICAM1, sICAM3 and leukocyte count, two hours after chocolate intake. As we did not observe a difference between HFC and NFC ingestion, these changes are likely caused by the high energy, sugar or fat content of chocolate. We therefore can neither verify nor rebut that chocolate intake acutely improves vascular function [2]. Similar changes as after acute chocolate consumption were observed three hours after high-fat shake consumption. Since this high-fat challenge test was implemented at the end of both interventions only, we could not determine if 4-week chocolate consumption, independent of the type, affected the capacity to respond to a high-fat challenge. Our observed postprandial high-fat responses are in line with our previous findings that a high-fat shake affects vascular function and leukocyte adherence capacity [34].

No differences were observed between NFC and HFC consumption on measures of vascular health. This observation is supported by a meta-analysis, in which the authors found that chronic chocolate or cocoa powder intake improved FMD regardless of the dose consumed [2]. Our results indicate that flavanol enriched chocolate was not healthier than regular dark chocolate with respect to vascular health markers. This cannot be explained by maximal flavanol absorption capacity in the gut, considering the 5 fold increase in plasma (–)-epicatechins 2 hours after intake and the 6.5 fold increase in 24-hour urine flavanol concentrations after 4-week intake of HFC compared to NFC intake. But the maximal effect on vascular health might have been reached by the flavanol dose in the NFC. So, if the vascular health effects are due to flavanols, it might still be relevant from a public's health prospective to increase chocolate flavanol content in order to reduce saturated fat and sugar intake per portion. However, this strategy would only work if adding flavanols would not negatively affect the high standards of chocolate flavour.

The EFSA approved health claim is largely based on studies using cocoa powder beverages. We specifically choose chocolate since it is the most commonly consumed cocoa derived product. Chocolate is also an energy dense product with high contents of sugar and fat. It must be noted that we observed a small, but significant increase in plasma glucose and FFA concentrations after 4-week chocolate intake. But despite these unfavourable trades of chocolate, we did observe clear improvements in vascular function markers. As no differences between both chocolates were observed we cannot rule out an intervention effect. However as vascular function outcomes returned to baseline after the washout period, subjects remained weight stable and the infringement in habits and life style were minimal, the results evidently points

towards an effect of chocolate intake on vascular function. Chocolate contains many potentially bioactive constituents in addition to flavanols [35]. These components or the synergy of these components may also be responsible for the observed effects.

An important aspect of this study was the independent analyses of chocolate flavanol content. Flavanol concentrations strongly deviated from the original product sheets. Apparently these concentrations are not constant and vary between batches. Previous chocolate intervention studies barely report self-analysed chocolate flavanol content and rely on the product sheets obtained from the manufacturer. Deviating concentrations in previously published studies can therefore be expected. We acknowledge that the NFC, which reflected regular dark chocolate, was relatively high in flavanols. Nonetheless this chocolate was produced by the company as a regular consumed dark chocolate and these concentrations can therefore be expected in common dark chocolate.

This study underlines and elaborate on the approved EFSA health claim by demonstrating that dark chocolate not only improves endothelium-dependent vasodilatation but also augmentation index. We additionally provide new insights on how chocolate affects the endothelium by demonstrating that chocolate consumption lowers the adherence capacity of leukocytes in the circulation. Extra flavanols did not result in an additional beneficial effect on these outcome measures, which might indicate that the maximal vascular response capacity has been reached, and/or that other chocolate components are relevant for vascular health effects. Increasing the flavanol content of chocolate may be relevant to reduce portion sizes and to decrease saturated fat and sugar consumption. However, additional flavanols do affect flavour and thereby negatively affecting motivation to eat chocolate. Therefore, chocolate-producing companies that want to increase the amount of flavanols in chocolate will have to deal with the preservation of taste.

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Conflict of interest

None.

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SUPPLEMENT

Supplemental Table S6.1 Nutritional values of the high fat shake

Energy	990 kcal
Protein	10 g
Carbohydrates	19 g
Fat	95 g
Saturated fatty acids	8 g
Mono unsaturated fatty acids	79 g
Poly unsaturated fatty acids	8 g
ALA	0 g
EPA	0 g
DHA	0 g

ALA = alpha-linolenic acid (C18:3); EPA = eicosapentaenoic acid (C20:5); DHA = docosahexaenoic acid (C22:6).

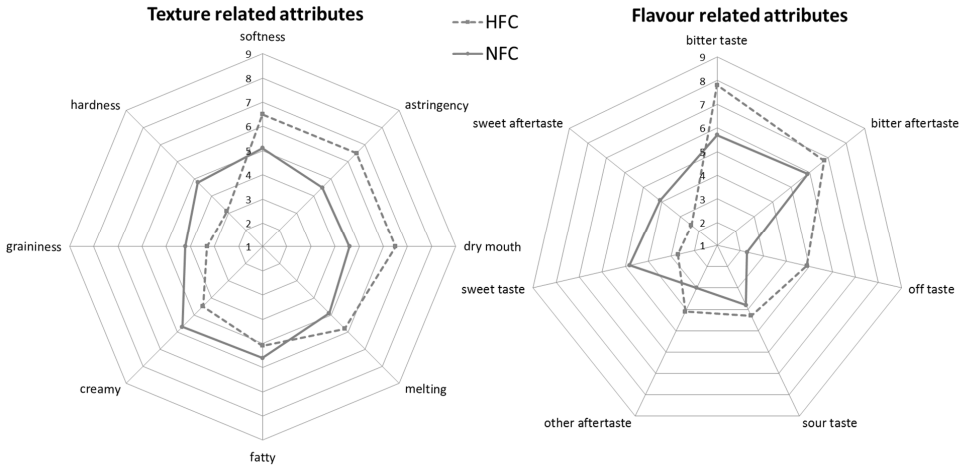
Supplemental Table S6.2 Baseline characteristics of participants

	Study part I (*n*=29)	Study part II (*n*=41)
Age (years)	64 ± 4	63 ± 5
Weight (kg)	89.7 ± 10.5	87.8 ± 10.5
Height (m)	1.79 ± 0.05	1.78 ± 0.06
BMI (kg/m^2)	27.8 ± 2.65	27.6 ± 2.31

Supplemental Table S6.3 Mean outcomes of afternoon consumption motivation before and after 4-week daily consumption

