# IDENTIFICATION OF EFFECT AND EXPOSURE BIOMARKERS IN HUMAN DIETARY INTERVENTION STUDIES

## USING VARIOUS OMICS APPROACHES

CHARLOTTE C.J.R. MICHIELSEN

## Propositions

1. Classical biomarkers alone are incapable of capturing diet-induced effects. (this thesis)

2. MicroRNA expression is not ready to be used as a diet effect biomarker. (this thesis)

3. Despite the fact that we know men and women are different, it is remarkable how rarely women are included in molecular and physiological nutrition studies.

4. Writing scientific articles is in essence the art of omission without losing vital information.

5. The coronavirus pandemic has challenged the boundaries of what is normal.

6. In the public debate there is often no room for nuance, causing non-experts to have the upper hand.

Propositions belonging to the thesis, entitled

Identification of effect and exposure biomarkers in human dietary intervention studies using various omics approaches

Charlotte C.J.R. Michielsen
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Charlotte C.J.R. Michielsen

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## CONTENTS

# 1

# General introduction

Even though the World Health Organization began sounding the alarm in the 1990s, and despite massive efforts being undertaken in the meantime (1, 2), the prevalence of overweight and obesity has continued to increase globally, rising into pandemic proportions. In line with trends seen all over the world, more than half of the Dutch population is overweight, with almost 15% even suffering from obesity[1]. Obesity is often associated with several metabolic disturbances, such as elevated glucose levels, hypertension, insulin resistance and abnormal lipid metabolism, which lead to increased risks of developing several metabolic diseases, such as type 2 diabetes mellitus, fatty liver disease, and cardiovascular disease (3, 4). Therefore, obesity represents a major health challenge. To prevent or even reverse obesity and associated diseases, nutrition may be a pivotal player. However, despite the fact that many nutrition studies have examined the effects of dietary intake and weight loss, so far this has not blunted the ever-increasing obesity numbers. Consequently, there is a need for a paradigm shift within nutritional research.

## NEW APPROACHES IN NUTRITIONAL RESEARCH TO IDENTIFY DIET EFFECT BIOMARKERS

Traditional nutrition studies have focused on changes in only a few physiological intermediate endpoints, e.g. fasting levels of cholesterol, triglycerides (TG) and insulin, while obesity and associated metabolic diseases are complex and caused by a combination of multiple factors, such as genetic and environmental factors (5). Focussing on a few biomarkers only, does therefore not capture the complete health status of the body. A more comprehensive biomarker profile will be more characteristic of the health status of an individual compared to single markers (6). Additionally, so called classical biomarkers, e.g. fasting levels of glucose, insulin, total cholesterol and TG, are designed to diagnose a disease, or to examine the effects of pharmacological treatments when the disease has already manifested (7, 8). However, when examining the effects of nutrition the focus is primarily on prevention of the disease and on improvements of health, rather than on diagnosing or curing the disease once it is already clearly present (**Figure 1**). For this, it is necessary to identify subjects who are at risk for developing a disease as early as possible. This means measuring nearer to the onset of a disease when dynamics in pathways may have changed, but the system is still able to return to a healthy state with minimal intervention, i.e. dietary interventions. Hence, by identifying biomarkers that reflect these

1 Gezondheidsenquête / Leefstijlmonitor CBS and RIVM, 2019

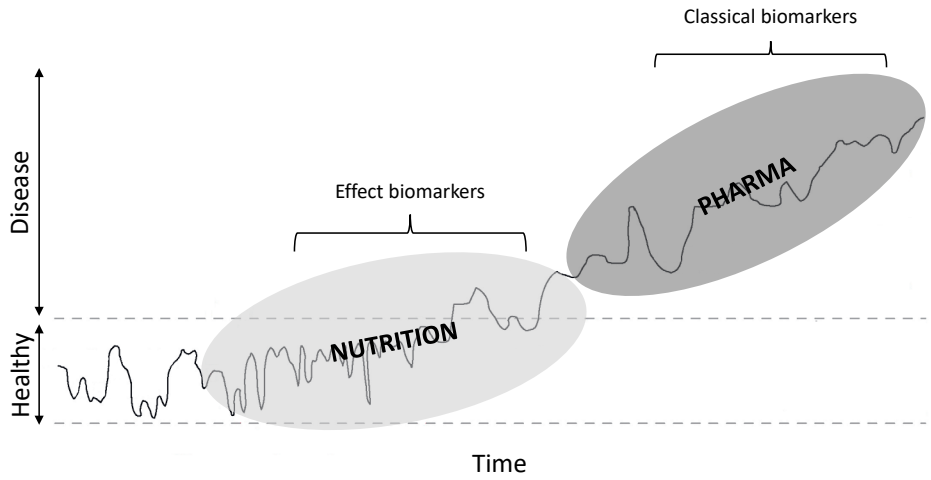


**Figure 1.** Development of complex multifactorial diseases, from a healthy to a disease state. Classical biomarkers are designed to assess the effects of pharmacological treatments when a subject is already in a disease state. Nutrition primarily plays a role in disease prevention and health improvements. To detect early perturbations from a healthy state, and to assess the more subtle and dispersed effects of nutrition, a shift is needed from classical biomarkers to effect biomarkers. Figure was adapted from Afman *et al.* (6) and van der Greef *et al.* (8).

early perturbations from a healthy state. For example, in the study by van Dijk *et al*. (9), a couple of seemingly healthy subjects with a normal BMI (<25 kg/m$^2$) clustered together among subjects with a high BMI (>30 kg/m$^2$), based on marker profiles of plasma proteins. As such, these diverging profiles may potentially be useful for early detection of obesity-linked disease development. Besides the difference in health and disease state focus, nutrition also causes more subtle and dispersed effects compared to the effects that can be expected from a pharmacological treatment (10). This is because nutrients and foods have a great variation in composition, concentration, as well as in complexity, leading to differences in affinities and specificities of numerous biological targets (6, 11). Pharmacological treatments on the other hand are often pure compounds that are administered in small doses, with high specificity for one or a few clear biological targets. By only taking into account changes in classical circulating biomarkers, the more subtle effects of nutrition may be missed. To measure and understand these more subtle and dispersed effects of nutrition, biomarkers are needed that are capable of detecting both subtle nutrition effects, as well as early perturbations from a healthy state. These biomarkers are called 'effect biomarkers', and refer to the functional response of the human body to an exposure (12), for example exposure to nutrition. The developments in the omics

field have enabled measurements of large numbers of parameters simultaneously, thereby paving the way to explore and identify these diet-induced effect biomarkers.

Omics measurements can be applied to each of the different layers of the central dogma of molecular biology, namely DNA, RNA, proteins, and metabolites. Genome-wide gene expression analysis is called 'transcriptomics' (10), and provides a comprehensive view on the expression of genes at a specified point in time in a given sample. Transcriptomics not only comprises the analyses of mRNA, but also includes expression changes in several shorter RNA sequences, such as microRNAs. MicroRNAs are small non-coding RNA molecules, that post-transcriptionally regulate gene expression typically by (partial) complementary binding to a target gene (13, 14). Profiling of microRNA expression changes is relatively unexplored in the field of nutrition and may help to identify effect biomarkers. Ideally, human samples from several organs important in cardiometabolic health should be investigated. However, due to ethical and practical issues, this is not feasible in human nutrition studies, wherein often healthy subjects participate. As a consequence, sampling is limited to more easily accessible tissues. In the context of obesity and associated metabolic abnormalities, especially the adipose tissue is an interesting tissue to examine, as it has been shown to be a key organ affected early in the development of obesity (15). The adipose tissue is therefore an exciting organ to identify possible effect biomarkers, and it may also give valuable insights in the metabolic effects of dietary interventions. The latter is exemplified by a study of van Dijk *et al.* (16), where a diet high in SFA induced a proinflammatory gene expression profile in subcutaneous adipose tissue samples, whereas a diet high in MUFA caused a more anti-inflammatory profile.

The consequences of cellular changes in metabolic organs and tissues will ultimately be reflected in the blood by secretion of proteins and metabolites in the circulation. Therefore, exploring metabolite profiles could also lead to the identification of effect biomarkers. The analysis of global metabolite profiles under a given set of conditions is called 'metabolomics' (17), and as metabolites reflect endpoints of cellular changes (18), they are interesting markers to examine. Unlike for transcriptomics studies, cellular tissue is not required for metabolomics studies. Therefore, easily accessible fluids can be used, such as urine or blood. Based on serum metabolomics, a distinction could be made between lean and obese individuals. Compared to the lean individuals, the obese individuals showed

an increase in proinflammatory metabolites and a decrease in metabolites linked with antioxidant properties (19). Also in terms of nutrition-induced effects, metabolomics studies have shown promising results. For example, Lankinen and colleagues (20) found a decrease in several proinflammatory lipid species after 8-week consumption of fatty fish. As circulating metabolites have shown to respond to nutrition, as well as to changes in health status, metabolomics is a promising technique to further explore for the identification of diet-induced effect biomarkers.

***Omics in nutrition research***

Not only can omics techniques be used to identify diet effect biomarkers, it can simultaneously increase our understanding of metabolic pathways that are affected by nutrition. Nutrients can be viewed as signaling molecules that can influence cellular gene expression, and thereby subsequently influence metabolite production. Nutrients can regulate gene expression via binding and activating transcription factors. The transcription factors act as nutrient sensors, by changing the level of expression of specific target genes in response to changes in dietary intake (21). Especially the dietary fatty acids have shown to have potent effects on gene expression and subsequent metabolite production. Diets rich in polyunsaturated fatty acids (PUFAs), especially the marine-based omega-3 fatty acids eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), have shown to be putative ligands for the transcription factor: peroxisome proliferator-activated receptor (PPAR) (22, 23). PPAR activation leads to the regulation of several genes involved in numerous lipid metabolic pathways, including fatty acid oxidation, fatty acid elongation and desaturation, and lipoprotein assembly and transport in several organs (24), thereby altering circulating metabolite levels of amongst others, TG-rich lipoproteins. Apart from activation through changes in dietary fatty acids intake, PPARs can also be activated upon fasting. During fasting, free fatty acids are released from the adipose tissue into the circulation where they travel to the liver. In the liver the free fatty acids can either be (partly) oxidized and converted into ketone bodies, or they can activate PPARα (21), one of the isotypes of PPAR (25). One well known gene target of PPARα is *ANGPTL4*. The resulting protein ANGPTL4 can subsequently inhibit the actions of an enzyme called lipoprotein lipase (LPL) (26, 27). LPL normally hydrolyzes circulating TG into non-esterified fatty acids that can subsequently be released into tissues. During a fasted state circulating TG levels are low, therefore LPL activity is inhibited, and hydrolyzation of circulating TG is decreased (28, 29). Interestingly, the functionality of PPAR during fasting has mostly been investigated in animal studies, and has yet to be confirmed in human studies.

## BIOMARKERS OF FOOD INTAKE

Apart from the proposed new approach from classical biomarkers to diet effect biomarkers, nutrition research faces another challenge. In most types of nutrition studies dietary intake cannot be controlled. However, to unravel true links between nutritional intake and health, it is essential to accurately assess dietary exposure. Currently, often self-reporting methods are used to assess dietary intake, such as 24 hour dietary recalls, food diaries and food-frequency questionnaires. However, these traditional instruments are subjective measures and contain well-known intake biases, such as recall and reporting biases, and do not take into account differences in metabolism (30, 31). Consequently, an accurate and objective measurement of dietary intake is needed, such as dietary exposure biomarkers, i.e. food intake biomarkers. An example of a relatively well-validated exposure biomarker are the alkylresorcinols for assessing whole grain wheat and rye intake (32-37). However, for most foods and dietary patterns, validated and accurate exposure biomarkers are limited or lacking (38). Also here omics approaches may advance the field. With the use of these techniques, in specific metabolomics, new biomarkers of food intake can be identified, which subsequently can be used in conjunction with the conventional methods to improve the assessment of dietary exposure (39, 40). Within this context, efforts are being undertaken to identify and evaluate (new) biomarkers of food intake (38, 41-43). To obtain a well-validated biomarker of food intake that can be used in nutrition research, the biomarker should be evaluated, e.g. on specificity and sensitivity (38), using three approaches: 1) the metabolite should be changed after an acute or chronic exposure to a specific food of interest, 2) the metabolite should be changed after exposure to a specific dietary pattern, and 3) the metabolite should be different between consumers and non-consumers in observational studies (44-46). Often studies that determine dietary exposure biomarkers only use one of these approaches, resulting in a plethora of articles with identified but seemingly not well-validated exposure biomarkers. Therefore, it would be very valuable if information on already identified exposure biomarkers from these different studies could be brought together through systematic literature searches.

## AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to identify potential biomarkers for diet-induced changes, i.e. effect biomarkers, as well as biomarkers of food intake, i.e. exposure biomarkers, using omics techniques. In **Chapters 2** to **5**, explorations of dietary exposure and effect biomarkers using different dietary

strategies in combination with various omics approaches are described. In **Chapter 2**, we compared the effects of a non-selective dietary PPAR agonist, n-3 long chain polyunsaturated fatty acids, to the effects of a synthetic selective PPAR agonist, fenofibrate, on plasma metabolomics. This was done in a 6-week randomized double-blind, placebo-controlled crossover intervention trial in twenty overweight and obese individuals. In **Chapter 3**, we examined the contribution of MUFA within a Mediterranean type diet on serum metabolites, using a targeted NMR platform. For this, we performed a randomized fully controlled three-arm parallel dietary intervention trial for 8 weeks, in healthy subjects at risk of the metabolic syndrome. Next, in **Chapter 4**, we explored the effects of two energy-restricted diets on microRNA expression in human subcutaneous adipose tissue. We did this in a parallel-designed 12 week 25% energy restricted dietary intervention, in which the two energy-restricted diets differed in diet quality. **Chapter 5** describes the effects of a prolonged fast on clearance mechanisms of plasma TG. This thesis is concluded with a review that focusses on exposure biomarkers for cocoa and liquorice products, **Chapter 6**. An overall discussion of chapters 2 to 6 is presented in **Chapter 7**.

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# 2

## Comparative analysis of the effects of fish oil and fenofibrate on plasma metabolomic profiles in overweight and obese individuals

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IN PREPARATION

## ABSTRACT

**Scope:** It is known that the drug fenofibrate as well as dietary fish oils can effectively lower triglyceride (TG) concentrations. However, a more detailed comparative analysis of the effects of fenofibrate and fish oil on plasma metabolome is missing.

**Methods and results:** Twenty overweight and obese subjects participated in a randomized, double-blind, placebo-controlled cross-over intervention trial and received in random order 3.7 g/d n-3 fatty acids, 200 mg fenofibrate, or placebo treatment for 6 weeks separated by a wash-out period of at least 2-weeks. A total of 442 plasma metabolites, including 22 ratios, were measured via GC-MS, and two LC-MS platforms. Among the treatments, 237 metabolites were significantly different ($q<0.05$). 22 metabolites were changed in the same direction by both fish oil and fenofibrate, and included a decrease in several relatively saturated TG species. 33 metabolites were changed specifically by fenofibrate, and included a decrease in total cholesterol, and total lyso-phosphatidylcholine (LPC). 54 metabolites were changed by fish oil, and included an increase in unsaturated TG-, LPC-, phosphatidylcholine-, and cholesterol ester-species.

**Conclusion:** Both fenofibrate as well as fish oil altered the metabolic plasma profile markedly by reducing the saturated state of the TG fraction. Fish oil additionally increased unsaturated lipid species, which was likely caused by an increased incorporation of the consumed unsaturated fatty acids in these circulating lipid species, and possibly by the combined effects of several fish oil induced regulatory pathways. Both fenofibrate and fish oil resulted in a similar beneficial decrease in relatively saturated TG species, that have been associated with a decreased risk for developing cardiovascular disease (CVD). Interestingly, the fish oil induced increase in the unsaturated lipid fraction has also been associated with a reduced CVD risk, and thereby points towards the power of a nutrient to change a lipid profile in a potentially beneficial way.