		Time (days)					*P*-value		
Motivational score	Treatment	1	7	14	21	27	Treatment	Time (day)	Interaction
Desire for chocolate	NFC	5.6±2.2	5.2±2.2	4.8±2.7	5.4±2.2	5.2±2.1	0.020	0.10	0.98
	HFC	3.8±2.2	3.5±2.0	3.1±2.0	2.8±1.7	3.2±2.0			
Chance choice for chocolate	NFC	5.7±2.2	5.6±2.2	5.0±2.6	5.7±2.2	5.3±2.3	<0.001	0.91	0.28
	HFC	3.2±1.9	2.8±1.9	3.2±1.9	2.5±1.7	2.9±1.6			
Pleasantness of the chocolate	NFC	7.0±1.4	6.5±1.7	6.2±2.0	6.4±1.9	6.2±2.0	<0.001	0.03	0.78
	HFC	5.1±1.4	4.2±2.3	4.1±2.0	3.7±1.9	4.1±2.0			
Wanting to finish	NFC	7.3±1.3	6.4±1.8	6.0±2.3	6.4±1.9	6.1±2.0	<0.001	0.047	0.47
	HFC	4.8±1.8	4.0±2.4	3.9±1.9	3.6±2.1	3.5±2.0			

Only data of the first intervention period shown; HFC (n=18) and NFC (n=25). Data was collected on a 9-point scale and presented as mean ± SEM.



Supplemental Figure S6.1 Radar charts of the sensory profile of both intervention chocolates.

Chapter 7

General discussion

Recapturing aim

The ability of a person to respond to a metabolic challenge can reflect the capacity to adapt and might give a better indication of health and disease risk than fasting measures [1,2]. In this thesis we aimed to identify early markers for reduced endothelial function by using metabolic challenges. We applied different high fat (HF) challenges as a tool to trigger endothelial response capacity and to magnify differences in health status. To identify markers of early perturbations in endothelial health, we applied high-throughput metabolomic and transcriptomic tools in addition to an extensive phenotyping of vascular function parameters. Furthermore, we evaluated whether these potential early markers are reversible and can be improved after an intervention with a dietary anti-stressor.

Using a high-fat challenge test to trigger endothelial response capacity

A HF challenge is able to activate endothelial cells and leukocytes and is an acute source of dietary stress for the endothelium. The ability to respond to a HF challenge can be seen as measurement of endothelial flexibility. The endothelial response after a HF challenge may therefore be useful to detect early perturbations in endothelial health, even before this can be revealed during fasting. In all studies described in this thesis, we challenged the endothelium by means of a HF challenge and measured several outcome parameters to determine the endothelial response. An overview of the outcome parameters affected by a HF challenge is depicted in **Table 7.1**.

In two separate studies we observed that in middle-aged men, a HF challenge elicits a decrease in augmentation index (AIX), an increase in plasma concentrations of IL-8, sICAM1, sICAM3 and sVCAM1 and an increase in leukocyte adherence marker expression (chapters 3 and 6). In chapter 6 we additionally measured flow mediated dilation (FMD), and showed that those observations were accompanied with a decrease in FMD. We performed a similar study with young healthy men (chapter 2) and also observed a postprandial decrease in FMD after HF shake consumption. However, this decrease was also found after the consumption of an average breakfast shake. Interestingly, the decrease in FMD in young healthy men of 0.5%-point after the HF shake (chapter 2) was less pronounced than the decrease in FMD observed in middle-aged overweight men of 1.8%-point (chapter 6). Beside the smaller postprandial decrease in FMD, the AIX values in young healthy men were negative, meaning that no augmented central systolic blood pressure was present in this young study population. Furthermore, in

Table 7.1 Overview of the main outcomes of endothelial health markers after the high-fat challenges described in this thesis

		Chapter 2		Chapter 3			Chapter 6	
Intervention shake				HF			High-MUFA	
		HF/HE	Regular breakfast	SFA	MUFA	PUFA	NFC	HFC
Amount of fat		95g	15g	95g	95g	95g	95g	95g
Subjects		Lean young men (*n*=20)		Lean (*n*=18) + Obese (*n*=18) middle-aged men			Overweight middle-aged men (*n*=42)	
Time points		T= 0, 3 and 6h		T= 0, 2 and 4h			T= 0 and 3h	
Vascular function								
FMD		↓	↓		N/A		↓	↓
AIX		↓	↓	↓	↓	↓	↓	↓
Plasma markers								
SAA		↓	-	↓	↓	↓	-	-
IL-6		↑	↑	↓	↓	↓	↓	↓
TNFα		-		-	-	-	↑	↑
TM		↓	↓	↓	↓	↓	-	-
E-selectin		↓	↓	-	-	-	-	-
P-selectin		-	-	↑	-	-	↑	↑
IL-8		↑	-	↑	↑	↑	↑	↑
sICAM1		-	-	↑	↑	↑	↑	↑
sICAM3		-	-	↑	↑	↑	↑	↑
sVCAM1		↓	-	↑	↑	↑	↑	↑
Leukocyte surface markers								
Neutrophils	CD11a	N/A		↑	↑	↑	N/A	
	CD11b	N/A		↑	↑	↑	↑	↑
	CD11c	N/A			N/A		-	-
	CD62L	N/A		↑	↑	↑	↑	↑
Monocytes	CD11a	N/A		↑	↑	↑	N/A	
	CD11b	N/A		↑	↑	↑	↑	↑
	CD11c	N/A			N/A		↑	↑
	CD62L	N/A		↑	↑	↑	↑	↑
Leukocytes	CD11a	N/A		↓ lean	↓ lean	↓ lean	N/A	
	CD11b	N/A		↓ lean	↓ lean	↓ lean	↑	↑
	CD11c	N/A			N/A		↑	↑
	CD62L	N/A		↑	↑	↑	-	-
Leukocyte count		N/A		Shift to more neutrophils*			↑	↑

HF, high-fat; HE, high energy; SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly- unsaturated fatty acids; NFC, normal flavanol chocolate background; HFC, high flavanol chocolate background; FMD, flow mediated dilation; AIX, augmentation index; TM, thrombomodulin; ↑, increased after HF challenge; ↓, decreased after HF challenge; N/A, not measured in the study. * relative count, in proportion to lymphocytes and monocytes.

young healthy men, HF challenge intake did not elicit an increase in plasma sICAM1, ICAM3 and VCAM1, whereas it did in middle-aged men (chapter 3 and 6). The modest reduction in FMD, together with the absence of a clear increase in soluble adhesion markers after a HF challenge, suggests that young healthy men are able to handle a high-fat and high-energy load in such a way that it does not adversely affect endothelial function. This can be explained by an adequate metabolic and endothelial response capacity in young men, which is likely diminished in middle-age men. This finding is supported by many studies that did not observe a postprandial impairment in endothelial function after a HF meal in healthy younger aged volunteers [3-9]. Studies comparing the FMD response after a HF meal between middle-aged and young volunteers in a single study have not been performed yet. Due to adequate endothelial responses in young men, the HF challenge test in young men seems less applicable to identify early perturbations in endothelial health.

Fatty acid type of the HF challenge

In chapters 3, 4 and 5 we challenged lean and obese middle-aged men with a HF challenge, either high in saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) or n3 poly-unsaturated fatty acids (PUFA). We observed that MUFA intake elicited the highest postprandial plasma triglyceride (TG) concentrations. The higher postprandial rise of TG after MUFA intake compared to SFA intake was in line with previous observations [10-12]. MUFA intake also caused a more pronounced decrease in AIX compared to SFA and n3 PUFA intake (chapter 3). A more pronounced postprandial decrease in AIX after MUFA intake has not been reported before. Although we did not compare the postprandial impact of different fatty acids on FMD, one study showed that consumption of high oleic sunflower oil (high MUFA, rich in oleic acid) caused a more pronounced postprandial reduction in FMD compared to shea butter (high SFA, rich in stearic acid) consumption [13]. Based on the more pronounced postprandial rise of TG and more pronounced effects on vascular function after MUFA consumption, we selected the MUFA challenge for PBMCs transcriptome analysis. We compared gene expression changes after MUFA intake to those induced by a challenge of commonly consumed fatty acids, i.e. the SFA challenge (chapter 5). Transcriptome analysis showed that a high MUFA challenge changed the expression of more genes than a high SFA challenge did, especially in obese subjects. Taken together, we hypothesize that MUFAs are more potent fatty acids to trigger the vascular response capacity and to initiate gene expression changes in PBMCs, than SFAs or n3 PUFAs. For this reason, we chose the high MUFA challenge to trigger the endothelial response capacity after consumption of a dietary anti-stressor (chapter 6).

The total amount of fat in the HF challenge

All HF intervention shakes described in this thesis contained 95g of fat. Although the fatty acids used in our studies are commonly consumed and therefore expected to be well tolerated, this amount of fat is relatively high and does not reflect a common meal. Yet, the HF challenges described in this thesis were used as a tool to trigger the endothelial response capacity and can be compared to an oral glucose tolerance test (OGTT) in which a glucose load of 75g is provided to determine how quickly glucose is cleared from the blood [14]. In the OGTT the amount of glucose is also relatively high and does not reflect a common meal. The question remains if we need these high amounts of fat to elicit an endothelial response. The optimal amount of fat to trigger the endothelium is currently unknown and previous postprandial HF meal challenges described in the literature vary considerably in the amount of fat or adjust the amount of fat for body surface area (reviewed by Hall *et al.*[15] and Jackson *et al.*[16]). This makes comparisons between postprandial challenge studies difficult. In addition, as also observed in this thesis and summarized above, the required amount of fat to challenge endothelial function may depend on age, health status of the human volunteer and type of fat.

Factors affecting vascular function parameters

Measures of vascular function, such as FMD, have large variations in reproducibility [17]. Furthermore, small effect sizes can be expected in short term nutritional interventions. A reduction in the variation is of pivotal importance to detect small changes in endothelial health induced by relatively short-term nutritional intervention studies. Several factors are known to induce variation, such as changes in circadian rhythm [18,19]. For example, we observed a difference in postprandial FMD response between subjects that started the intervention at 07.30 a.m. compared to subjects that started at 09.30 a.m. (chapter 2, data not shown). To minimize variation induced by circadian rhythm, we recommend that the vascular measurements for each subject should be performed at the same time on each intervention day. Another aspect that may induce variation is the last meal consumed before the measurements. Although baseline measurements should be performed during fasting, the fat from the last meal consumed may still be in the intestinal tract and become available upon the second meal [20]. This is however based on studies that evaluated the impact of a HF breakfast on the TG response of a following meal 5 hours later, i.e. lunch. Nevertheless, to minimize the possible effect of fatty acid spill-over from the previous day, we recommend that subjects consume a standardized low-fat evening meal prior to each study day. Many other factors are also known to influence endothelial function including, age, use of certain medications such as blood pressure or cholesterol lowering drugs,

cigarette smoking, habitual dietary intake, gender, ethnicity, disease status and exercise [21]. To minimize between-subject variation, the target population should be as homogenous as possible. To minimize within-person variation, one might consider to replicate observations within the same individual in combination with a cross-over design. The latter strategy will enhance power and enabled us to detect the small postprandial changes in FMD in young men (chapter 2).

Potential mechanisms of endothelial triggering by a HF challenge

In the introduction we described that one of the current hypothesis is that elevated TRLs in the postprandial state can activate leukocytes and endothelial cells and thereby affect endothelial function [22]. In chapter 3 we showed that a high-fat MUFA challenge induced the most pronounced plasma TG response compared to a high-fat SFA and n3 PUFA challenge, especially in obese subjects. However, despite this clear difference in postprandial plasma TG, the HF-induced response in plasma markers involved in cellular adherence and leukocyte cell surface activation markers was independent of fatty acid type consumed or being lean or obese. In addition, we correlated changes in plasma TG concentrations with changes in plasma markers involved in cellular adherence and leukocyte cell surface activation markers, but did not observe conclusive correlations (chapter 3). Furthermore, in healthy young men (chapter 2), marked differences in plasma TG response between the HF/HE shake and the average breakfast shake were observed, but no differences in FMD or other markers of endothelial activation, except for IL-8 were seen (chapter 2). Our findings suggest that besides the magnitude of the postprandial TG response, other factors are involved in triggering the endothelial response capacity. A possible factor that may contribute to the postprandial inflammatory and vascular response is endotoxin [23,24]. Previous studies demonstrated that HF meal intake causes a postprandial increase in plasma endotoxins levels. Endotoxins derive from gram negative gut bacteria and can be transported through the gut wall during chylomicron release in the lymph [25]. Once endotoxins are in the circulation, they may activate inflammatory processes in endothelial cells and leukocytes [25]. A study in mice showed that high fat diet-induced changes in gut microbiota were associated with an increase in plasma endotoxin levels [26]. These findings suggest that also gut microbiota composition may be of importance in the endotoxin induced inflammatory response. It is known that gut microbiota composition is altered in people who are obese [27] and it has been demonstrated that the postprandial increase in plasma endotoxins upon HF intake is higher in obese subjects with an impaired glucose tolerance and type 2 diabetic subjects if compared to normal weight healthy subjects [23]. In chapter 3 we compared the postprandial responses of 13

cytokines involved in cellular adhesion, coagulation and systemic inflammation between lean and obese subjects, but did not observe a clear difference between the groups. This might be due to the fact that the obese subjects were relatively healthy and did not have diabetes. Still, by using more sensitive transcriptomic tools, we observed changes in gene expression profiles involved in immune function in PBMCs after a HF challenge between lean and obese subjects (chapter 5), but whether these changes are due to endotoxin or to HF challenges per se remains to be elucidated.