## INTRODUCTION

Elevated levels of plasma triglycerides are increasingly recognized as an important, independent, risk factor for cardiovascular disease (1-3), and it is one of the features of the metabolic syndrome. Lowering plasma triglyceride levels may therefore be a therapeutic target to lower cardiovascular disease risk.

A class of drugs that effectively lowers plasma triglycerides are fibrates. Fenofibrate in particular is one of the most commonly used fibrates (4). It selectively activates the ligand-dependent nuclear transcription factor peroxisome proliferator-activated receptor alpha (PPARα). Activation of PPARα leads to regulation of genes involved in lipid metabolism including fatty acid oxidation and lipoprotein assembly and transport. As a result, fenofibrate inhibits synthesis and secretion of triglycerides by the liver, and stimulates degradation of TG-rich lipoproteins, thereby not only reducing TG, but also very-low and low density lipoprotein cholesterol (5-7). Besides the therapeutic approach, plasma triglycerides can also effectively be lowered via dietary strategies in the form of dietary n-3 fatty acids (8-10). Fish oil specifically contains high amounts of n-3 fatty acids in the form of eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Similar to fenofibrate, these dietary n-3 fatty acids can act as ligands for transcription factor PPARα (11, 12). In addition, they can bind with high affinity to PPARγ and PPARδ, two other isotypes of PPARs that are also involved in lipid metabolism (13). However, while the TG lowering effects of fenofibrate have been attributed to PPARα activation, the TG lowering effects of fish oil likely occur independent of PPARα activation, as significant TG reductions have been observed PPARα deficient mice models upon fish oil (14). Instead, the TG lowering effects of fish oil are likely caused via a reduction of ApoB synthase in the liver, thereby impairing VLDL assembly and secretion (15-18), resulting in reduced TG levels.

The TG lowering effects of both fenofibrate and fish oils are well known (8, 19). Indeed, a placebo-controlled cross-over study by Bragt *et al.* (20) demonstrated that both fenofibrate and fish oil reduced plasma triglyceride concentration. Additionally, they observed a reduction in large VLDL, and sE-selectin, and an increase in HDL cholesterol by both fenofibrate and fish oil. However, a more detailed comparative analysis of the effects of fenofibrate and fish oil on plasma metabolomic profiles, including several lipid classes and lipid species, was not performed. To gain a more comprehensive insight into biochemical changes underlying the observed effects of fenofibrate and fish oil on cardiovascular

risk factors, and to explore which changes are shared between fenofibrate and fish oil, we performed metabolic profiling using gas chromatography - mass spectrometry (GC-MS) as well as liquid chromatography (LC)-MS platforms, in plasma samples from the study by Bragt and colleagues (20).

## MATERIALS AND METHODS

### *Subjects*

For the current manuscript, we used samples collected during a randomized, double-blind, placebo controlled crossover designed human intervention study, examining the effects of fenofibrate and fish oil on inflammatory parameters, vascular function and lipoprotein profiles in overweight and obese subjects. This study was performed in 2007 at Maastricht University and was approved by the Medical Ethics Committee of Maastricht University and was registered at EudraCT 2006-005743-28. An extensive description of the study design, recruitment, methods and results of the primary outcome measures can be found elsewhere (20). In short, 26 subjects (BMI ≥ 27 kg/m$^2$) were assigned in random order to a fish oil, a fenofibrate, and a placebo intervention for 6 weeks with a wash-out period of at least 2 weeks in between the intervention periods. During the fish oil intervention, subjects consumed 8 fish oil capsules daily (Marinol C-38™, Lipid Nutrition, Wormerveer, the Netherlands), providing approximately 3.7 g/d n-3 LCPUFA (1.7 g/d EPA and 1.2 g/d DHA,) and 2 capsules placebo-matching fenofibrate (200 mg/d cellulose). During the fenofibrate period, subjects consumed 2 capsules providing 200 mg/d micronized fenofibrate (Lipanthyl®, Fournier Laboratories, Dijon, France) and 8 placebo-matching fish oil capsules (containing 80% High Oleic Sunflower Oil, HOSO). During the placebo period, subjects received 8 HOSO capsules and 2 cellulose capsules.

### *Blood sampling*

In week 5 and 6 of each intervention period fasting EDTA, heparin, and NaF blood samples were taken in BD vacutainer® tubes (Becton Dickinson Company, NJ, USA). Plasma aliquots were snap-frozen and stored at -80°C until further analyses.

### *Plasma metabolic profiling*

Plasma samples from weeks 5 and 6 were pooled and changes in plasma free fatty acids (TNO, Zeist, the Netherlands) were analysed using liquid chromatography mass spectrometry (LC-MS) as described in (21). Changes in other plasma lipids were analysed using another LC-MS platform (University of

Leiden, The Netherlands), as described in (22). Further changes in metabolite profiles were analysed using gas chromatography mass spectrometry (GC-MS) (TNO, Zeist, the Netherlands), as described in (21). A total of 442 plasma metabolites, including 22 ratios, were quantified, of which 401 could be identified, including a wide variety of chemical classes (**Table S1: Supplemental Material**).



***Metabolic profiling analysis***

For the analysis of the metabolomics data, log transformed data was used. Main effects at the end of the intervention periods were tested with an ANOVA, with treatment as fixed factor and subject number as random factor. Subsequently, linear mixed models were used to assess between diet effects. Significant metabolites were selected using the false discovery rate (FDR) adjusted F-statistic (23) q-value <0.05. Linear mixed model analysis was performed using the *lme4* (24) and *emmeans* (25) R libraries. Furthermore, it was examined whether observed changes for fish oil and fenofibrate relative to the placebo group could be separated by using sparse partial least squares discriminant analysis (sPLS-DA). The sPLS-DA model was made using the *caret* (26) and *spls* (27) R libraries. Optimal hyperparameters were evaluated using grid search and selected based on the highest area under the receiver operating characteristic curve (AUROC) during five times repeated five-fold cross-validation. All analyses were done using R v4.0.2 (28).

## RESULTS

Ten men and ten women completed the trial. Compliance of the subjects was good, as calculated mean daily intakes of capsules was 95% or higher during all three intervention periods. Baseline characteristics of the study population, and the effects on the main outcome parameters have been reported elsewhere (20).

***Effects on metabolic profiles***

Two subjects had missing plasma samples after the fenofibrate intervention, and one subject had missing plasma samples after the placebo intervention. Their remaining samples were included in the univariate analysis. In total 442 metabolites, including 22 ratios, were measured using the FFA (42), GC-MS (204) and LC-MS (196) platforms. Of these, the precise identity of 41 metabolites is not established yet (all GCMS data). SPLS-DA analysis lead to a best fitting model consisting of 1 component, a kappa of 0.5, an eta of 0.9, and an area under the ROC curve of 1, indicating a perfect

ability to place the right subject in the right treatment group. Selected predictors in the sPLS model for the separation between the fenofibrate and fish oil interventions were: C20:5/C20:3w6 ratio, C20:4w6/ C20:5 ratio, C20:5/C20:4w3 ratio, C22:6, 'unknown_59b', phosphatidylcholine (PC)36:5, PC(38:6), PC(40:6), and triglyceride TG(40:6).

Subsequent univariate analysis on the 442 metabolites showed that after adjustment for multiple testing, 237 metabolites were significantly different among the treatments (ANOVA, q-value <0.05, **Figure 1**). Compared to placebo, 85 metabolites were significantly different after the fish oil treatment, and 102 metabolites were significantly different after the fenofibrate treatment.

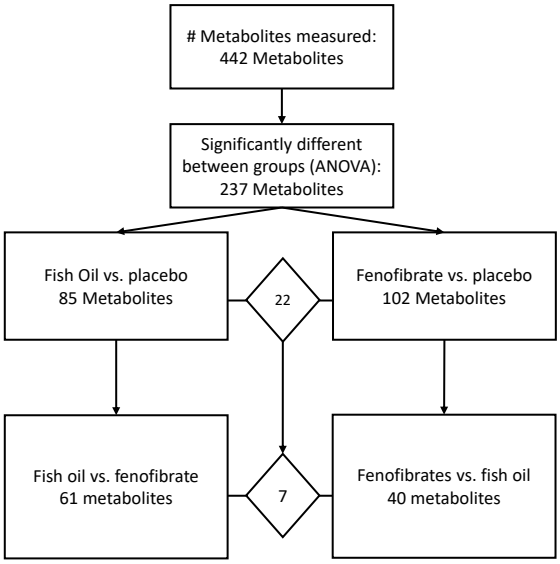



**Figure 1.** Flow chart of the number of metabolites differentially changed between the interventions. Main effects at the end of each intervention period were tested with an ANOVA, using treatment as fixed factor and subject number as random factor. Linear mixed models were used to assess between diet effects. Significant metabolites were selected using false discovery rate adjusted F-statistic (q-value <0.05). Numbers in the diamond shaped figures represent the overlapping metabolites.

### *Metabolites affected by both fish oil and fenofibrate*

Compared to placebo, 22 metabolites were significantly different after both fish oil and fenofibrate treatment. Of these, 15 were decreased after both fish oil and fenofibrate treatment compared to placebo (**Table 1a**). This included a decrease in total triglycerides and ten triglyceride species, with sizes ranging from 50 up to 58 C-atoms, and double bonds ranging from 1 double bond to 5 double bonds, indicating relatively saturated TGs, compared to the other measured TG species that contained

up to 18 double bonds. The other 7 metabolites were significantly oppositely affected by fish oil and fenofibrate treatment (**Table 1b**). This included the LPC(16:1) / LPC(16:0) ratio, which was significantly decreased by the fish oil treatment and significantly increased by the fenofibrate treatment. The other six metabolites were all significantly increased by fish oil and significantly decreased by fenofibrate treatment and included several (lyso)phosphatidylcholines containing a high number of double bonds.

***Metabolites affected by fenofibrate only***

Of the 102 metabolites that were significantly different by fenofibrate compared to placebo, 40 metabolites were also significantly different from fish oil (Figure 1). Of these 40 metabolites, 33 remained unchanged after fish oil treatment compared to placebo, and were therefore specifically changed by fenofibrate (**Table 2**). Fenofibrate decreased total cholesterol and several saturated (lyso)phosphatidylcholines (LPC), including the total sum of LPC, and total sum of lysophosphatidylethanolamines (LPE). Besides these reductions, fenofibrate decreased uric acid and its derivative methyluric acid, as well as ascorbic acid, the ratio of tryptophan to other amino acids, 1,5-anhydro-D-glucitol, and 2,3,4-trihydroxybutanoic acid, and increased 2,4-dihydroxybutanoic acid, and 2,3-dihydroxybutanoic acid.

***Metabolites affected by fish oil only***

Of the 85 metabolites that were significantly different by fish oil compared to placebo, 61 metabolites were also significantly different from fenofibrate (Figure 1). Of these 61 metabolites, 54 remained unchanged after fenofibrate treatment compared to placebo, and were therefore specifically changed by fish oil **Table 3**. Fish oil increased phosphatidylcholines containing 5 double bonds or more, triglycerides species containing 6 double bonds or more, and cholesterol esters 20:5 and 22:6. In addition, fish oil increased the total sum of cholesterol esters, campesterol, and the campesterol to cholesterol ratio, and several omega 3 and 6 fatty acids and their ratios. Lastly, three unknown compounds were increased by fish oil. Based on the molecular weight, one of these compounds (compound 'unknown 59b') is possibly related to vitamin E, which might represent the tocopherol content of the fish oil capsules.

## DISCUSSION

In this study, we characterized and compared the effects of the selective PPARα agonist fenofibrate (200 mg/d) with a dietary non-selective PPARα agonist n-3 LCPUFAs (3.7 g/d) on plasma metabolic

**Table 1.** Metabolites affected by both fenofibrate and fish oil. A. Shared effects of fenofibrate and fish oil treatments on the metabolites. B. Metabolites significantly oppositely affected by fenofibrate and fish oil. Main effects at the end of each intervention period were tested with an ANOVA, using treatment as fixed factor and subject number as random factor. Linear mixed models were used to assess between diet effects. Significant metabolites were selected using false discovery rate adjusted F-statistic (q-value <0.05). On the right of each table, the individual log fold changes are presented. These changes are presented on a colour scale ranging from blue (decreased, LR ≤ -0.1) to red (increased, LR ≥ 0.1). Individuals are depicted in the same colour in all tables. Grey boxes indicate missing values.

**A**

| | | ANOVA | Fenofibrate vs. placebo | | Fish oil vs. placebo | | Fish oil vs. Fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | *q* -value | logFC | *q* -value | logFC | *q* -value | logFC | *q* -value |
| Metabolites | C22:4 | **0.005** | -0.013 | **0.046** | -0.014 | **0.024** | -0.001 | NS |
| | LPC(20:4) | **0.001** | -0.042 | **0.005** | -0.029 | **0.049** | 0.013 | NS |
| | PC(36:2) | **0.001** | -0.206 | **0.009** | -0.166 | **0.025** | 0.039 | NS |
| | PE(38:4) | **<0.001** | -0.075 | **0.025** | -0.099 | **0.001** | -0.024 | NS |
| | TG | **<0.001** | -0.682 | **<0.001** | -0.402 | **0.020** | 0.280 | NS |
| | TG(50:1) | **<0.001** | -0.687 | **0.001** | -0.436 | **0.027** | 0.251 | NS |
| | TG(50:2) | **<0.001** | -0.544 | **0.001** | -0.416 | **0.004** | 0.128 | NS |
| | TG(50:3) | **<0.001** | -0.357 | **0.002** | -0.250 | **0.015** | 0.106 | NS |
| | TG(52:2) | **<0.001** | -0.682 | **0.002** | -0.576 | **0.004** | 0.106 | NS |
| | TG(52:3) | **<0.001** | -0.600 | **<0.001** | -0.401 | **0.008** | 0.199 | NS |
| | TG(54:3) | **<0.001** | -0.498 | **0.002** | -0.423 | **0.003** | 0.074 | NS |
| | TG(54:4) | **<0.001** | -0.470 | **0.002** | -0.370 | **0.006** | 0.101 | NS |
| | TG(55:3) | **0.005** | -0.006 | **0.024** | -0.005 | **0.048** | 0.001 | NS |
| | TG(56:4) | **0.004** | -0.041 | **0.026** | -0.037 | **0.040** | 0.004 | NS |
| | TG(58:5) | **0.004** | -0.003 | **0.029** | -0.003 | **0.026** | 0.000 | NS |

**B**

| | | ANOVA | Fenofibrate vs. placebo | | Fish oil vs. placebo | | Fish oil vs. Fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | *q* -value | logFC | *q* -value | logFC | *q* -value | logFC | *q* -value |
| Metabolites | alk_PC(40:6) | **<0.001** | -0.004 | **0.016** | 0.005 | **0.004** | 0.009 | **<0.001** |
| | LPC(16:1)/LPC(16:0) | **<0.001** | 0.004 | **0.044** | -0.005 | **0.025** | -0.009 | **<0.001** |
| | LPC(22:6) | **<0.001** | -0.021 | **0.031** | 0.036 | **<0.001** | 0.058 | **<0.001** |
| | LPE(22:6) | **<0.001** | -0.004 | **0.027** | 0.004 | **0.009** | 0.008 | **<0.001** |
| | PC(36:0) | **<0.001** | -0.001 | **0.010** | 0.001 | **0.049** | 0.001 | **<0.001** |
| | PC(38:6) | **<0.001** | -0.207 | **0.024** | 0.473 | **<0.001** | 0.680 | **<0.001** |
| | PC(40:7) | **<0.001** | -0.018 | **0.025** | 0.015 | **0.049** | 0.033 | **<0.001** |