The composition of postprandial TRLs may also contribute to the postprandial inflammatory and vascular response. Postprandial TRLs differ in apolipoprotein composition, lipid content and probably also in oxylipin composition compared to fasting TRLs (chapter 4) [28-30]. These changes in TRL composition may create more atherogenic TRLs in the postprandial phase. Still, other causes not related to the HF meal may also have contributed to the activation of the endothelium and leukocytes in our studies. As we did not compare the postprandial HF response in middle-aged men to a response to a common meal, we cannot rule out if postprandial changes after these shakes were due to the high energy content, the meal intake per se or by changes of circadian rhythm (chapters 3 to 6). Although, the latter effect is hard to control for in postprandial challenge studies, due to the strong integration between the circadian rhythm and meal intake [31].

Markers of early perturbations in endothelial health

In this thesis we demonstrate that endothelial function, as estimated by FMD, is impaired after a dietary stressor, i.e. a HF challenge (chapter 2 and 6) and is improved after consumption of a dietary anti-stressor, i.e. dark chocolate (chapter 6). Although FMD is still the most accepted and established method to study endothelial function, it has serious drawbacks. FMD is time consuming, technically challenging and shows relatively large variations in reproducibility. The latter is also proven by the fact that FMD outcomes in similar target populations vary considerably between research centres. In addition, early perturbations in endothelial health may not always be detected by FMD. Therefore, the question remains whether other measures of vascular function are more sensitive and easier to use than FMD and, additionally, also reflect endothelial health. In the current thesis we used different techniques to measure endothelial health. Besides measures of vascular function, we measured 13 cytokines in plasma involved in cellular adhesion, coagulation and systemic inflammation, determined the protein expression of leukocyte cell surface activation markers and applied high-through put nutrigenomics techniques such as metabolomic and transcriptomic tools. By using such a comprehensive characterizing approach we identified potential leads for early biomarkers that may be suitable to reflect perturbations in endothelial health.

Vascular markers of endothelial perturbations

Besides FMD as measurement of vascular function, we measured the augmentation index (AIX) in all intervention studies (chapters 2, 3 and 6). The AIX can be relative easily and fast deduced from a pulse wave analysis of the radial artery, which makes it an interesting vascular measure to implement in complex study designs. The AIX is an estimate of stiffness and wave reflection and a lower AIX in the fasting state is associated with a lower risk of cardiovascular disease (CVD) [32]. The question remains if the AIX is also a potential early marker for endothelial function. The AIX is largely determined by the structural composition of the artery wall [33,34]. Short term induced changes in AIX are not caused by structural changes, but are most likely caused by changes in vascular tone, which is partly regulated by the endothelium. Yet, vascular tone can also be regulated by other factors such as autonomic nerves. The rapid postprandial changes in AIX after HF meal intake (chapters 3 and 6) were probably due to fast neuronal or hormone mediated peripheral vasodilation in response to nutrient intake [35]. Although these postprandial changes in AIX may still be of importance for vascular health, they may not always reflect changes in endothelial function. Changes in AIX under fasting conditions are more likely to reflect changes in endothelial function, because the acute effects of a meal can largely be ruled out [35]. This was also nicely reflected by our chocolate intervention study, where under fasting conditions, we observed that the decrease in AIX after 4-week daily intake of dark chocolate was accompanied with an improvement in FMD (chapter 6). Taken together, in both long term and postprandial studies, we consider the AIX to be a valuable outcome measure in addition to, but not instead of other endothelial markers, such as FMD.

Blood markers of endothelial perturbations

To identify potential early perturbations in endothelial health in the blood, we measured 13 cytokines in plasma involved in cellular adhesion, coagulation and systemic inflammation. In young men, we observed that high-fat/high-energy (HF/HE) shake consumption increased plasma IL-8 concentrations, compared to an average breakfast control shake, while no increases were observed in the more classical markers of endothelial function such as soluble adhesion molecules sICAM1, sICAM3 and sVCAM1. This increase in plasma IL-8 was paralleled by an increased gene expression of IL-8 in PBMCs (chapter 2). In middle-aged men as well, we observed an increase in plasma IL-8 concentrations after high SFA, MUFA and n3 PUFA intake (chapters 3 and 6). Similar to the effects observed in young healthy volunteers (chapter 2), this increase in plasma IL-8 was accompanied by an increase in PBMC gene expression of IL-8 [36]. IL-8 is produced by leukocytes and endothelial cells [37] and activates and recruits