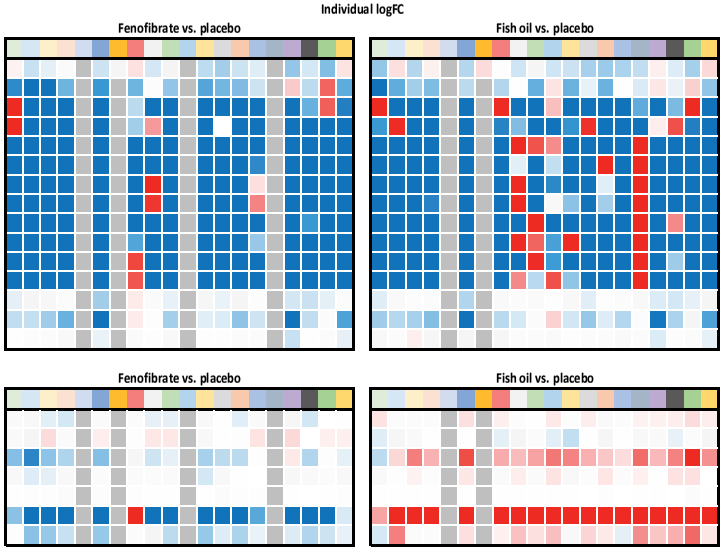


**Table 2.** Metabolites affected by fenofibrate only. Main effects at the end of each intervention period were tested with an ANOVA, using treatment as fixed factor and subject number as random factor. Linear mixed models were used to assess between diet effects. Significant metabolites were selected using false discovery rate adjusted F-statistic (q-value <0.05). On the right of each table, the individual log fold changes are presented. These changes are presented on a colour scale ranging from blue (decreased, LR ≤ -0.1) to red (increased, LR ≥ 0.1). Individuals are depicted in the same colour in all tables. Grey boxes indicate missing values.

| | | ANOVA | Fenofibrate vs. placebo | | Fish oil vs. placebo | | Fish oil vs. Fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | *q*-value | logFC | *q*-value | logFC | *q*-value | logFC | *q*-value |
| Metabolites | 1,5-Anhydro-D-Glucitol | **<0.001** | -0.158 | **<0.001** | -0.037 | NS | 0.122 | **0.001** |
| | 2,3,4-Trihydroxybutanoic acid | **0.005** | -0.014 | **0.040** | 0.002 | NS | 0.016 | **0.009** |
| | 2,3-Dihydroxybutanoic acid | **<0.001** | 0.003 | **0.002** | 0.000 | NS | -0.003 | **<0.001** |
| | 2,4-Dihydroxybutanoic acid | **<0.001** | 0.015 | **<0.001** | -0.002 | NS | -0.018 | **<0.001** |
| | 4-oxo-proline | **<0.001** | -0.009 | **0.004** | 0.000 | NS | 0.008 | **0.002** |
| | alk LPC(16:1) | **0.009** | -0.003 | **0.043** | 0.000 | NS | 0.002 | **0.044** |
| | ascorbic acid | **0.002** | -0.036 | **0.021** | 0.000 | NS | 0.036 | **0.009** |
| | C18:0 Fatty acid | **0.002** | -0.052 | **0.020** | 0.001 | NS | 0.053 | **0.007** |
| | C18:2 / C20:3w6 ratio | **<0.001** | -0.966 | **0.024** | 0.375 | NS | 1.341 | **<0.001** |
| | C20:4w6 / C20:3w6 ratio | **<0.001** | -0.693 | **0.049** | 0.522 | NS | 1.215 | **<0.001** |
| | CholE18:2 | **0.002** | -0.012 | **0.014** | -0.002 | NS | 0.010 | **0.018** |
| | Cholesterol | **<0.001** | -0.070 | **0.005** | 0.009 | NS | 0.079 | **<0.001** |
| | DG(44:9) | **<0.001** | -0.002 | **0.034** | 0.002 | NS | 0.004 | **<0.001** |
| | LPC sum | **<0.001** | -0.244 | **0.004** | -0.035 | NS | 0.209 | **0.005** |
| | LPC(16:0) | **<0.001** | -0.198 | **0.004** | -0.006 | NS | 0.192 | **0.001** |
| | LPC(18:0) | **<0.001** | -0.154 | **0.002** | 0.004 | NS | 0.158 | **<0.001** |
| | LPE sum | **<0.001** | -0.013 | **0.004** | -0.001 | NS | 0.011 | **0.003** |
| | LPE(18:0) | **<0.001** | -0.006 | **0.005** | 0.000 | NS | 0.006 | **0.001** |
| | methyl uric acid, isomeer 1 | **<0.001** | -0.001 | **0.013** | 0.000 | 0.072 | 0.001 | **<0.001** |
| | methyl uric acid, isomeer 2 | **<0.001** | -0.001 | **0.005** | 0.000 | NS | 0.001 | **<0.001** |
| | P7478_uk 05 | **0.002** | 0.015 | **0.024** | -0.001 | NS | -0.016 | **0.006** |
| | P7502_uk 05 | **<0.001** | 0.002 | **0.004** | 0.000 | NS | -0.003 | **<0.001** |
| | P7881_uk 22 | **0.001** | 0.003 | **0.014** | 0.000 | NS | -0.003 | **0.009** |
| | PC(32:0) | **<0.001** | -0.029 | **0.034** | 0.017 | NS | 0.046 | **<0.001** |
| | PC(40:8) | **<0.001** | -0.004 | **0.025** | 0.003 | NS | 0.006 | **<0.001** |
| | SM(20:0) | **0.004** | -0.019 | **0.026** | -0.002 | NS | 0.018 | **0.025** |
| | SM(21:0) | **0.007** | -0.009 | **0.040** | -0.001 | NS | 0.008 | **0.037** |
| | SM(d16:1/20:0) | **0.005** | -0.057 | **0.043** | 0.004 | NS | 0.061 | **0.018** |
| | TG(40:8) | **<0.001** | -0.003 | **0.030** | 0.002 | NS | 0.005 | **<0.001** |
| | TG(52:4) | **<0.001** | -0.582 | **0.000** | -0.295 | 0.050 | 0.287 | **0.044** |
| | Tryptophan / other amino acids | **0.007** | -0.025 | **0.036** | -0.001 | NS | 0.024 | **0.031** |
| | Uric acid | **<0.001** | -0.759 | **<0.001** | 0.020 | NS | 0.780 | **<0.001** |
| | Uridine | **<0.001** | 0.014 | **0.001** | -0.003 | NS | -0.017 | **<0.001** |

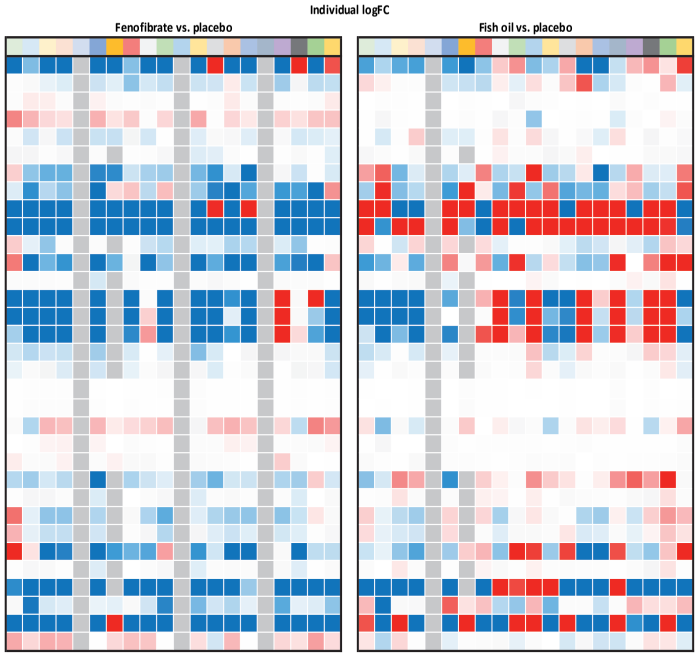
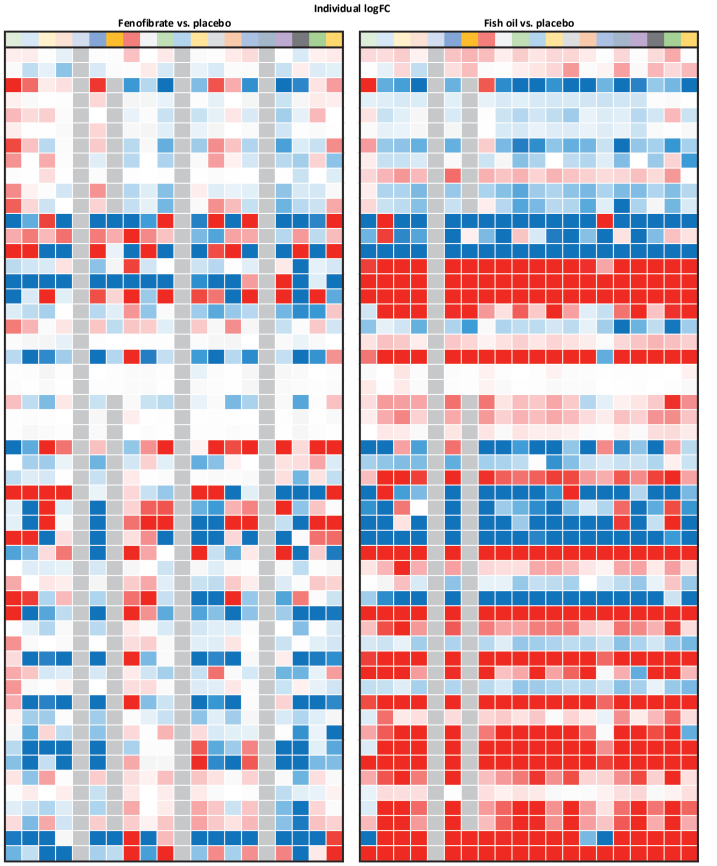


**Table 3.** Metabolites affected by fish oil only. Main effects at the end of each intervention period were tested with an ANOVA, using treatment as fixed factor and subject number as random factor. Linear mixed models were used to assess between diet effects. Significant metabolites were selected using false discovery rate adjusted F-statistic (q-value <0.05). On the right of each table, the individual log fold changes are presented. These changes are presented on a colour scale ranging from blue (decreased, LR ≤ -0.1) to red (increased, LR ≥ 0.1). Individuals are depicted in the same colour in all tables. Grey boxes indicate missing values.

| | | ANOVA | Fenofibrate vs. placebo | | Fish oil vs. placebo | | Fish oil vs. Fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | *q* -value | logFC | *q* -value | logFC | *q* -value | logFC | *q* -value |
| Metabolites | 32006/01.07.02 uk x 21 | **<0.001** | 0.004 | NS | 0.014 | **<0.001** | 0.010 | **0.001** |
| | 32006\01.08.02 uk x 55 | **<0.001** | -0.008 | NS | 0.010 | **0.039** | 0.018 | **<0.001** |
| | alk PC sum | **0.004** | -0.015 | NS | -0.082 | **0.015** | -0.067 | **0.044** |
| | alk PC(34:1) | **<0.001** | -0.001 | NS | -0.010 | **<0.001** | -0.009 | **<0.001** |
| | alk PC(34:3) | **0.001** | 0.000 | NS | -0.012 | **0.010** | -0.013 | **0.005** |
| | alk PC(36:2) | **<0.001** | -0.001 | NS | -0.007 | **<0.001** | -0.006 | **0.001** |
| | alk PC(36:4) | **<0.001** | -0.008 | NS | -0.047 | **0.001** | -0.039 | **0.006** |
| | alk PC(36:5) | **0.004** | -0.002 | NS | -0.019 | **0.020** | -0.017 | **0.032** |
| | alk PC(36:6) | **<0.001** | -0.001 | NS | 0.025 | **<0.001** | 0.026 | **<0.001** |
| | alk PC(38:4) | **<0.001** | -0.004 | NS | -0.035 | **<0.001** | -0.030 | **<0.001** |
| | alk PC(38:5) | **0.002** | -0.005 | NS | -0.029 | **0.010** | -0.024 | **0.034** |
| | C16-C18 sum + C20-C22 sum | **<0.001** | -0.151 | NS | -0.578 | **<0.001** | -0.427 | **0.002** |
| | C20:3w9 / C20:4w6 ratio | **<0.001** | 0.027 | NS | -0.050 | **0.019** | -0.076 | **<0.001** |
| | C20:4w6 / C20:5 ratio | **<0.001** | 0.082 | NS | -1.752 | **<0.001** | -1.834 | **<0.001** |
| | C20:5 | **<0.001** | -0.012 | NS | 0.209 | **<0.001** | 0.221 | **<0.001** |
| | C20:5 / C20:3w6 ratio | **<0.001** | -0.666 | NS | 2.558 | **<0.001** | 3.224 | **<0.001** |
| | C20:5 / C20:4w3 ratio | **<0.001** | -0.034 | NS | 1.406 | **<0.001** | 1.439 | **<0.001** |
| | C22:5w3 | **<0.001** | -0.025 | NS | 0.060 | **<0.001** | 0.085 | **<0.001** |
| | C22:5w6 / C22:6w3 ratio | **<0.001** | 0.009 | NS | -0.037 | **<0.001** | -0.046 | **<0.001** |
| | C22:6 Fatty acid | **<0.001** | -0.003 | NS | 0.014 | **<0.001** | 0.017 | **<0.001** |
| | C22:6w3 | **<0.001** | -0.054 | NS | 0.258 | **<0.001** | 0.312 | **<0.001** |
| | Campesterol | **<0.001** | -0.002 | NS | 0.002 | **0.039** | 0.004 | **<0.001** |
| | campesterol /cholesterol ratio | **<0.001** | -0.001 | NS | 0.002 | **0.012** | 0.003 | **<0.001** |
| | CholE sum | **<0.001** | -0.015 | NS | 0.026 | **0.003** | 0.041 | **<0.001** |
| | CholE20:5 | **<0.001** | -0.001 | NS | 0.026 | **<0.001** | 0.027 | **<0.001** |
| | CholE22:6 | **<0.001** | -0.002 | NS | 0.006 | **<0.001** | 0.008 | **<0.001** |
| | LPC(18:1)/LPC(18:0) ratio | **<0.001** | 0.060 | NS | -0.098 | **0.020** | -0.158 | **<0.001** |
| | LPC(20:3) | **<0.001** | -0.009 | NS | -0.030 | **<0.001** | -0.021 | **0.001** |
| | LPC(20:5) | **<0.001** | -0.007 | NS | 0.082 | **<0.001** | 0.089 | **<0.001** |
| | LPC(36:4)/LPC(16:0) ratio | **0.002** | 0.043 | NS | -0.151 | **0.049** | -0.193 | **0.004** |
| | PC(32:1) | **0.007** | -0.001 | NS | -0.057 | **0.048** | -0.056 | **0.037** |
| | PC(34:1) | **0.002** | 0.004 | NS | -0.202 | **0.019** | -0.206 | **0.010** |
| | PC(36:3) | **<0.001** | -0.069 | NS | -0.379 | **<0.001** | -0.310 | **<0.001** |
| | PC(36:5) | **<0.001** | -0.041 | NS | 0.862 | **<0.001** | 0.903 | **<0.001** |
| | PC(36:6) | **<0.001** | -0.006 | NS | 0.026 | **<0.001** | 0.032 | **<0.001** |
| | PC(38:2) | **0.001** | 0.004 | NS | -0.014 | **0.025** | -0.018 | **0.002** |
| | PC(38:3) | **<0.001** | 0.022 | NS | -0.198 | **<0.001** | -0.220 | **<0.001** |
| | PC(38:5) | **<0.001** | -0.068 | NS | 0.457 | **<0.001** | 0.525 | **<0.001** |
| | PC(38:7) | **<0.001** | -0.009 | NS | 0.046 | **<0.001** | 0.055 | **<0.001** |
| | PC(40:4) | **<0.001** | 0.001 | NS | -0.026 | **<0.001** | -0.027 | **<0.001** |
| | PC(40:6) | **<0.001** | -0.086 | NS | 0.296 | **<0.001** | 0.382 | **<0.001** |
| | PE(38:7) | **<0.001** | -0.008 | NS | 0.077 | **<0.001** | 0.086 | **<0.001** |
| | TG(40:4) | **<0.001** | 0.000 | NS | -0.027 | **<0.001** | -0.027 | **<0.001** |
| | TG(40:6) | **<0.001** | -0.084 | NS | 0.295 | **<0.001** | 0.378 | **<0.001** |
| | TG(40:7) | **<0.001** | -0.015 | 0.053 | 0.016 | **0.025** | 0.031 | **<0.001** |
| | TG(54:7) | **<0.001** | -0.018 | NS | 0.109 | **<0.001** | 0.127 | **<0.001** |
| | TG(56:7) | **<0.001** | -0.126 | NS | 0.385 | **<0.001** | 0.510 | **<0.001** |
| | TG(56:8) | **<0.001** | -0.075 | NS | 0.342 | **<0.001** | 0.417 | **<0.001** |
| | TG(58:10) | **<0.001** | 0.003 | NS | 0.059 | **<0.001** | 0.056 | **<0.001** |
| | TG(58:6) | **<0.001** | -0.001 | NS | 0.008 | **0.013** | 0.009 | **0.002** |
| | TG(58:8) | **<0.001** | -0.011 | NS | 0.077 | **<0.001** | 0.088 | **<0.001** |
| | TG(58:9) | **<0.001** | 0.000 | NS | 0.073 | **<0.001** | 0.073 | **<0.001** |
| | Totaal omega3 fatty acids | **<0.001** | -0.162 | 0.079 | 0.311 | **<0.001** | 0.473 | **<0.001** |
| | unknown 59b | **<0.001** | -0.032 | NS | 0.774 | **<0.001** | 0.806 | **<0.001** |



profiles in overweight and obese subjects. We demonstrated that both fish oil as well as fenofibrate alter the metabolic plasma profile markedly, with clear intervention specific and shared effects between the two treatments.