neutrophils [38] and triggers monocyte adhesion to the endothelium [39,40]. Taken together, these findings suggest that IL-8 may be a potential marker for acute endothelial activation upon a dietary stressor such as a HF meal. In addition to the increase in IL-8, we observed that a HF challenge increased plasma concentrations of soluble adhesion molecules sICAM1, sICAM3 and sVCAM1, but only in middle-aged men (chapters 3 and 6). These soluble adhesion molecules are involved in the recruitment and adherence of leukocytes to the endothelium [41]. A HF challenge also increased the number of leukocytes in the circulation (chapter 6), indicating an activation of the systemic immune response [42]. Furthermore, in two separate studies we observed that a HF challenge increased leukocyte cell surface integrin and selectin expression (chapter 3 and 6). These integrins and selectins are able to interact with cell adhesion molecules (CAMs) present on the endothelium, thereby facilitating the adherence of leukocytes to the endothelium [41]. In summary, in two separate studies we observed, that a HF challenge increased plasma soluble adhesion molecules, increased leukocyte cell surface integrin and selectin expression and increased the number of leukocytes in the circulation in middle-aged men. These findings evidently point towards an activated state of cellular adherence in the circulation after a HF challenge. This activated state of cellular adherence after a HF challenge was accompanied by a decrease in FMD and AIX (chapter 6). Since endothelial health is determined by a complex interplay of endothelial cells, leukocytes and factors circulating in the plasma, the assessment of endothelial function based on a single outcome measure, such as FMD, would provide an oversimplified picture of endothelial health status. Based on the findings in this thesis we recommend that endothelial health is better determined by means of a biomarker profile consisting of the vascular measures FMD and AIX, a subset of soluble adhesion molecules in the plasma, leukocyte counts and cell surface integrin and selectin expression. As prove of principle, we investigated whether this biomarker profile could be beneficially changed by a dietary anti-stressor (chapter 6). We showed that the increase in fasting FMD after 4-week daily intake of dark chocolate was paralleled by a decrease in AIX and a less activated state of cellular adherence, reflected by the decrease in plasma concentrations of sICAM1 and sICAM3, the decreased leukocyte cell surface integrin and selectin expression and the decrease in leukocyte count, all pointing towards an improvement of our identified biomarker profile for endothelial health. Still, this biomarker profile is a combination of functional measures and blood markers as we are not able to determine the intracellular effects in the vascular wall due to the limited accessibility to endothelial cells in a pre-clinical setting. The fact that factors involved in leukocyte adherence to the endothelium are affected by dietary stressors and are reversibly affected after an intervention with a dietary anti-stressor, point towards a potential role of leukocytes in early perturbations of endothelial health. The use of transcriptome analysis in a subset of those leukocytes, the PBMCs, allowed the identification of stress related and

metabolic compensatory pathways affected by HF challenges differing in fatty acid type. For example, the SFA induced changes in expression of genes involved in cholesterol biosynthesis, cholesterol influx and efflux, may point towards an intracellular situation of excess cholesterol in the PBMCs after SFA intake. Abundant intracellular cholesterol in monocytes is stored in lipid droplets and excess storage may eventually lead to foam cell formation [43]. Further exploration of the effects of SFA on cholesterol homeostasis in the monocyte fraction of PBMCs, especially after repeated exposure, may increase our understanding on the mechanisms behind the more atherogenic effect of diets high in SFA compared to diets high in MUFAs. The MUFA challenge up-regulated the expression of many peroxisome proliferator-activated receptor α (PPARα) target genes, including genes involved in β-oxidation. Up-regulation of the β-oxidation may reduce the amount of fatty acids available for other processes such as intracellular lipid accumulation, an important process involved in the formation of lipid loaded macrophages [44]. In contrary to what can be expected from activation of PPARα, i.e. a down-regulation in expression of genes involved in inflammation, MUFA intake up-regulated the expression of genes involved in inflammation. This might be explained by the unsaturated double bond in MUFAs, which makes them more susceptible to oxidative stress compared to SFAs. A MUFA challenge may therefore elicit higher stress and subsequent higher inflammatory response in PBMCs compared to a SFA challenge. We hypothesize that repeated moderate exposure to such stress-inducing fatty acids, will activate the transcriptional response, hence increasing the cellular capacity to adapt. This may improve the resilience capacity of cells, ultimately leading to positive health effects and potentially reducing the risk of CVD on the long run. Future studies are needed to elucidate how repeated moderate exposure to these fatty acids will lead to longer-term adaptations, and what their effect is on endothelial health.

Besides exploring the use of transcriptomics techniques in PBMCs, we also applied metabolomic tools on plasma samples to determine whether a HF challenge is able to affect circulating oxylipin profiles (chapter 4). Oxylipins are involved in both pro- and anti-inflammatory processes and are well known for their local effects, but recent evidence suggests that these molecules may also exert systemic effects. We showed that oxylipin profiles are changed by the intake of a HF challenge test. Also, the postprandial changes of many oxylipins were affected by dietary fatty acid composition. Considering their involvement in pro- and anti-inflammatory processes, these findings suggest that postprandial changes in circulating oxylipins may have an effect on endothelial health. This study was explorative and little is known yet on the effect of circulating oxylipins on endothelial cells. Future research is needed to elucidate their potential role in endothelial function and CVD risk.

How to measure health status

In chapters 3, 4 and 5 we comprehensively characterized middle-aged lean and obese men before and after a HF challenge. Under fasting conditions, obese individuals had higher plasma insulin and TG levels compared to lean individuals. Obese individuals also had higher fasting concentrations of plasma C-reactive protein (CRP), serum amyloid A (SAA) and von Willebrand factor (vWF) compared to lean individuals (chapter 3). Both CRP and SAA are produced predominantly by the liver and can be considered markers of systemic inflammation [45,46]. vWF can be produced by endothelial cells and plays a major role in blood coagulation. Increased concentrations of CRP, SAA and vWF have been associated with a higher risk of CVD and belong to the classical and coarse biomarkers for CVD [47]. In addition to these plasma markers, gene expression profile analysis in PBMCs identified 294 genes that were significantly differently expressed between lean and obese subjects under fasting conditions (chapter 5).

Hence, quite some differences were already present between lean and obese subjects under fasting conditions. We applied a HF challenge with the ultimate goal to magnify differences between lean and obese subjects. With respect to plasma, we observed that obese individuals had elevated TG and insulin responses after HF intake. The postprandial responses of 13 cytokines involved in cellular adhesion, coagulation and systemic inflammation and oxylipins profiles in plasma were not different between lean and obese (chapter 3 and 4). When only taking the plasma pool into account, differences between lean and obese subjects were only observed with regard to metabolic markers and not inflammatory or endothelial markers. Of note, most circulating inflammatory factors can be secreted by several different organs. Postprandial responses of those markers in plasma may therefore not always reflect the cellular response capacity of the endothelium or leukocytes.

With respect to the differences in cellular responses between lean and obese subjects, we observed that lymphocyte CD11a and CD11b expression after a HF challenge was decreased in lean, but not in obese subjects (chapter 3). This may be due to a reduced capacity of lymphocytes in obese subjects to respond adequately to a HF load. Differences in postprandial cellular responses between lean and obese subjects became especially explicit by the application of transcriptomics in PBMCs (chapter 5). While prior to the challenge, under fasting conditions, 294 genes were significantly differently expressed between lean and obese subjects, this amount was increased to 607 genes after the SFA challenge and to 2,516 genes after the MUFA challenge. Several biological pathways that may be perturbed in obese subjects were identified. We found that SFA intake elicited a pro-thrombotic gene expression profile in obese but not in lean subjects. Furthermore, after MUFA intake, the expression of GPI-anchored protein

synthesis genes was up-regulated while expression of various G-protein coupled receptors was down-regulated in obese relative to lean subjects. GPI-anchored proteins on leukocytes regulate integrin-mediated cell adhesion [48,49] and many GPCRs are known to mediate both inflammatory and anti-inflammatory processes [50]. Changes in expression of genes involved in haemostasis and immune function in PBMCs after a HF challenge may be one of the first detectable hallmarks for small disturbances in health status. Whether these findings are consistent and to what extent those changes are linked to endothelial health remains to be elucidated.