Fenofibrate and the fish oil intervention reduced total triglycerides (TGs), and several TG species containing less than five double bonds. The total TG lowering effects of fenofibrate as well as fish oil are well documented (8, 19). The effects of fenofibrate have previously been ascribed to 1) increased TG clearance by activation of lipoprotein lipase via PPAR mediated decrease of apo C-III gene expression (29-32), and 2) a decreased TG production and secretion from the liver via upregulation of fatty acid beta oxidation via PPAR activation (33). The TG lowering effects of fish oil on the other hand have previously been ascribed to a reduction of ApoB synthase in the liver, thereby impairing VLDL assembly and secretion (15-18). We here demonstrate that besides a decrease in total TG, both fenofibrate and fish oil reduced specifically the TG species containing fewer than 5 double bonds, indicating a remodelling of the TG species towards a lower saturation status of the TG fraction. As higher proportions of saturated fatty acids have been positively associated with obesity and insulin resistance (34-37), the observed shift towards a lower saturated state points towards a more beneficial profile. In addition, TG species with a high saturated state have shown consistent and strong positive associations with cardiovascular disease risk (38). The observed decrease in saturated TG species might have been caused by an increased activity of enzymes responsible for desaturation of fatty acids, such as FADS1, FADS2 and SCD1, which are known targets of the transcriptional regulator PPAR (39). In line with this, the fenofibrate intervention decreased the C18:2/C20:3w6 ratio, which is used as an estimate for an increase in the activity of the enzyme delta 6 desaturase (D6D), encoded by the FADS2 gene. The fish oil intervention did not decrease this ratio, but did increase the C20:4w6/C20:3w6 ratio, which is used as an estimate for increased activity delta 5 desaturase (D5D). This enzyme, encoded by the FADS1 gene, is required for the synthesis of highly unsaturated fatty acids (40). An increase in D5D activity, as estimated by this ratio, has been described upon replacing saturated fatty acids with PUFA (41). As fenofibrate decreased this ratio, the question remains whether this effect can indeed be attributed to PPAR activation. Especially since fish oil is not only a ligand for PPARα, but can also activate other metabolic pathways involved in lipid metabolism (42). For example, fish oils have shown to inhibit the sterol regulatory element-binding protein 1c (SREBP-1c) (43), and can thereby inhibit *de novo* lipogenesis in the liver. This might also partially explain the observed decrease in the relatively

saturated TG species.

Thus, both fenofibrate and fish oil reduced the total TG class, and the saturated status of the TG fraction. The effect of fenofibrate is likely explained by PPAR mediated expression of several genes involved in desaturase enzyme activities of fatty acids, such as FADS1, FADS2 and SCD1. The effects of fish oils are likely mediated via multiple mechanistic pathways.

Furthermore, fenofibrate decreased the total sum of LPC species. LPC species have been found to be increased in atherosclerotic plaques (44), and have shown positive associations with obesity and features of subcutaneous obesity (45). Fenofibrate specifically decreased total cholesterol and one cholesterol ester, namely cholesterol ester(18:2); cholesteryl linoleate. Cholesteryl linoleate has been found to be the main cholesterol species in atherosclerotic plaques (44), and a decrease may therefore point towards a more beneficial fatty acid profile. The observed decrease in total cholesterol might in part have been mediated via PPAR activated expression of ATP-binding cassette transporter 1 (ABCA1) (39), leading to an increased reverse cholesterol transport (46). Fenofibrate also decreased uric acid, as well as its derivative methyluric acid, which are well-known effects, caused by an increase in renal urate excretion (47-49). Lastly, fenofibrate decreased 1,5-anydro-D-glucitol, a marker of glycaemic control in diabetes patients (50), which has not previously been reported to be decreased by fenofibrate.

Fish oil intervention reduced total TG, and caused a shift within the TG class by both decreasing the more saturated TG species, as well as increasing unsaturated TG species. Next to lower proportions of the more saturated fatty acids, higher proportions of longer chain omega-3 and 6 polyunsaturated fatty acids in plasma TG have been inversely associated with obesity and insulin resistance (34-37, 51). This might thus point towards an even more beneficial profile (34, 52) compared to the profile induced by fenofibrate treatment. In line with our results, similar increases in desaturation of TG species in the circulation have previously been observed in several dietary intervention studies examining the effects of either fish oils or fatty fish intake (53, 54).

Apart from the PPAR mediated activation of FADS1 and the activation of other regulatory pathways (e.g. SREBP-1c), the observed increase by fish oil in unsaturated TG-, PC-, LPC-, and cholesterol

ester-species may have been caused by an increased supply of fatty acids containing a high amount of double bonds via the fish oil, that can subsequently be incorporated in circulating lipid species. This substantiates the suggestion that the average dietary fatty acid composition in the circulation represents the dietary intake during the last 3 to 6 weeks (41, 55). In line with our results, the increase in cholesterol ester(20:5) was also observed after 8 weeks of consumption of a healthy diet containing fatty fish in subjects with features of the metabolic syndrome (56), and after 7 weeks of a lower dose of fish oil (1.6 g/d EPA+DHA) (57). Similar to our study, the latter study also reported an increase in LPC(22:6) (57). The exact consequences of the changes in unsaturated PC- and LPC-species for cardiovascular health remain to be elucidated.

Thus, apart from the shared effects of fenofibrate and fish oil on total TG and the more saturated TG species, the fish oil intervention additionally increased several unsaturated TGs, PCs, LPCs, and cholesterol ester species. This is likely mainly due to an increased incorporation in the circulating lipid species of the consumed unsaturated fatty acids, EPA and DHA, but might also partly be regulated by PPAR mediated activation of genes involved in fatty acid desaturase activity.

Next to EPA and DHA, the fish oil capsules contained a mix of natural tocopherols totalling to an amount of around 21 mg per day (~32 IU), which was potentially reflected in the metabolic profiling by compound ‘unknow 59b’. As vitamin E was not present in the fibrate supplement, effects of vitamin E in the fish oil intervention cannot be completely excluded. The amount of fish oil (3.7 grams) taken daily was very high in comparison to the recommended daily dietary intake of EPA and DHA, of 0.2 gram as set by the Dutch Health Council. This might partly explain why the observed effects on total TG and saturated TG species were comparable between the fenofibrate and the fish oil intervention. For the treatment of patients with hypertriglyceridemia, high doses (2-4 g/day total EPA+DHA) are recommended by the American Heart Association (9), and in the landmark REDUCE-IT trial (58) a dose of 4 g/day n-3 fatty acids has shown to be effective as secondary prevention of cardiovascular events in high-risk patients treated with statin.

A limitation of the methods used in this study is that we could only identify the sum compositions of the lipid species by the used platforms, and not the precise identity of the molecular lipid species (59). The strength of this study lies in its design; by including both the fenofibrate as well as the fish oil

treatment in a crossover study, we could directly compare the effects of a pharmacological treatment to the effects of a nutrient intervention. Lastly, by combining two LC-MS platforms and one GC-MS platform, we obtained a comprehensive view of the individual metabolic changes caused by fish oil and fenofibrate in the circulation.

In summary, the current study in which we compared the effects of a 6 week intervention with either fenofibrate or fish oil, revealed shared and specific effects of both interventions on plasma metabolome, especially on the lipid metabolites. Both fenofibrate and fish oil reduced the total TG class, and the saturated status of the TG fraction. The decrease in the relatively saturated TG species by fenofibrate can almost entirely be attributed to PPARα activation, while this effect in the fish oil intervention is potentially mediated via the activation of other mechanistic pathways. The fish oil intervention additionally increased several unsaturated TGs, PCs, LPCs, and cholesterol ester species. This is likely caused by an increased incorporation in the circulating lipid species of the consumed unsaturated fatty acids, EPA and DHA, but might also partly be regulated by the combined effects of PPAR mediated activation of genes involved in fatty acid desaturase activity. In conclusion, both fenofibrate, a drug, and fish oil, a nutrient, resulted in a similar beneficial decrease in relatively saturated TG species, that has been associated with a decreased risk for developing CVD. Interestingly, the fish oil consumption additionally induced an increase in the unsaturated lipid fraction, which has also been associated with a reduced CVD risk. This points towards the power of a nutrient to change a lipid profile in a potentially beneficial way.

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## CONFILCT OF INTEREST

There is no conflict of interest.

## AUTHOR CONTRIBUTIONS

*M.C.E.B* and *R.P.M.* designed and executed the study. *C.C.J.R.M.* and *R.W.J.H.* analysed the data.

*S.W.* cleaned the metabolomics dataset and calculated the metabolite ratios. *C.C.J.R.M.* wrote the manuscript, which was critically reviewed and improved by *S.W., R.P.M.,* and *L.A.A.* All authors read and approved the final manuscript.

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## LIST OF ABBREVIATIONS

| Abbreviation | Full description |
|---|---|
| ABCA1 | ATP-binding cassette transporter 1 |
| CVD | Cardiovascular Disease |
| D5D | Delta 5 Desaturase |
| D6D | Delta 6 Desaturase |
| DHA | Docosahexaenoic Acid |
| EPA | Eicosapentaenoic Acid |
| FDR | False Discovery Rate |
| FFA | Free Fatty Acids |
| GCMS | Gas Chromatography - Mass Spectrometry |
| HDL | High Density Lipoprotein |
| HOSO | High Oleic Sunflower Oil |
| LCMS | Liquid Chromatography - Mass Spectrometry |
| LCPUFA | Long Chain Polyunsaturated Fatty Acids |
| LPC | Lyso-Phosphatidylcholine |
| LPE | Lyso-Phosphatidylethanolamine |
| PC | Phosphatidylcholine |
| PPAR | Peroxisome Proliferator-Activated Receptor |
| ROC | Receiver Operating Characteristic |
| sPLS-DA | sparse Partial Least Squares Discriminant Analysis |
| SREBP-1c | Sterol Regulatory Element-Binding Protein 1c |
| TG | Triglyceride |
| VLDL | Very Low Density Lipoprotein |

## SUPPLEMENTAL MATERIAL

**Supplemental Table 1.** Overview of the measured metabolites. Platform 1 = GCMS, Platform 2 = LCMS polar, Platform 3 = LCMS FFA. *Continues on the next pages.*