Recommendations for future research

Although the scope of this thesis was to evaluate factors involved in vascular function and leukocyte adherence, our findings in several chapters suggest that also coagulatory processes are affected after a HF challenge. First, in middle-aged men, we observed a decrease in plasma thrombomodulin (TM) after the intake of a HF challenge (chapter 3 and 6). TM is secreted by the endothelium and inhibits platelet adhesion and coagulation. A decrease in TM causes a more pro-thrombotic environment [51]. Secondly, in chapter 3 we observed an increase in plasma P-selectin after SFA consumption, which was not observed after MUFA or n3 PUFA consumption. P-selectin is expressed both by platelets and endothelial cells and modulates adhesion of leukocytes and platelets to the endothelium [52]. The observed postprandial SFA induced increase in P-selectin might therefore enhance P-selectin modulated leukocyte adherence to the endothelium in the postprandial state. Thirdly, after SFA intake, we observed an up-regulation of expression of genes involved in the activation of platelets in obese subjects, whereas a down-regulation was seen in lean subjects (chapter 5). All together, these findings point towards a HF induced pro-thrombotic environment, especially after SFA consumption. Therefore, it is of interest to further explore the effects of a high SFA challenge, on markers of platelet activation and coagulation, such as platelet aggregation, ex vivo bleeding time and platelet activation marker expression, particular in obese subjects.

In this thesis we propose that changes in endothelial function may best be determined by a biomarker profile consisting of the vascular measures FMD and AIX combined with a subset of soluble adhesion molecules in the plasma, leukocyte counts and cell surface integrin and selectin expression. Still, long-term studies are needed to confirm these findings and to elucidate to what extent changes in this biomarker profile are predictive for CVD risk. We also demonstrate that a HF challenge triggers the endothelial response capacity and has the potential to detect small differences in health status. However, we did not observe a difference response to a HF challenge

after an intervention with two different doses of a dietary anti-stressor (chapter 6). As we did not incorporate a HF challenge before the intervention, we were unable to determine whether the dietary anti-stressor, independent of dose, would have affected the capacity to respond to a HF challenge. This stresses the importance of applying a HF challenge test before and after a nutritional intervention. The sensitivity will increase even more if these HF challenge tests are combined with high-throughput nutrigenomics tools, as we identified these tools to be highly suitable to detect small differences in health status after a HF challenge test. Such tools are also suitable to identify biological pathways that may have been affected by the intervention.

Final conclusion

In this thesis we extensively characterized the postprandial response to a HF challenge in human subjects with different disease risk profiles and optimized the HF challenge test. We identified MUFAs as most potent fatty acids to trigger the vascular and cellular response capacity, which makes it the optimal fatty acid type to use in a HF challenge test. We demonstrated that besides functional measures of vascular function, also plasma and cellular factors involved in leukocyte adhesion to the endothelium are adversely affected by dietary stressors and are beneficially affected by a dietary anti-stressor. Therefore, we conclude that endothelial health can be more comprehensively measured by means of a biomarker profile consisting not only of the vascular function measures FMD and AIX, but also of a subset of soluble adhesion molecules in the plasma, leukocyte counts and cell surface integrin and selectin expression. To identify potential new leads for biomarkers, we applied whole genome gene expression profiling, combined with the HF challenge test which enabled us to detect small differences in health status. Furthermore, we identified metabolic and inflammatory pathways that are specifically affected by either MUFAs or SFAs. These findings increased our understanding on how a SFA or MUFA challenge exert their distinct effects on stress related and metabolic compensatory cellular processes and provided us with new potential leads to detect early perturbations in endothelial health.

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Nederlandse samenvatting

Hart- en vaatziekten behoren tot de meest voorkomende chronische ziektes wereldwijd. De onderliggende pathofysiologie voor het krijgen van hart- en vaatziekten is veelal atherosclerose, een sluipend ziekteproces waarbij vet en ontstekingscellen zich ophopen aan de binnenkant van de slagaders. Een belangrijke voorbode voor het krijgen van atherosclerose is het hebben van niet goed werkende endotheelcellen, ook wel endotheeldysfunctie genoemd. Endotheelcellen bekleden de binnenkant van de bloedvaten en spelen een belangrijke rol in de verwijding en de vernauwing van bloedvaten, in ontstekingsprocessen en in de bloedstolling. Endotheeldysfunctie wordt gekenmerkt door een vernauwing van de bloedvaten, een milde vorm van ontsteking en verhoogde aanwezigheid van stollingsfactoren. Er zijn verschillende voedingstoffen die een effect op de endotheelfunctie kunnen hebben. Deze kunnen gekwalificeerd worden als positief of negatief voor het endotheel.

Een voorbeeld van een negatief voedingscomponent voor het endotheel is de inname van een grote hoeveelheid vet. De inname van grote hoeveelheden vet kan tijdelijk de vaatfunctie verslechteren en zorgen voor een voorbijgaande milde ontstekingsreactie. De mate waarin een persoon kan reageren op een maaltijd met veel vet, weerspiegelt de flexibiliteit en daarmee ook de gezondheid van onder andere het endotheel. Het meten van de endotheelfunctie na het blootstellen van personen aan zo'n hoog-vetmaaltijd kan daarom mogelijk gebruikt worden om vroege verstoringen in het endotheel aan te tonen, eerder dan onder nuchtere condities.

Hoewel hart- en vaatziekten vaak mensen treffen van middelbare of oudere leeftijd, begint endotheeldysfunctie vaak al op vroegere leeftijd. Endotheeldysfunctie in een vroeg stadium is nog omkeerbaar en het vroeg kunnen opsporen van verstoringen is daarom van cruciaal belang. Echter, de toegankelijkheid van het endotheel bemoeilijkt het onderzoek bij mensen. Bij onderzoek met mensen is men aangewezen tot het meten van vaatfunctie op een niet invasieve manier. Op dit moment is de meest gebruikte en geaccepteerde manier om endotheelfunctie te bepalen een ultrasoundmeting genaamd 'flow mediated dilation', ofwel FMD. Deze meting is erg lastig, duurt lang en heeft veel variatie. De vraag blijft of er ook andere gevoeligere metingen zijn, die makkelijker uit zijn te voeren. Een mogelijke andere manier om de gezondheid van bloedvaten te bepalen is door het meten van de bloeddrukgolf. Aan de hand van de bloeddrukgolf kunnen we de augmentatie-index (AIX) berekenen, een maat voor bloeddrukgolfreflecties en een mogelijke indicatie van slagaderlijke stijfheid.