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| 1,2,4-Trihydroxybenzene | 1 | **0.076** | -0.001 | **0.174** | 0.000 | 1.000 | 0.000 | **0.271** |
| 1,2DG-MwND592 | 1 | **0.033** | -0.010 | **0.062** | -0.005 | 0.607 | 0.005 | 0.528 |
| 1,2DG-MwND618 | 1 | **0.008** | -0.003 | **0.129** | -0.004 | **0.025** | -0.001 | 0.892 |
| 1,2DG-MwND620 | 1 | **0.090** | -0.006 | **0.160** | -0.004 | 0.653 | 0.003 | 0.793 |
| 1,2-dipalmitoylglycerol | 1 | 0.713 | -0.004 | 0.887 | 0.000 | 1.000 | 0.004 | 0.919 |
| 1,3DG-MwND592 | 1 | **0.118** | -0.027 | **0.207** | -0.019 | 0.557 | 0.008 | 0.935 |
| 1,3DG-MwND620 | 1 | **0.056** | -0.003 | **0.109** | -0.001 | 0.840 | 0.002 | **0.499** |
| 1,5-Anhydro-D-Glucitol | 1 | **0.000** | -0.158 | **0.000** | -0.037 | 0.672 | 0.122 | **0.001** |
| 10227\01.03 uk x 20 | 1 | **0.169** | -0.001 | **0.252** | 0.000 | 1.000 | 0.000 | 0.622 |
| 10227\01.03 uk x 25 | 1 | **0.321** | 0.000 | **0.451** | 0.000 | 0.850 | 0.000 | 0.989 |
| 1-Methylhistidine | 1 | **0.339** | -0.018 | 0.881 | -0.034 | 0.577 | -0.016 | 0.926 |
| 1-Monooleoylglycerol | 1 | **0.156** | -0.001 | **0.295** | 0.000 | 0.521 | 0.000 | 0.999 |
| 1-Monopalmitoylglycerol | 1 | **0.141** | -0.001 | 0.641 | 0.001 | 0.943 | 0.003 | **0.238** |
| 1-Monostearoylglycerol | 1 | 0.643 | 0.000 | 0.951 | 0.000 | 1.000 | 0.001 | 0.825 |
| 2,3,4-Trihydroxybutanoic acid | 1 | **0.005** | -0.014 | **0.040** | 0.002 | 1.000 | 0.016 | **0.009** |
| 2,3-Dihydroxybutanoic acid | 1 | **0.000** | 0.003 | **0.002** | 0.000 | 1.000 | -0.003 | **0.000** |
| 2,4-Dihydroxybutanoic acid | 1 | **0.000** | 0.015 | **0.000** | -0.002 | 0.943 | -0.018 | **0.000** |
| 2-Hydroxybutanoic acid | 1 | **0.340** | -0.009 | 1.000 | -0.040 | 0.613 | -0.031 | 0.735 |
| 2-hydroxyhippuric acid | 1 | **0.017** | 0.001 | 0.853 | -0.003 | **0.235** | -0.005 | **0.037** |
| 2-Hydroxypentanoic acid | 1 | 0.558 | -0.002 | 0.991 | -0.004 | 0.849 | -0.003 | 0.935 |
| 2-Hydroxypiperidine | 1 | **0.323** | -0.001 | 0.547 | 0.000 | 1.000 | 0.001 | 0.581 |
| 2-monopalmitoylglycero | 1 | **0.002** | 0.000 | **0.073** | 0.000 | 0.736 | 0.000 | **0.003** |
| 31944 uk 02 | 1 | **0.011** | -0.054 | **0.132** | 0.017 | 1.000 | 0.071 | **0.023** |
| 31944 uk 07 | 1 | **0.147** | 0.000 | 0.526 | 0.000 | 1.000 | 0.000 | **0.265** |
| 31944 uk 11 | 1 | **0.358** | 0.000 | 0.574 | 0.000 | 0.704 | 0.000 | 0.999 |
| 31944 uk 12 | 1 | **0.064** | 0.014 | 0.929 | 0.051 | **0.165** | 0.037 | **0.374** |
| 31944 uk 15 | 1 | **0.443** | 0.000 | 0.986 | -0.001 | 0.698 | 0.000 | 0.892 |
| 32006/01.07.02 uk x 21 | 1 | **0.000** | 0.004 | **0.387** | 0.014 | **0.000** | 0.010 | **0.001** |
| 32006\01.08.02 uk x 10 | 1 | **0.040** | -0.001 | **0.288** | 0.000 | 0.996 | 0.001 | **0.071** |
| 32006\01.08.02 uk x 25 | 1 | 0.982 | 0.000 | 1.000 | 0.000 | 1.000 | 0.000 | 0.999 |
| 32006\01.08.02 uk x 40 | 1 | **0.475** | -0.014 | 0.951 | 0.016 | 1.000 | 0.030 | 0.654 |
| 32006\01.08.02 uk x 5 | 1 | **0.045** | 0.019 | **0.121** | 0.003 | 1.000 | -0.016 | **0.198** |
| 32006\01.08.02 uk x 55 | 1 | **0.000** | -0.008 | **0.174** | 0.010 | **0.039** | 0.018 | **0.000** |
| 32006\01.08.02 uk x 60 | 1 | **0.055** | -0.001 | 0.885 | 0.003 | **0.443** | 0.005 | **0.102** |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| 3-Amino-2-piperidinon | 1 | **0.054** | 0.002 | **0.073** | 0.001 | **0.498** | -0.001 | 0.695 |
| 3-Hydroxybutanoic acid | 1 | 0.834 | -0.023 | 0.945 | -0.015 | 1.000 | 0.008 | 0.999 |
| 3-Methyl-2-oxo-valeric acid | 1 | **0.186** | 0.001 | 0.656 | -0.001 | 1.000 | -0.001 | **0.298** |
| 3-Methylhistidine | 1 | **0.310** | 0.004 | 0.945 | 0.010 | 0.550 | 0.006 | 0.837 |
| 4-Deoxyglucose | 1 | **0.373** | -0.002 | **0.491** | -0.001 | 1.000 | 0.001 | 0.892 |
| 4-hydroxyglutamate semialdehyde | 1 | **0.373** | 0.001 | 0.532 | 0.000 | 1.000 | -0.001 | 0.711 |
| 4-hydroxyglutamate semialdehyde | 1 | **0.063** | 0.001 | **0.132** | 0.001 | **0.384** | 0.000 | 0.937 |
| 4-Methyl-2-oxovaleric acid | 1 | **0.083** | 0.003 | **0.427** | -0.001 | 0.990 | -0.004 | **0.125** |
| 4-oxo-proline | 1 | **0.000** | -0.009 | **0.004** | 0.000 | 1.000 | 0.008 | **0.002** |
| AA | 1 | **0.226** | 0.065 | **0.375** | 0.009 | 1.000 | -0.056 | 0.524 |
| Acetoacetate peak 1 | 1 | 0.869 | 0.000 | 1.000 | 0.000 | 1.000 | -0.001 | 0.979 |
| Acetoacetate peak 2 | 1 | 0.793 | 0.001 | 1.000 | -0.001 | 1.000 | -0.002 | 0.923 |
| Alanine | 1 | **0.335** | 0.029 | 0.881 | 0.054 | 0.572 | 0.025 | 0.923 |
| alk_LPC | 2 | **0.010** | -0.006 | **0.034** | -0.001 | 1.000 | 0.005 | **0.072** |
| alk_LPC_16_0 | 2 | **0.055** | -0.001 | **0.245** | 0.000 | 1.000 | 0.002 | **0.112** |
| alk_LPC_16_1 | 2 | **0.009** | -0.003 | **0.043** | 0.000 | 1.000 | 0.002 | **0.044** |
| alk_LPC_18_1 | 2 | **0.010** | -0.002 | **0.027** | -0.001 | **0.183** | 0.001 | 0.733 |
| alk_PC | 2 | **0.004** | -0.015 | 0.929 | -0.082 | **0.015** | -0.067 | **0.044** |
| alk_PC_34_1 | 2 | **0.000** | -0.001 | 0.853 | -0.010 | **0.000** | -0.009 | **0.000** |
| alk_PC_34_2 | 2 | **0.026** | -0.001 | 1.000 | -0.012 | **0.102** | -0.010 | **0.137** |
| alk_PC_34_3 | 2 | **0.001** | 0.000 | 1.000 | -0.012 | **0.010** | -0.013 | **0.005** |
| alk_PC_36_2 | 2 | **0.000** | -0.001 | 0.780 | -0.007 | **0.000** | -0.006 | **0.001** |
| alk_PC_36_3 | 2 | **0.012** | 0.000 | 1.000 | -0.004 | **0.059** | -0.004 | **0.076** |
| alk_PC_36_4 | 2 | **0.000** | -0.008 | 0.881 | -0.047 | **0.001** | -0.039 | **0.006** |
| alk_PC_36_5 | 2 | **0.004** | -0.002 | 0.999 | -0.019 | **0.020** | -0.017 | **0.032** |
| alk_PC_36_6 | 2 | **0.000** | -0.001 | 0.985 | 0.025 | **0.000** | 0.026 | **0.000** |
| alk_PC_38_4 | 2 | **0.000** | -0.004 | 0.800 | -0.035 | **0.000** | -0.030 | **0.000** |
| alk_PC_38_5 | 2 | **0.002** | -0.005 | 0.912 | -0.029 | **0.010** | -0.024 | **0.034** |
| alk_PC_38_6 | 2 | **0.002** | 0.006 | 0.705 | 0.022 | **0.009** | 0.015 | **0.074** |
| alk_PC_40_6 | 2 | **0.000** | -0.004 | **0.016** | 0.005 | **0.004** | 0.009 | **0.000** |
| alk_TG | 2 | **0.043** | -0.007 | **0.082** | -0.004 | 0.558 | 0.003 | 0.652 |
| alk_TG_42_6 | 2 | **0.012** | 0.000 | 0.951 | 0.002 | **0.132** | 0.003 | **0.029** |
| alk_TG_50_0 | 2 | **0.077** | -0.001 | **0.415** | -0.001 | **0.183** | 0.000 | 0.918 |
| alk_TG_50_1 | 2 | **0.022** | -0.003 | **0.046** | -0.002 | **0.419** | 0.001 | 0.634 |
| alk_TG_50_2 | 2 | **0.394** | 0.001 | 0.694 | 0.000 | 1.000 | -0.001 | 0.622 |
| alk_TG_52_0 | 2 | **0.093** | -0.002 | **0.174** | -0.001 | 0.538 | 0.001 | 0.919 |
| alk_TG_52_1 | 2 | **0.088** | -0.002 | **0.205** | -0.002 | **0.359** | 0.000 | 0.999 |
| alk_TG_58_2 | 2 | **0.189** | 0.000 | 0.616 | 0.000 | **0.384** | 0.000 | 0.935 |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| alpha-Ketoglutaric acid | 1 | **0.055** | 0.001 | **0.139** | 0.000 | 1.000 | 0.000 | **0.232** |
| Aminomalonic acid | 1 | 0.647 | 0.010 | 0.891 | 0.011 | 0.943 | 0.002 | 0.999 |
| Arachidonic acid | 1 | **0.009** | -0.003 | **0.027** | -0.001 | 0.924 | 0.002 | **0.112** |
| ascorbic acid | 1 | **0.002** | -0.036 | **0.021** | 0.000 | 1.000 | 0.036 | **0.009** |
| Benzyl alcohol | 1 | **0.046** | 0.001 | **0.121** | 0.000 | 1.000 | -0.001 | **0.208** |
| Beta-Alanine | 1 | 0.985 | 0.000 | 1.000 | 0.000 | 1.000 | 0.000 | 0.999 |
| C10:0 Fatty acid | 1 | **0.085** | -0.005 | **0.165** | -0.001 | 1.000 | 0.003 | **0.367** |
| C12:0 Fatty acid | 1 | **0.111** | -0.002 | **0.174** | -0.001 | 0.686 | 0.001 | 0.802 |
| C12_0 | 3 | **0.118** | -0.063 | **0.179** | -0.029 | 0.874 | 0.034 | 0.658 |
| C14:0 Fatty acid | 1 | **0.005** | -0.031 | **0.015** | -0.015 | **0.424** | 0.016 | **0.271** |
| C14_0 | 3 | **0.004** | -0.224 | **0.014** | -0.083 | 0.611 | 0.140 | **0.128** |
| C16:0 Fatty acid | 1 | **0.003** | -0.179 | **0.013** | -0.055 | 0.746 | 0.124 | **0.074** |
| C16:1 Fatty acid | 1 | **0.029** | -0.067 | **0.055** | -0.037 | 0.506 | 0.030 | 0.606 |
| C16_0 | 3 | **0.008** | -0.354 | **0.024** | -0.141 | 0.619 | 0.213 | **0.225** |
| C16_0_C18_2 | 3 | **0.396** | 0.031 | 0.809 | 0.046 | 0.653 | 0.015 | 0.999 |
| C16_1 | 3 | **0.014** | -0.286 | **0.033** | -0.159 | **0.397** | 0.127 | 0.532 |
| C16_1_C16_0 | 3 | **0.077** | -0.021 | **0.266** | -0.023 | **0.233** | -0.002 | 0.999 |
| C18:0 Fatty acid | 1 | **0.002** | -0.052 | **0.020** | 0.001 | 1.000 | 0.053 | **0.007** |
| C18:1 Fatty acid | 1 | **0.022** | -0.214 | **0.046** | -0.132 | **0.367** | 0.082 | 0.682 |
| C18:1 fatty acid, isomer of oleic acid | 1 | **0.017** | -0.012 | **0.036** | -0.006 | 0.593 | 0.007 | **0.367** |
| C18:2 Fatty acid | 1 | **0.001** | -0.034 | **0.005** | -0.014 | **0.443** | 0.020 | **0.091** |
| C18_0 | 3 | **0.018** | -0.189 | **0.050** | -0.035 | 1.000 | 0.155 | **0.114** |
| C18_1 | 3 | **0.021** | -0.384 | **0.049** | -0.264 | **0.275** | 0.120 | 0.831 |
| C18_1_C18_0 | 3 | **0.036** | -0.131 | **0.295** | -0.187 | **0.090** | -0.056 | 0.892 |
| C18_2 | 3 | **0.008** | -0.355 | **0.023** | -0.175 | **0.432** | 0.180 | **0.346** |
| C18_2_C20_3w6 | 3 | **0.000** | -0.966 | **0.024** | 0.375 | 0.636 | 1.341 | **0.000** |
| C18_3-w3 | 3 | **0.011** | -0.124 | **0.031** | -0.032 | 0.959 | 0.092 | **0.114** |
| C18_3-w6 | 3 | **0.017** | -0.026 | **0.046** | -0.019 | **0.204** | 0.007 | 0.892 |
| C18_3w6_C18_2 | 3 | **0.026** | 0.002 | 0.572 | -0.002 | **0.479** | -0.004 | **0.041** |
| C20:1 fatty acid | 1 | **0.148** | -0.003 | **0.236** | -0.001 | 1.000 | 0.002 | 0.577 |
| C20_0 | 3 | **0.345** | -0.008 | 0.548 | -0.001 | 1.000 | 0.008 | 0.642 |
| C20_1 | 3 | **0.066** | -0.040 | **0.129** | -0.012 | 1.000 | 0.028 | **0.374** |
| C20_1_C20_0 | 3 | 0.660 | -0.104 | 0.887 | -0.114 | 0.975 | -0.010 | 0.999 |
| C20_2 | 3 | **0.052** | -0.022 | **0.093** | -0.010 | 0.759 | 0.012 | 0.506 |
| C20_3-w6 | 3 | **0.007** | 0.004 | **0.241** | -0.003 | 0.506 | -0.007 | **0.008** |
| C20_3-w9 | 3 | **0.481** | -0.017 | 0.792 | -0.020 | 0.789 | -0.003 | 0.999 |
| C20_3w9_C20_2 | 3 | **0.052** | 0.141 | **0.331** | -0.062 | 0.996 | -0.203 | **0.086** |
| C20_3w9_C20_4w6 | 3 | **0.000** | 0.027 | **0.325** | -0.050 | **0.019** | -0.076 | **0.000** |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| C20_4-w3 | 3 | **0.008** | -0.008 | 0.501 | 0.011 | **0.294** | 0.019 | **0.013** |
| C20_4-w6 | 3 | **0.349** | -0.050 | 0.564 | -0.001 | 1.000 | 0.049 | 0.626 |
| C20_4w6_C20_3w6 | 3 | **0.000** | -0.693 | **0.049** | 0.522 | **0.204** | 1.215 | **0.000** |
| C20_4w6_C20_5 | 3 | **0.000** | 0.082 | 0.934 | -1.752 | **0.000** | -1.834 | **0.000** |
| C20_5 | 3 | **0.000** | -0.012 | 0.972 | 0.209 | **0.000** | 0.221 | **0.000** |
| C20_5_C20_3w6 | 3 | **0.000** | -0.666 | **0.121** | 2.558 | **0.000** | 3.224 | **0.000** |
| C20_5_C20_4w3 | 3 | **0.000** | -0.034 | 0.986 | 1.406 | **0.000** | 1.439 | **0.000** |
| C22:6 Fatty acid | 1 | **0.000** | -0.003 | **0.353** | 0.014 | **0.000** | 0.017 | **0.000** |
| C22_4 | 3 | **0.005** | -0.013 | **0.046** | -0.014 | **0.024** | -0.001 | 0.999 |
| C22_5-w3 | 3 | **0.000** | -0.025 | **0.236** | 0.060 | **0.000** | 0.085 | **0.000** |
| C22_5-w6 | 3 | 0.878 | -0.001 | 0.964 | 0.000 | 1.000 | 0.000 | 0.999 |
| C22_5w6_C22_6w3 | 3 | **0.000** | 0.009 | 0.548 | -0.037 | **0.000** | -0.046 | **0.000** |
| C22_6-w3 | 3 | **0.000** | -0.054 | **0.450** | 0.258 | **0.000** | 0.312 | **0.000** |
| Caffeine | 1 | **0.024** | -0.066 | **0.415** | 0.056 | 0.622 | 0.122 | **0.037** |
| Campesterol | 1 | **0.000** | -0.002 | **0.168** | 0.002 | **0.039** | 0.004 | **0.000** |
| campesterol_cholesterol | 1 | **0.000** | -0.001 | 0.679 | 0.002 | **0.012** | 0.003 | **0.000** |
| Car | 1 | **0.382** | -0.006 | 1.000 | 0.024 | 0.850 | 0.030 | 0.614 |
| ChE | 2 | **0.000** | -0.015 | **0.153** | 0.026 | **0.003** | 0.041 | **0.000** |
| CholE_18_1 | 2 | **0.192** | 0.002 | **0.284** | 0.001 | 0.884 | -0.001 | 0.831 |
| CholE_18_2 | 2 | **0.002** | -0.012 | **0.014** | -0.002 | 1.000 | 0.010 | **0.018** |
| CholE_18_3 | 2 | **0.017** | 0.001 | **0.303** | -0.001 | 0.673 | -0.001 | **0.023** |
| CholE_20_4 | 2 | **0.041** | -0.005 | **0.078** | -0.003 | 0.550 | 0.002 | 0.643 |
| CholE_20_5 | 2 | **0.000** | -0.001 | 1.000 | 0.026 | **0.000** | 0.027 | **0.000** |
| CholE_22_6 | 2 | **0.000** | -0.002 | **0.372** | 0.006 | **0.000** | 0.008 | **0.000** |
| Cholesterol | 1 | **0.000** | -0.070 | **0.005** | 0.009 | 1.000 | 0.079 | **0.000** |
| Citric acid | 1 | **0.484** | 0.034 | 0.898 | -0.022 | 1.000 | -0.056 | 0.661 |
| Creatinine | 1 | **0.139** | 0.000 | 0.991 | 0.000 | 0.540 | 0.000 | **0.271** |
| D(+) Glyceric acid | 1 | **0.484** | -0.001 | 0.887 | 0.000 | 1.000 | 0.001 | 0.660 |
| D-(+)-Xylose peak1 | 1 | 0.501 | -0.011 | 0.753 | -0.011 | 0.858 | 0.000 | 0.999 |
| DCHP | 1 | **0.426** | 0.000 | 0.562 | 0.000 | 1.000 | 0.000 | 0.858 |
| DG | 1 | **0.083** | -0.044 | **0.154** | -0.028 | 0.577 | 0.016 | 0.863 |
| DG | 2 | **0.006** | -0.028 | **0.020** | -0.017 | **0.237** | 0.012 | 0.502 |
| DG_36_2_ | 2 | **0.005** | -0.026 | **0.021** | -0.019 | **0.122** | 0.008 | 0.754 |
| DG_36_3_ | 2 | 0.669 | 0.000 | 0.912 | 0.000 | 1.000 | 0.000 | 0.872 |
| DG_36_4_ | 2 | 0.882 | 0.000 | 1.000 | 0.000 | 1.000 | 0.000 | 0.999 |
| DG_42_7 | 2 | **0.050** | 0.000 | 0.973 | 0.000 | **0.153** | 0.000 | **0.280** |
| DG_44_9 | 2 | **0.000** | -0.002 | **0.034** | 0.002 | **0.232** | 0.004 | **0.000** |
| D-Glucose peak2 | 1 | **0.183** | -0.007 | 1.000 | 0.031 | 0.588 | 0.037 | **0.357** |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| D-Glutamic acid | 1 | **0.160** | 0.047 | **0.300** | 0.005 | 1.000 | -0.042 | **0.406** |
| dihydroxybutanoic acid | 1 | **0.032** | 0.003 | **0.288** | -0.001 | 0.919 | -0.004 | **0.052** |
| DL-alpha-Aminoadipic acid | 1 | 0.793 | 0.000 | 0.986 | 0.000 | 1.000 | 0.000 | 0.923 |
| D-Maltose peak1 | 1 | **0.404** | 0.002 | 0.905 | -0.002 | 1.000 | -0.004 | 0.581 |
| D-Mannose | 1 | 0.516 | 0.009 | 0.641 | 0.004 | 1.000 | -0.005 | 0.934 |
| D-Ribose | 1 | 0.800 | -0.001 | 0.914 | -0.001 | 1.000 | 0.000 | 0.999 |
| D-Ribulose- or D-Xylulose | 1 | **0.163** | 0.002 | **0.382** | 0.000 | 1.000 | -0.002 | **0.340** |
| Erythronic acid | 1 | **0.014** | 0.002 | **0.067** | 0.000 | 1.000 | -0.002 | **0.043** |
| FA | 1 | **0.007** | -0.300 | **0.021** | -0.128 | 0.554 | 0.172 | **0.245** |
| FAC16:0 sn-G-3-PC-degr. | 1 | **0.000** | -0.126 | **0.062** | 0.075 | **0.443** | 0.201 | **0.001** |
| FAC16:0-alpha-LPA-degr. | 1 | **0.141** | -0.027 | **0.381** | 0.004 | 1.000 | 0.031 | **0.284** |
| FAC18:0 sn-G-3-PC-degr. | 1 | **0.015** | -0.059 | **0.449** | 0.062 | 0.506 | 0.121 | **0.025** |
| FAC18:2 sn-G-3-PC-degr. | 1 | **0.044** | -0.012 | 0.548 | -0.023 | **0.099** | -0.011 | 0.622 |
| FFA_TOTAL | 3 | **0.012** | -0.388 | **0.029** | -0.176 | 0.557 | 0.212 | **0.334** |
| Fructose | 1 | **0.226** | 0.002 | 0.809 | 0.003 | **0.426** | 0.002 | 0.872 |
| Fumaric acid | 1 | **0.135** | -0.001 | **0.300** | -0.001 | **0.412** | 0.000 | 0.999 |
| Glutamic acid internal amide | 1 | **0.135** | 0.093 | **0.207** | 0.041 | 0.908 | -0.051 | 0.672 |
| Glycerol | 1 | **0.079** | -0.052 | **0.139** | -0.020 | 0.937 | 0.033 | **0.498** |
| Glycine | 1 | **0.077** | 0.118 | **0.136** | 0.049 | 0.874 | -0.069 | 0.548 |
| Glycolic acid | 1 | 0.737 | 0.001 | 0.885 | 0.000 | 1.000 | 0.000 | 0.999 |
| Hexadecanoic-methylester | 1 | **0.367** | 0.000 | 0.999 | 0.001 | 0.874 | 0.002 | 0.578 |
| HypoXanthine | 1 | **0.264** | -0.002 | **0.377** | -0.001 | 0.874 | 0.001 | 0.930 |
| Indole-3-propionic acid | 1 | **0.470** | 0.019 | 0.598 | 0.012 | 1.000 | -0.007 | 0.989 |
| Inositol | 1 | 0.509 | 0.004 | 0.638 | 0.002 | 1.000 | -0.002 | 0.923 |
| KB | 1 | 0.857 | -0.023 | 0.951 | -0.016 | 1.000 | 0.006 | 0.999 |
| L-(+)-Arabinose | 1 | **0.355** | 0.002 | 0.605 | 0.000 | 1.000 | -0.002 | 0.593 |
| L-4-Hydroxyproline | 1 | **0.057** | 0.046 | **0.118** | 0.012 | 1.000 | -0.035 | **0.301** |
| Lactic acid | 1 | 0.982 | 0.007 | 1.000 | -0.014 | 1.000 | -0.021 | 0.999 |
| lactic acid - oximer | 1 | 0.892 | 0.000 | 0.988 | 0.000 | 1.000 | 0.000 | 0.999 |
| L-Asparagine | 1 | **0.156** | 0.009 | **0.296** | 0.001 | 1.000 | -0.009 | **0.363** |
| L-Aspartic acid | 1 | **0.410** | 0.016 | 0.616 | 0.001 | 1.000 | -0.015 | 0.685 |
| LCB 16:1-16:0 SM-degr. | 1 | **0.040** | -0.046 | **0.157** | 0.002 | 1.000 | 0.049 | **0.108** |
| LCB 16:1-18:0 SM-degr. | 1 | **0.021** | -0.100 | **0.085** | -0.002 | 1.000 | 0.098 | **0.076** |
| LCB 16:1-20:0 SM-degr. | 1 | **0.005** | -0.057 | **0.043** | 0.004 | 1.000 | 0.061 | **0.018** |
| LCB 16:1-22:0 SM-degr. | 1 | **0.037** | -0.022 | **0.081** | -0.016 | **0.333** | 0.006 | 0.892 |
| LCB 16:1-24:1 SM-degr. | 1 | **0.014** | -0.033 | **0.454** | 0.036 | **0.476** | 0.070 | **0.024** |
| LCB 17:1-16:0 SM-degr. | 1 | **0.041** | -0.022 | **0.230** | 0.006 | 1.000 | 0.028 | **0.077** |
| LCB 17:1-18:0 SM-degr. | 1 | **0.016** | -0.008 | **0.146** | 0.002 | 1.000 | 0.010 | **0.034** |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| LCB 17:1-24:0 SM-degr. | 1 | **0.002** | -0.002 | 0.601 | 0.004 | **0.090** | 0.005 | **0.004** |
| LCB 18:0-16:0 SM-degr. | 1 | **0.186** | -0.009 | 0.800 | 0.010 | 0.903 | 0.019 | **0.300** |
| LCB 18:1-16:0 SM-degr. | 1 | **0.310** | -0.098 | **0.415** | -0.049 | 0.990 | 0.049 | 0.892 |
| LCB 18:1-17:0 SM-degr. | 1 | **0.045** | -0.005 | **0.236** | 0.001 | 1.000 | 0.006 | **0.089** |
| LCB 18:1-18:0 SM-degr. | 1 | **0.428** | -0.051 | 0.601 | -0.007 | 1.000 | 0.044 | 0.739 |
| LCB 18:1-22:0 SM-degr. | 1 | **0.061** | -0.005 | 0.898 | -0.016 | **0.153** | -0.011 | **0.394** |
| LCB 18:1-23:0 SM-degr. | 1 | 0.787 | 0.001 | 0.919 | 0.000 | 1.000 | -0.001 | 0.964 |
| LCB 18:1-24:0+LCB 18:1-24:1SM | 1 | **0.065** | 0.041 | **0.415** | 0.061 | **0.149** | 0.020 | 0.892 |
| LCB 18:2-16:0 SM-degr. | 1 | **0.254** | -0.058 | **0.360** | -0.032 | 0.908 | 0.026 | 0.899 |
| LCB 18:2-18:0 SM-degr. | 1 | **0.141** | -0.054 | **0.288** | -0.005 | 1.000 | 0.049 | **0.374** |
| LCB 18:2-20:0 SM-degr. | 1 | **0.106** | -0.033 | **0.225** | -0.004 | 1.000 | 0.029 | **0.334** |
| LCB 18:2-24:0+LCB 18:2-24:1SM | 1 | **0.090** | 0.016 | 0.951 | 0.060 | **0.224** | 0.044 | **0.428** |
| L-Cysteine | 1 | **0.043** | 0.025 | **0.079** | 0.008 | 0.943 | -0.017 | **0.316** |
| L-Cystine | 1 | **0.011** | 0.200 | **0.388** | -0.193 | 0.526 | -0.393 | **0.021** |
| L-Glutamine | 1 | 0.787 | 0.023 | 0.903 | 0.013 | 1.000 | -0.010 | 0.999 |
| L-Histidine | 1 | **0.467** | 0.059 | 0.717 | -0.005 | 1.000 | -0.064 | 0.691 |
| L-Isoleucine | 1 | 0.688 | -0.017 | 0.951 | 0.008 | 1.000 | 0.025 | 0.872 |
| L-Leucine | 1 | 0.589 | -0.033 | 0.755 | -0.007 | 1.000 | 0.027 | 0.892 |
| L-Lysine | 1 | **0.323** | 0.058 | **0.427** | 0.025 | 1.000 | -0.033 | 0.846 |
| L-Methionine | 1 | **0.118** | 0.016 | **0.248** | 0.001 | 1.000 | -0.016 | **0.301** |
| L-Ornithine | 1 | **0.324** | 0.059 | **0.482** | 0.011 | 1.000 | -0.048 | 0.670 |
| LPC | 1 | **0.001** | -0.127 | **0.120** | 0.081 | 0.512 | 0.208 | **0.002** |
| LPC | 2 | **0.000** | -0.244 | **0.004** | -0.035 | 1.000 | 0.209 | **0.005** |
| LPC_16_0 | 2 | **0.000** | -0.198 | **0.004** | -0.006 | 1.000 | 0.192 | **0.001** |
| LPC_16_1 | 2 | **0.047** | -0.006 | 0.564 | -0.012 | **0.107** | -0.006 | 0.612 |
| LPC_16_1_LPC_16_0 | 2 | **0.000** | 0.004 | **0.044** | -0.005 | **0.025** | -0.009 | **0.000** |
| LPC_18_0 | 2 | **0.000** | -0.154 | **0.002** | 0.004 | 1.000 | 0.158 | **0.000** |
| LPC_18_1 | 2 | **0.036** | -0.081 | **0.160** | -0.086 | **0.130** | -0.006 | 0.999 |
| LPC_18_1_LPC_18_0 | 2 | **0.000** | 0.060 | **0.236** | -0.098 | **0.020** | -0.158 | **0.000** |
| LPC_18_2 | 2 | **0.018** | -0.129 | **0.039** | -0.075 | **0.384** | 0.054 | 0.606 |
| LPC_18_3 | 2 | **0.108** | -0.002 | 0.836 | -0.005 | **0.235** | -0.003 | 0.614 |
| LPC_20_0 | 2 | **0.012** | -0.001 | **0.029** | 0.000 | **0.443** | 0.000 | **0.426** |
| LPC_20_1 | 2 | **0.037** | -0.002 | **0.100** | 0.000 | 1.000 | 0.002 | **0.165** |
| LPC_20_2 | 2 | **0.024** | -0.001 | 0.507 | -0.002 | **0.054** | -0.001 | **0.495** |
| LPC_20_3 | 2 | **0.000** | -0.009 | **0.299** | -0.030 | **0.000** | -0.021 | **0.001** |
| LPC_20_4 | 2 | **0.001** | -0.042 | **0.005** | -0.029 | **0.049** | 0.013 | 0.606 |
| LPC_20_5 | 2 | **0.000** | -0.007 | 0.945 | 0.082 | **0.000** | 0.089 | **0.000** |
| LPC_22_6 | 2 | **0.000** | -0.021 | **0.031** | 0.036 | **0.000** | 0.058 | **0.000** |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| LPC_36_4_LPC_16_0 | 2 | **0.002** | 0.043 | 0.856 | -0.151 | **0.049** | -0.193 | **0.004** |
| LPE | 2 | **0.000** | -0.013 | **0.004** | -0.001 | 1.000 | 0.011 | **0.003** |
| LPE_18_0 | 2 | **0.000** | -0.006 | **0.005** | 0.000 | 1.000 | 0.006 | **0.001** |
| LPE_18_1 | 2 | **0.012** | -0.002 | **0.139** | -0.002 | **0.036** | -0.001 | 0.918 |
| LPE_20_4 | 2 | **0.005** | -0.002 | **0.301** | -0.003 | **0.012** | -0.002 | **0.358** |
| LPE_22_6 | 2 | **0.000** | -0.004 | **0.027** | 0.004 | **0.009** | 0.008 | **0.000** |
| L-Phenylalanine | 1 | **0.322** | 0.015 | 0.504 | 0.001 | 1.000 | -0.014 | 0.602 |
| L-Proline | 1 | **0.155** | 0.065 | **0.237** | 0.040 | 0.689 | -0.025 | 0.892 |
| L-Serine | 1 | **0.064** | 0.167 | **0.151** | 0.026 | 1.000 | -0.141 | **0.269** |
| L-Threonine | 1 | **0.058** | 0.033 | **0.482** | -0.024 | 0.849 | -0.057 | **0.094** |
| L-Tryptophan | 1 | **0.135** | -0.089 | **0.300** | 0.000 | 1.000 | 0.089 | **0.320** |
| L-Tyrosine | 1 | **0.043** | 0.152 | **0.073** | 0.068 | 0.707 | -0.084 | **0.481** |
| L-Valine | 1 | **0.186** | -0.053 | **0.458** | 0.010 | 1.000 | 0.063 | **0.346** |
| lysine | 1 | 0.882 | 0.000 | 1.000 | 0.000 | 1.000 | -0.001 | 0.989 |
| malic acid | 1 | **0.297** | -0.003 | 0.564 | -0.003 | 0.581 | 0.000 | 0.999 |
| Meso-erythrytol | 1 | **0.485** | 0.001 | 0.954 | -0.001 | 1.000 | -0.002 | 0.664 |
| methyl uric acid | 1 | **0.000** | -0.001 | **0.013** | 0.000 | **0.072** | 0.001 | **0.000** |
| MG | 1 | **0.200** | -0.003 | 0.564 | 0.001 | 1.000 | 0.004 | **0.340** |
| Monomethylphosphate | 1 | **0.335** | -0.033 | 0.504 | -0.005 | 1.000 | 0.029 | 0.652 |
| Myo-inositol | 1 | **0.140** | 0.072 | **0.236** | 0.051 | 0.588 | -0.021 | 0.954 |
| Myo-inositol-1,2-cyclic phosphate | 1 | **0.079** | -0.029 | **0.174** | -0.004 | 1.000 | 0.025 | **0.294** |
| Myo-inositolphosphate | 1 | **0.156** | 0.004 | 0.881 | -0.006 | 0.736 | -0.010 | **0.270** |
| N-Acetylaminomalonic acid | 1 | 0.982 | 0.000 | 1.000 | 0.000 | 1.000 | 0.000 | 0.999 |
| N-carboxyl-alanine | 1 | 0.685 | 0.003 | 0.999 | -0.004 | 1.000 | -0.008 | 0.865 |
| N-carboxyproline | 1 | **0.245** | 0.003 | 0.885 | -0.004 | 0.908 | -0.007 | **0.374** |
| Nicotinamide | 1 | **0.041** | 0.000 | **0.281** | 0.000 | 1.000 | 0.000 | **0.076** |
| N-methyl-4(?)-hydroxyproline | 1 | 0.506 | 0.000 | 1.000 | 0.004 | 0.894 | 0.004 | 0.780 |
| OA | 1 | 0.931 | 0.018 | 1.000 | -0.019 | 1.000 | -0.037 | 0.999 |
| o-Phosphorylethanolamine | 1 | **0.331** | 0.000 | 1.000 | -0.002 | 0.725 | -0.002 | 0.581 |
| P7478_uk03 | 1 | 0.793 | 0.001 | 0.914 | 0.000 | 1.000 | -0.001 | 0.989 |
| P7478_uk04 | 1 | **0.033** | 0.000 | **0.194** | 0.000 | 1.000 | 0.000 | **0.059** |
| P7478_uk05 | 1 | **0.002** | 0.015 | **0.024** | -0.001 | 1.000 | -0.016 | **0.006** |
| P7478_uk15 | 1 | **0.075** | 0.001 | **0.136** | 0.000 | 1.000 | -0.001 | **0.393** |
| P7478_uk19 | 1 | **0.000** | -0.001 | **0.005** | 0.000 | **0.231** | 0.001 | **0.000** |
| P7502_UK02 | 1 | **0.037** | 0.001 | 0.968 | -0.002 | **0.284** | -0.003 | **0.090** |
| P7502_UK05 | 1 | **0.000** | 0.002 | **0.004** | 0.000 | 0.925 | -0.003 | **0.000** |
| P7881_uk 01 | 1 | **0.002** | -0.022 | **0.062** | 0.008 | 0.840 | 0.030 | **0.003** |
| P7881_uk 11 | 1 | **0.001** | -0.012 | **0.078** | 0.007 | **0.481** | 0.019 | **0.001** |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| P7881_uk 20 | 1 | **0.008** | -0.007 | **0.174** | 0.004 | 0.760 | 0.011 | **0.014** |
| P7881_uk 22 | 1 | **0.001** | 0.003 | **0.014** | 0.000 | 1.000 | -0.003 | **0.009** |
| P7881_uk 28 | 1 | **0.244** | -0.009 | 0.717 | 0.005 | 1.000 | 0.014 | **0.374** |
| P7881_uk 30 | 1 | **0.146** | 0.002 | 0.999 | -0.006 | 0.550 | -0.008 | **0.298** |
| P7881_uk 38 | 1 | **0.141** | -0.006 | **0.285** | -0.001 | 1.000 | 0.005 | **0.367** |
| P7881_uk 46 | 1 | **0.382** | 0.000 | 0.999 | 0.001 | 0.894 | 0.002 | 0.593 |
| P7881_uk 48 | 1 | 0.853 | -0.001 | 1.000 | -0.004 | 1.000 | -0.003 | 0.998 |
| P7881_uk 51 | 1 | **0.144** | -0.003 | 0.562 | -0.005 | **0.275** | -0.002 | 0.901 |
| PC | 2 | **0.007** | -0.179 | **0.024** | -0.060 | 0.746 | 0.119 | **0.124** |
| PC_32_0 | 2 | **0.000** | -0.029 | **0.034** | 0.017 | **0.384** | 0.046 | **0.000** |
| PC_32_1 | 2 | **0.007** | -0.001 | 1.000 | -0.057 | **0.048** | -0.056 | **0.037** |
| PC_32_2 | 2 | **0.001** | -0.022 | **0.005** | -0.014 | **0.091** | 0.008 | **0.413** |
| PC_34_0nr2 | 2 | 0.670 | 0.000 | 0.951 | 0.001 | 0.943 | 0.000 | 0.999 |
| PC_34_1 | 2 | **0.002** | 0.004 | 1.000 | -0.202 | **0.019** | -0.206 | **0.010** |
| PC_34_2 | 2 | **0.006** | -0.248 | **0.023** | -0.180 | **0.118** | 0.069 | 0.793 |
| PC_34_3 | 2 | **0.000** | -0.040 | **0.087** | -0.080 | **0.000** | -0.041 | **0.053** |
| PC_34_4 | 2 | **0.118** | -0.007 | **0.300** | 0.000 | 1.000 | 0.008 | **0.280** |
| PC_36_0 | 2 | **0.000** | -0.001 | **0.010** | 0.001 | **0.049** | 0.001 | **0.000** |
| PC_36_1 | 2 | **0.013** | 0.046 | 0.569 | -0.068 | **0.334** | -0.114 | **0.020** |
| PC_36_2 | 2 | **0.001** | -0.206 | **0.009** | -0.166 | **0.025** | 0.039 | 0.892 |
| PC_36_3 | 2 | **0.000** | -0.069 | 0.507 | -0.379 | **0.000** | -0.310 | **0.000** |
| PC_36_4 | 2 | **0.001** | -0.141 | **0.063** | -0.200 | **0.003** | -0.059 | 0.652 |
| PC_36_5 | 2 | **0.000** | -0.041 | 0.968 | 0.862 | **0.000** | 0.903 | **0.000** |
| PC_36_6 | 2 | **0.000** | -0.006 | 0.645 | 0.026 | **0.000** | 0.032 | **0.000** |
| PC_38_2 | 2 | **0.001** | 0.004 | 0.804 | -0.014 | **0.025** | -0.018 | **0.002** |
| PC_38_3 | 2 | **0.000** | 0.022 | 0.881 | -0.198 | **0.000** | -0.220 | **0.000** |
| PC_38_4 | 2 | **0.010** | -0.117 | **0.075** | -0.129 | **0.040** | -0.012 | 0.999 |
| PC_38_4_LPC_18_0 | 2 | **0.002** | 0.096 | **0.491** | -0.161 | **0.145** | -0.256 | **0.003** |
| PC_38_5 | 2 | **0.000** | -0.068 | 0.601 | 0.457 | **0.000** | 0.525 | **0.000** |
| PC_38_6 | 2 | **0.000** | -0.207 | **0.024** | 0.473 | **0.000** | 0.680 | **0.000** |
| PC_38_7 | 2 | **0.000** | -0.009 | **0.415** | 0.046 | **0.000** | 0.055 | **0.000** |
| PC_40_4 | 2 | **0.000** | 0.001 | 1.000 | -0.026 | **0.000** | -0.027 | **0.000** |
| PC_40_6 | 2 | **0.000** | -0.086 | **0.165** | 0.296 | **0.000** | 0.382 | **0.000** |
| PC_40_7 | 2 | **0.000** | -0.018 | **0.025** | 0.015 | **0.049** | 0.033 | **0.000** |
| PC_40_8 | 2 | **0.000** | -0.004 | **0.025** | 0.003 | **0.125** | 0.006 | **0.000** |
| PC_LPC | 2 | **0.173** | 0.089 | **0.483** | -0.022 | 1.000 | -0.112 | **0.308** |
| PE | 2 | **0.001** | -0.233 | **0.005** | -0.141 | **0.117** | 0.091 | **0.388** |
| PE_36_3 | 2 | **0.010** | -0.025 | **0.194** | -0.038 | **0.025** | -0.013 | 0.722 |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| PE_36_4 | 2 | **0.001** | -0.029 | **0.133** | -0.048 | **0.003** | -0.020 | **0.375** |
| PE_36_5 | 2 | **0.202** | -0.004 | 1.000 | -0.024 | **0.456** | -0.020 | 0.553 |
| PE_38_2 | 2 | **0.005** | -0.193 | **0.013** | -0.104 | **0.268** | 0.088 | **0.346** |
| PE_38_4 | 2 | **0.000** | -0.075 | **0.025** | -0.099 | **0.001** | -0.024 | 0.711 |
| PE_38_5 | 2 | **0.080** | -0.026 | 0.621 | -0.050 | **0.169** | -0.025 | 0.677 |
| PE_38_6 | 2 | **0.008** | -0.064 | **0.136** | 0.023 | 0.943 | 0.086 | **0.016** |
| PE_38_7 | 2 | **0.000** | -0.008 | 0.951 | 0.077 | **0.000** | 0.086 | **0.000** |
| Phosphate | 1 | **0.064** | -0.017 | 1.000 | -0.121 | **0.200** | -0.104 | **0.286** |
| Pipecolinic acid | 1 | **0.250** | 0.001 | **0.495** | 0.000 | 1.000 | -0.001 | **0.466** |
| Pseudo uridine | 1 | **0.022** | 0.011 | **0.046** | 0.004 | 0.894 | -0.007 | **0.240** |
| Pyruvic acid | 1 | **0.441** | 0.019 | 0.593 | 0.004 | 1.000 | -0.015 | 0.797 |
| S7010 ukx08 | 1 | 0.612 | -0.001 | 1.000 | -0.003 | 0.925 | -0.003 | 0.901 |
| ser_gly | 1 | **0.156** | 0.048 | **0.443** | -0.012 | 1.000 | -0.060 | **0.284** |
| Sitosterol | 1 | **0.001** | -0.002 | **0.160** | 0.001 | **0.334** | 0.003 | **0.002** |
| sitosterol_cholesterol | 1 | **0.033** | 0.000 | 0.885 | 0.001 | **0.310** | 0.002 | **0.065** |
| SM | 2 | **0.036** | -0.086 | **0.168** | 0.008 | 1.000 | 0.094 | **0.090** |
| SM_14_0 | 2 | **0.034** | -0.045 | **0.247** | 0.015 | 1.000 | 0.060 | **0.066** |
| SM_15_0 | 2 | **0.015** | -0.004 | **0.034** | -0.002 | 0.622 | 0.002 | **0.301** |
| SM_16_0 | 2 | **0.038** | -0.051 | **0.073** | -0.026 | 0.592 | 0.025 | 0.577 |
| SM_16_1 | 2 | **0.065** | -0.014 | **0.211** | -0.014 | **0.231** | 0.000 | 0.999 |
| SM_16_1_SM_16_0 | 2 | **0.043** | 0.007 | **0.355** | -0.004 | 0.943 | -0.010 | **0.072** |
| SM_17_0 | 2 | **0.043** | -0.002 | **0.160** | 0.000 | 1.000 | 0.002 | **0.114** |
| SM_18_0 | 2 | **0.027** | -0.018 | **0.209** | 0.005 | 1.000 | 0.024 | **0.056** |
| SM_18_2 | 2 | **0.213** | -0.004 | **0.359** | -0.003 | 0.611 | 0.001 | 0.999 |
| SM_20_0 | 2 | **0.004** | -0.019 | **0.026** | -0.002 | 1.000 | 0.018 | **0.025** |
| SM_20_1 | 2 | **0.140** | -0.004 | **0.237** | -0.001 | 1.000 | 0.003 | 0.502 |
| SM_21_0 | 2 | **0.007** | -0.009 | **0.040** | -0.001 | 1.000 | 0.008 | **0.037** |
| SM_22_0 | 2 | **0.056** | -0.031 | **0.105** | -0.016 | 0.637 | 0.015 | 0.634 |
| SM_22_1 | 2 | **0.014** | -0.026 | **0.046** | -0.005 | 1.000 | 0.021 | **0.107** |
| SM_23_0 | 2 | **0.031** | -0.015 | **0.174** | 0.002 | 1.000 | 0.017 | **0.072** |
| SM_23_1 | 2 | **0.014** | -0.035 | **0.427** | 0.035 | 0.534 | 0.069 | **0.023** |
| SM_24_0 | 2 | **0.239** | -0.024 | **0.347** | -0.010 | 1.000 | 0.014 | 0.768 |
| SM_24_1 | 2 | **0.021** | -0.016 | 0.881 | 0.043 | **0.275** | 0.059 | **0.044** |
| SM_24_2 | 2 | **0.056** | -0.014 | 0.564 | 0.013 | 0.736 | 0.027 | **0.091** |
| SM_25_1 | 2 | **0.012** | -0.003 | **0.272** | 0.002 | 0.673 | 0.005 | **0.018** |
| S-methyl-L-cysteine | 1 | 0.899 | 0.000 | 1.000 | 0.001 | 1.000 | 0.001 | 0.993 |
| sn-Glycerol-3-Phosphate | 1 | **0.419** | -0.009 | 0.549 | -0.006 | 0.943 | 0.003 | 0.993 |
| SPM | 1 | **0.260** | -0.097 | **0.397** | -0.022 | 1.000 | 0.075 | 0.634 |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| stearoylamide | 1 | 0.969 | -0.003 | 1.000 | 0.003 | 1.000 | 0.006 | 0.999 |
| Succinic acid | 1 | **0.323** | 0.000 | 0.770 | 0.000 | 1.000 | 0.000 | **0.481** |
| Sucrose | 1 | **0.474** | 0.007 | 0.804 | -0.002 | 1.000 | -0.009 | 0.674 |
| sum_0 | 3 | **0.008** | -0.336 | **0.024** | -0.123 | 0.686 | 0.213 | **0.180** |
| sum_1 | 3 | **0.021** | -0.400 | **0.047** | -0.270 | **0.284** | 0.130 | 0.800 |
| sum_2_plus | 3 | **0.008** | -0.342 | **0.029** | -0.060 | 1.000 | 0.282 | **0.063** |
| sum_w3 | 3 | **0.000** | -0.162 | **0.079** | 0.311 | **0.000** | 0.473 | **0.000** |
| sum_w6 | 3 | **0.011** | -0.340 | **0.026** | -0.169 | **0.465** | 0.171 | **0.375** |
| sum_w9_plus | 3 | **0.015** | -0.380 | **0.033** | -0.201 | **0.443** | 0.178 | **0.490** |
| sumC16C18_sumC20C22 | 3 | **0.000** | -0.151 | **0.489** | -0.578 | **0.000** | -0.427 | **0.002** |
| TG | 2 | **0.000** | -0.682 | **0.000** | -0.402 | **0.020** | 0.280 | **0.124** |
| TG_40_4 | 2 | **0.000** | 0.000 | 1.000 | -0.027 | **0.000** | -0.027 | **0.000** |
| TG_40_5 | 2 | **0.228** | 0.009 | 0.995 | 0.038 | **0.481** | 0.028 | 0.634 |
| TG_40_6 | 2 | **0.000** | -0.084 | **0.178** | 0.295 | **0.000** | 0.378 | **0.000** |
| TG_40_7 | 2 | **0.000** | -0.015 | **0.053** | 0.016 | **0.025** | 0.031 | **0.000** |
| TG_40_8 | 2 | **0.000** | -0.003 | **0.030** | 0.002 | **0.294** | 0.005 | **0.000** |
| TG_42_0 | 2 | **0.334** | -0.030 | **0.482** | -0.007 | 1.000 | 0.023 | 0.711 |
| TG_42_1 | 2 | **0.275** | -0.020 | **0.449** | -0.017 | 0.666 | 0.003 | 0.999 |
| TG_42_2 | 2 | **0.196** | -0.007 | **0.312** | -0.005 | 0.658 | 0.002 | 0.989 |
| TG_44_0 | 2 | **0.235** | -0.074 | **0.371** | -0.056 | 0.678 | 0.018 | 0.999 |
| TG_44_1 | 2 | **0.239** | -0.060 | **0.354** | -0.039 | 0.805 | 0.022 | 0.935 |
| TG_44_2 | 2 | **0.168** | -0.025 | **0.288** | -0.019 | 0.605 | 0.006 | 0.989 |
| TG_46_0 | 2 | **0.156** | -0.154 | **0.254** | -0.105 | 0.651 | 0.050 | 0.934 |
| TG_46_1 | 2 | **0.156** | -0.128 | **0.272** | -0.095 | 0.595 | 0.033 | 0.989 |
| TG_46_2 | 2 | **0.088** | -0.070 | **0.163** | -0.046 | 0.565 | 0.025 | 0.892 |
| TG_46_3 | 2 | **0.133** | -0.015 | **0.216** | -0.005 | 1.000 | 0.009 | 0.577 |
| TG_48_0 | 2 | **0.090** | -0.265 | **0.166** | -0.179 | 0.550 | 0.086 | 0.901 |
| TG_48_1 | 2 | **0.019** | -0.347 | **0.044** | -0.217 | **0.334** | 0.130 | 0.680 |
| TG_48_2 | 2 | **0.017** | -0.243 | **0.046** | -0.179 | **0.207** | 0.064 | 0.892 |
| TG_48_3 | 2 | **0.014** | -0.098 | **0.034** | -0.060 | **0.308** | 0.038 | 0.642 |
| TG_48_4 | 2 | **0.050** | -0.017 | **0.119** | -0.004 | 1.000 | 0.013 | **0.280** |
| TG_50_0 | 2 | **0.131** | -0.194 | **0.211** | -0.124 | 0.650 | 0.071 | 0.892 |
| TG_50_1 | 2 | **0.000** | -0.687 | **0.001** | -0.436 | **0.027** | 0.251 | **0.301** |
| TG_50_2 | 2 | **0.000** | -0.544 | **0.001** | -0.416 | **0.004** | 0.128 | 0.649 |
| TG_50_3 | 2 | **0.000** | -0.357 | **0.002** | -0.250 | **0.015** | 0.106 | **0.498** |
| TG_50_4 | 2 | **0.000** | -0.134 | **0.003** | -0.073 | **0.131** | 0.061 | **0.228** |
| TG_51_1 | 2 | **0.022** | -0.053 | **0.052** | -0.041 | **0.186** | 0.012 | 0.932 |
| TG_51_2 | 2 | **0.003** | -0.126 | **0.014** | -0.087 | **0.103** | 0.039 | 0.670 |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| TG_51_3 | 2 | **0.001** | -0.071 | **0.004** | -0.040 | **0.130** | 0.031 | **0.270** |
| TG_51_4 | 2 | **0.001** | -0.020 | **0.004** | -0.008 | **0.426** | 0.012 | **0.060** |
| TG_52_0 | 2 | **0.124** | -0.052 | **0.205** | -0.034 | 0.619 | 0.018 | 0.899 |
| TG_52_1 | 2 | **0.016** | -0.439 | **0.036** | -0.238 | **0.443** | 0.201 | 0.510 |
| TG_52_2 | 2 | **0.000** | -0.682 | **0.002** | -0.576 | **0.004** | 0.106 | 0.901 |
| TG_52_3 | 2 | **0.000** | -0.600 | **0.000** | -0.401 | **0.008** | 0.199 | **0.300** |
| TG_52_4 | 2 | **0.000** | -0.582 | **0.000** | -0.295 | **0.050** | 0.287 | **0.044** |
| TG_52_5 | 2 | **0.000** | -0.213 | **0.002** | -0.097 | **0.227** | 0.116 | **0.073** |
| TG_52_6 | 2 | **0.001** | -0.025 | **0.491** | 0.044 | **0.128** | 0.069 | **0.003** |
| TG_53_1 | 2 | **0.052** | -0.018 | **0.126** | -0.014 | **0.305** | 0.004 | 0.989 |
| TG_53_2 | 2 | **0.014** | -0.067 | **0.037** | -0.049 | **0.187** | 0.019 | 0.863 |
| TG_54_0 | 2 | **0.055** | -0.009 | **0.146** | -0.008 | **0.269** | 0.001 | 0.999 |
| TG_54_1 | 2 | **0.093** | -0.098 | **0.166** | -0.065 | 0.557 | 0.033 | 0.892 |
| TG_54_2 | 2 | **0.009** | -0.345 | **0.025** | -0.225 | **0.211** | 0.121 | 0.658 |
| TG_54_3 | 2 | **0.000** | -0.498 | **0.002** | -0.423 | **0.003** | 0.074 | 0.914 |
| TG_54_4 | 2 | **0.000** | -0.470 | **0.002** | -0.370 | **0.006** | 0.101 | 0.742 |
| TG_54_5 | 2 | **0.001** | -0.333 | **0.005** | -0.213 | **0.078** | 0.119 | **0.464** |
| TG_54_6 | 2 | **0.001** | -0.176 | **0.062** | 0.089 | 0.588 | 0.266 | **0.001** |
| TG_54_7 | 2 | **0.000** | -0.018 | 0.657 | 0.109 | **0.000** | 0.127 | **0.000** |
| TG_55_1 | 2 | **0.044** | -0.005 | **0.205** | -0.006 | **0.135** | -0.001 | 0.999 |
| TG_55_2 | 2 | **0.490** | -0.004 | 0.656 | -0.003 | 0.937 | 0.001 | 0.999 |
| TG_55_3 | 2 | **0.005** | -0.006 | **0.024** | -0.005 | **0.048** | 0.001 | 0.989 |
| TG_56_0 | 2 | **0.269** | -0.012 | 0.548 | -0.014 | 0.551 | -0.002 | 0.999 |
| TG_56_1 | 2 | **0.118** | -0.010 | **0.353** | -0.011 | **0.296** | -0.002 | 0.999 |
| TG_56_2 | 2 | **0.129** | -0.029 | **0.225** | -0.022 | 0.550 | 0.007 | 0.979 |
| TG_56_3 | 2 | **0.035** | -0.044 | **0.091** | -0.036 | **0.235** | 0.008 | 0.989 |
| TG_56_4 | 2 | **0.004** | -0.041 | **0.026** | -0.037 | **0.040** | 0.004 | 0.999 |
| TG_56_5 | 2 | **0.031** | -0.049 | **0.292** | -0.073 | **0.072** | -0.024 | 0.837 |
| TG_56_6 | 2 | **0.018** | -0.060 | **0.360** | 0.045 | 0.673 | 0.105 | **0.031** |
| TG_56_7 | 2 | **0.000** | -0.126 | **0.211** | 0.385 | **0.000** | 0.510 | **0.000** |
| TG_56_8 | 2 | **0.000** | -0.075 | **0.356** | 0.342 | **0.000** | 0.417 | **0.000** |
| TG_57_1 | 2 | **0.443** | -0.014 | 0.564 | -0.008 | 1.000 | 0.006 | 0.957 |
| TG_57_2 | 2 | **0.097** | -0.008 | **0.165** | -0.005 | 0.577 | 0.003 | 0.892 |
| TG_58_1 | 2 | **0.301** | -0.024 | **0.476** | -0.021 | 0.689 | 0.004 | 0.999 |
| TG_58_10 | 2 | **0.000** | 0.003 | 1.000 | 0.059 | **0.000** | 0.056 | **0.000** |
| TG_58_2 | 2 | **0.131** | -0.016 | **0.206** | -0.010 | 0.687 | 0.007 | 0.863 |
| TG_58_3 | 2 | **0.172** | -0.006 | **0.292** | -0.005 | 0.601 | 0.001 | 0.993 |
| TG_58_4 | 2 | **0.352** | -0.003 | **0.476** | -0.001 | 1.000 | 0.001 | 0.892 |