Naast het meten van vaatfunctie kan men ook bloed afnemen. In het bloed kan men aspecten bepalen die een rol spelen bij de gezondheid van het endotheel. Zo kan men bijvoorbeeld de eiwitexpressie van zogenaamde 'adhesiemoleculen' meten aan de buitenkant van witte bloedcellen, die een rol spelen bij het aanhechten aan het endotheel. Ook kan men in het plasma

eiwitten bepalen die faciliteren bij het hechten van witte bloedcellen aan het endotheel, of een rol spelen bij ontsteking en bloedstolling.

Daarnaast kunnen we ook gebruik maken van relatief nieuwe, gevoelige en uitgebreide screeningstechnieken zoals 'metaboloom'- en 'transcriptoom'-analyses. Met een metaboloom-analyse kan men 'oxilipin'-profielen meten. Oxylipins zijn afbraakproducten van vetzuren die een rol spelen bij ontsteking. Met een transcriptoomanalyse kan men de activiteit van al onze genen in één meting bepalen. Een verandering in de activiteit van genen kan inzichten verschaffen over welke processen er in het lichaam veranderen. Dit geeft ons informatie via welke mechanismen voeding een effect kan hebben op ontsteking, metabolisme en zo ook op endotheelfunctie. Perifere bloedmononucleaire cellen (PBMC's), een subpopulatie van witte bloedcellen, zijn relatief makkelijk verkrijgbaar, spelen een belangrijke rol bij ontsteking en kunnen interactie aangaan met het endotheel. PBMC's zijn daarom belangrijke cellen om transcriptoomonderzoek aan te doen.

Het doel van dit promotieonderzoek was om markers te identificeren die vroege verstoringen in endotheelfunctie weerspiegelen. Om potentiële vroege markers te identificeren hebben we deelnemers blootgesteld aan een stresstest voor het endotheel, namelijk een hoog-vet maaltijd met 95 gram vet.

In **hoofdstuk 2** hebben we de hoog-vet stresstest gevalideerd door bij jonge gezonde vrijwilligers de response na een vetrijke milkshake te vergelijken met die van een normaal ontbijt. Voor en na de inname van deze milkshakes hebben we verschillende vaatmetingen uitgevoerd en hebben we ontstekingsfactoren in het plasma gemeten. In deze jonge gezonde vrijwilligers zagen we dat de hoog-vet stresstest de endotheelfunctie tijdelijk verslechterde, gezien de daling in FMD. Deze effecten werden echter ook gevonden na het consumeren van een normaal ontbijt. Jonge gezonde vrijwilligers zijn daarom kennelijk nog in staat om op een adequate manier te reageren op een vetrijke maaltijd. De concentratie van interleukine-8, een eiwit betrokken bij het plakken van witte bloedcellen aan de vaatwand, was toegenomen na de consumptie van hoog-vet ten opzichte van de consumptie van een normaal ontbijt.

Om nieuwe markers voor vroege verstoringen in endotheelfunctie te identificeren hebben we hoog-vet stresstesten toegepast op twee type deelnemers van middelbare leeftijd met een verschillend risicoprofiel voor het krijgen van hart- en vaatziekten, namelijk slanke mensen en mensen met obesitas. Ook hebben we in deze studie de hoog-vet stresstesten geoptimaliseerd door 3 verschillende soorten hoog-vet testen toe te passen, namelijk met verzadigde vetten, enkelvoudig onverzadigde vetten of omega-3 meervoudig onverzadigde vetten (**hoofdstukken 3 tot en met 5**).

Na het toepassen van de hoog-vet testen bij mannen van middelbare leeftijd, zagen we in **hoofdstuk 3** dat de consumptie van een hoog-vet stresstest een daling induceerde van de AIX, een toename induceerde van de adhesiemoleculen aan de buitenkant van witte bloedcellen en een toename induceerde van eiwitten in het plasma die faciliteren bij de aanhechting van witte bloedcellen aan de vaatwand. De daling van de AIX en de toename van vetten in het bloed waren het sterkst na de inname van hoog-vet met enkelvoudig-onverzadigde vetten.

Ook hebben we in deze studie uitgebreide screeningstechnieken toegepast. **Hoofdstuk 4** beschrijft dat 'oxilipin'-profielen veranderden na het consumeren van een hoog-vet stresstest en dat de mate waarin deze profielen veranderden voor een groot deel afhankelijk was van het type vet dat geconsumeerd was. De toepassing van transcriptome analyse in PBMC's (**hoofdstuk 5**), onthulde dat de veranderingen in genexpressieprofielen na een hoog-vet stresstest anders zijn in slanke mensen dan in mensen met obesitas. Deze verschillen werden nog duidelijker na een hoog-vet stresstest met enkelvoudig-onverzadigde vetten. Ook zagen we dat verzadigd vet leidde tot een verlaging van activiteit van genen betrokken bij cholesterolopname en cholesterolaanmaak en een verhoging van de activiteit van genen betrokken bij cholesterolflux uit de cellen. De consumptie van enkelvoudig-onverzadigde vetten leidde tot een verhoging van de activiteit van genen die betrokken zijn bij de vetverbranding en bij ontsteking.

In **hoofdstuk 6** hebben we gekeken of de aspecten die veranderen na een hoog-vet test, geïdentificeerd in hoofdstuk 3, ook verbeterd kunnen worden na een interventie met een positief voedingsmiddel voor het endotheel, namelijk pure chocolade. Een 4-weekse interventie met pure chocolade zorgde voor een verbetering van de FMD, een verlaging van de AIX, een afname van adhesiemoleculen aan de buitenkant van witte bloedcellen en een afname van eiwitten in het plasma die faciliteren bij de aanhechting van witte bloedcellen aan de vaatwand. Er werd geen verschil gevonden tussen twee type chocolades met verschillende concentraties flavanolen.