| Metabolite name | Platform | ANOVA | fenofibrate vs. placebo | | fish oil vs. placebo | | fish oil vs. fenofibrate | |
|---|---|---|---|---|---|---|---|---|
| | | q-value | log(FC) | q-value | log(FC) | q-value | log(FC) | q-value |
| TG_58_5 | 2 | **0.004** | -0.003 | **0.029** | -0.003 | **0.026** | 0.000 | 0.999 |
| TG_58_6 | 2 | **0.000** | -0.001 | 0.972 | 0.008 | **0.013** | 0.009 | **0.002** |
| TG_58_8 | 2 | **0.000** | -0.011 | 0.806 | 0.077 | **0.000** | 0.088 | **0.000** |
| TG_58_9 | 2 | **0.000** | 0.000 | 1.000 | 0.073 | **0.000** | 0.073 | **0.000** |
| TG_59_0 | 2 | **0.381** | -0.003 | 0.889 | -0.005 | 0.619 | -0.002 | 0.934 |
| TG_59_1 | 2 | **0.079** | -0.018 | **0.211** | -0.017 | **0.308** | 0.001 | 0.999 |
| TG_59_2 | 2 | **0.149** | -0.007 | 0.559 | -0.011 | **0.310** | -0.004 | 0.930 |
| TG_59_3 | 2 | 0.588 | -0.001 | 0.718 | 0.000 | 1.000 | 0.000 | 0.989 |
| TG_60_1 | 2 | **0.156** | -0.009 | **0.452** | -0.011 | **0.359** | -0.002 | 0.999 |
| TG_60_18 | 2 | **0.251** | -0.005 | **0.395** | -0.004 | 0.689 | 0.001 | 0.999 |
| TG_60_2 | 2 | **0.156** | -0.011 | **0.355** | -0.011 | **0.426** | 0.000 | 0.999 |
| TG_60_3 | 2 | **0.077** | -0.005 | **0.387** | -0.007 | **0.183** | -0.002 | 0.930 |
| TG_62_3 | 2 | 0.667 | -0.001 | 0.945 | -0.001 | 0.943 | 0.000 | 0.999 |
| try_oAA | 1 | **0.007** | -0.025 | **0.036** | -0.001 | 1.000 | 0.024 | **0.031** |
| unknown 39b | 1 | **0.236** | 0.000 | 0.564 | 0.000 | 1.000 | 0.000 | **0.386** |
| unknown 39d | 1 | **0.033** | 0.000 | 0.912 | -0.002 | **0.090** | -0.001 | **0.270** |
| unknown 42 | 1 | **0.470** | 0.000 | 0.743 | 0.000 | 1.000 | 0.000 | 0.691 |
| unknown 47 | 1 | **0.319** | 0.000 | **0.420** | 0.000 | 0.990 | 0.000 | 0.892 |
| unknown 48 | 1 | **0.247** | 0.001 | 0.951 | 0.003 | **0.476** | 0.002 | 0.711 |
| unknown 52 | 1 | 0.966 | 0.000 | 1.000 | 0.000 | 1.000 | 0.000 | 0.999 |
| unknown 59b | 1 | **0.000** | -0.032 | 0.931 | 0.774 | **0.000** | 0.806 | **0.000** |
| unknown 59c | 1 | **0.196** | -0.003 | 0.951 | 0.007 | 0.698 | 0.010 | **0.340** |
| unknown 60 | 1 | **0.074** | -0.001 | 1.000 | 0.018 | **0.305** | 0.019 | **0.211** |
| unknown 60a | 1 | 0.836 | -0.002 | 1.000 | -0.017 | 1.000 | -0.015 | 0.989 |
| Ureum | 1 | **0.235** | 0.074 | 0.749 | -0.056 | 1.000 | -0.131 | **0.356** |
| Uric acid | 1 | **0.000** | -0.759 | **0.000** | 0.020 | 1.000 | 0.780 | **0.000** |
| Uridine | 1 | **0.000** | 0.014 | **0.001** | -0.003 | 0.911 | -0.017 | **0.000** |
| Vitamin E | 1 | **0.034** | -0.019 | **0.119** | -0.001 | 1.000 | 0.018 | **0.114** |