Tot slot bediscussiëren we in **hoofdstuk 7** de belangrijkste bevindingen van dit proefschrift. In dit proefschrift hebben we de effecten van een hoog-vet stresstest uitgebreid in kaart gebracht bij deelnemers met een verschillend risicoprofiel om hart- en vaatziekten te ontwikkelen. Daarnaast hebben we enkelvoudig-onverzadigde vetten gekwalificeerd als meest geschikte soort vet om de vasculaire functie te testen. We achten deze vetten dus het meest geschikt om te gebruiken in een hoog-vet stresstest.

Ook hebben we aangetoond dat de inname van negatief voedingsproduct voor het endotheel, namelijk hoog vet, factoren in het bloed verhoogt die een rol spelen bij de binding van witte bloedcellen aan de vaatwand. Deze factoren zijn verlaagd na de interventie met pure chocolade, een voedingsproduct welke een positieve werking heeft op endotheelcellen. Wij concluderen

daarom dat de gezondheid van het endotheel het best kan worden bepaald door vaatmetingen te combineren met het meten van factoren die een rol spelen bij het binden van witte bloedcellen aan de vaatwand.

Ook tonen we in dit proefschrift aan dat het gebruik van een hoog-vet stresstest in combinatie met het gebruik van gevoelige transcriptoomanalyse, verschillen in gezondheidstatus kan uitvergroten. Daarnaast hebben we met deze transcriptoomanalyse metabole en ontstekingsprocessen in kaart gebracht die specifiek veranderen na het consumeren van verzadigde of enkelvoudig-onverzadigde vetten. Deze uitkomsten vergroten onze kennis op het gebied van de werking van verschillende vetzuren in het lichaam met als uiteindelijk doel te begrijpen hoe de consumptie van vet onze gezondheid beïnvloedt.

Dankwoord

Zo… en dan nu eindelijk het dankwoord. Laten we beginnen met de mensen waarmee ik direct te maken kreeg toen ik dit avontuur begon en die er gedurende het hele project nauw bij betrokken zijn geweest.

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Naast de mensen van het eerste uur zijn er een hoop mensen die in de loop van mijn promotietraject een belangrijke rol zijn gaan spelen.

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About the author

CURRICULUM VITAE

Diederik Esser was born on March 7, 1981 in Woerden, the Netherlands. In 2000, he completed secondary school at the O.S.G. Sevenwolden (Athenaeum) in Heerenveen. Thereafter he started his bachelor in biology at the University of Groningen. He did his first master thesis at the department of medical-biomics of the university hospital of Groningen, where he investigated the protein composition of saliva and the possibilities to use saliva as a diagnostic fluid. He performed his second master thesis at the department of gastro-intestinal research of the Catholic University of Leuven (Belgium), where he investigated the effect of prebiotic intake on colon metabolism in healthy volunteers. Shortly after receiving his MSc. degree in Medical Biology, Diederik started his PhD project 'Diet and early changes in endothelial function' at the division of Human Nutrition of Wageningen University under the supervision of Prof.dr. Michael Müller and Dr. Lydia Afman. During his PhD project, which was sponsored by the Top Institute of Food and Nutrition (TIFN), he performed long-term and postprandial challenge studies in humans with a focus on endothelial function. During his PhD project, Diederik was also involved in teaching and supervising students during their master thesis. In addition, he was appointed in 2012 as a science advisor for the follow-up TIFN projects in cardiovascular health. Since January 2013, Diederik is appointed as a post-doctoral fellow at the division of Human Nutrition of Wageningen University.

LIST OF PUBLICATIONS

Esser D, van Dijk SJ, Oosterink E, Müller M, Afman LA.

Distinct changes in peripheral blood mononuclear cell gene expression profiles between lean and obese subjects after a high-fat challenge differing in fatty acid type (manuscript in preparation).

Esser D and Strassburg K (equal contribution), Vreeken R, Hankemeier T, Müller M, van Duynhoven J, Afman LA, Jacobs DM.

Postprandial fatty acid-specific changes in circulating oxylipins in lean and obese men after a high fat challenge (submitted for publication).

Esser D, Mars M, Oosterink E, Stalmach A, Müller M, Afman LA.

Dark chocolate improves vascular function and leukocyte adherence capacity; no additional effect of high flavanols content (submitted for publication).

Esser D, van Dijk SJ, Oosterink E, Müller M, Afman LA.

A high fat SFA, MUFA or n3 PUFA challenge affects the vascular response and initiates an activated state of cellular adherence in lean and obese middle aged men. Journal of Nutrition (2013), 143(6): 843-851.

Esser D, Oosterink E, Op 't Roodt J, Henry RMA, Stehouwer CDA, Müller M, Afman LA.

Vascular and inflammatory high fat meal responses in young healthy men; A discriminative role of IL-8 observed in a randomized trial. PLoS One (2013), 8(2): e53474.

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Esser D, Alvarez-Llamas G, de Vries MP, Weening D, Vonk RJ, Roelofsen H.

Sample stability and protein composition of saliva: implications for its use as diagnostic fluid. Biomarker Insights (2008), 3: 25-37.

OVERVIEW OF COMPLETED EDUCATIONAL ACTIVITIES

Discipline specific activities

Vascular measures training (Maastricht, The Netherlands, 2008)
NuGO week 2008 (Potsdam, Germany, 2008)
NuGO course 'Microarray analysis' (Maastricht, The Netherlands, 2009)
Masterclass 'Nutrigenomics' (Wageningen, The Netherlands, 2009)
Wageningen Nutritional Sciences Forum (Arnhem, The Netherlands, 2009)
NWO voedingsdagen (Deurne, 2008–2011)
NuGO week 2009 (Montecatini Terme, Italy, 2009)
ISFALL conference (Maastricht, The Netherlands, 2010)
Cardio Vasculaire Conferentie (Noordwijkerhout, The Netherlands, 2011)
NuGO week 2011 (Wageningen, The Netherlands, 2011)
TIFN 'cardiovasculaire dagen' (Utrecht, The Netherlands, 2012)

General activities

Netherlands Heart Foundation PhD training course (2009)
TIFN program day (2009)
Workshop: pictures, tables and infographics in your research (2010)
Workshop: how to write a word class paper (2011)
Master class 'Analysis using R' (2012)
TIFN workshop: De Vries Nutrition Solutions (2012)

Optionals

Preparation research proposal (2008)
Journal club Human Nutrition (2008–2010)
Journal club Nutrition, Metabolism and Genomics Group (2009–2010)
NMG scientific meetings (2008–2013)
Human Nutrition PhD tour to Mexico and USA (2011)

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