# 3

# Disentangling the Effects of Monounsaturated Fatty Acids from Other Components of a Mediterranean Diet on Serum Metabolite Profiles: A Randomized Fully Controlled Dietary Intervention in Healthy Subjects at Risk of the Metabolic Syndrome

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## ABSTRACT

**Scope:** The Mediterranean (MED) diet has been associated with a decreased risk of cardiovascular diseases. It is unclear whether this health effect can be mainly contributed to high intakes of MUFA, characteristic for the MED diet, or whether other components of a MED diet also play an important role.

**Methods and Results:** A randomized fully controlled parallel trial is performed to examine the effects of the consumption of a saturated fatty acid (SFA)-rich diet, a MUFA-rich diet, or a MED diet for 8 weeks on metabolite profiles, in 47 subjects at risk of the metabolic syndrome. A total of 162 serum metabolites were assessed before and after the intervention, by using a targeted NMR platform. 52 metabolites were changed during the intervention (false discovery rate [FDR] $p<0.05$). Both MUFA and MED diet decreased exactly the same fractions of LDL, including particle number, lipid, phospholipid and free cholesterol fraction (FDR $p<0.05$). The MED diet additionally decreased the larger subclasses of very-low-density lipoprotein (VLDL), several related VLDL fractions, VLDL-triglycerides, and serum-triglycerides (FDR $p<0.05$).

**Conclusion:** These findings clearly demonstrate that the MUFA component is responsible for reducing several LDL subclasses and fractions, and therefore causes an anti-atherogenic lipid profile. Interestingly, consumption of the other components in the MED diet show additional health effects.

## INTRODUCTION

Cardiovascular diseases (CVD) are the main cause of death worldwide (1). The development of CVD and other related metabolic disorders, are known to be affected by diet and especially by dietary fatty acids (2, 3). High intakes of saturated fatty acids (SFA) have been widely recognized to have detrimental health effects (1). Randomized controlled trials have demonstrated that a reduction in SFA intake can lead to a reduction in CVD events (4). In particular, replacing part of the saturated fatty acids (SFA) in the diet by MUFA has led to a decrease in inflammation (5), a reduction in triglycerides, total cholesterol, and LDL-cholesterol (3, 6), and an improvement in insulin sensitivity (7, 8); thereby positively affecting cardiovascular health. Furthermore, a higher intake of MUFA, when consumed in the form of olive oil, has been associated with a reduction in risk of all-cause mortality, cardiovascular mortality, and stroke (9).



The Mediterranean (MED) diet is characterized by a high consumption of MUFA mainly derived from olive oil (10). The consumption of this diet has been associated with a decreased risk of the metabolic syndrome and CVD (11). Additionally, the consumption of a MED diet has been shown to affect several risk factors of CVD, such as reductions in total cholesterol, LDL cholesterol, triglycerides, blood pressure, endothelial dysfunction, and insulin concentrations, and an increase in serum HDL cholesterol concentration (1, 3, 11-14). The question remains whether these observed health effects are caused by the MUFA component in this diet only, or whether other components of the MED diet have additional health effects.

Therefore, the objective of this study was to investigate the effects of partly replacing SFA by MUFA from olive oil in a Western type diet and the effects of a MED-type diet equally high in MUFA on concentrations of circulating lipids, lipoprotein particles, lipoprotein composition, and low-molecular-weight metabolites, including amino acids, in a parallel fully controlled-feeding trial in both men and women at risk of the metabolic syndrome. Since the MED-type diet is characterized not only by a high consumption of MUFA, but also by other potent dietary components such as nuts, legumes, fruits, fish, red wine, and unrefined cereals (3, 15-18), we hypothesized that the effect of a MED-type diet on the measured metabolites is more pronounced than the effect of partly replacing SFA by MUFA alone.

## MATERIALS AND METHODS

### *Subjects*

In total, 60 men and women participated in this study. The recruitment of subjects, inclusion and exclusion criteria, the study design, composition of the diets, and the primary outcome variable have previously been reported in more detail (3). In short, subjects ranged in age from 45 to 60 years old, and were included if they were at risk of developing the metabolic syndrome. The latter was defined as having a body mass index of ≥ 25 kg/m$^2$ or a waist circumference of ≥ 80 cm for women and ≥ 94 cm for men. Subjects were excluded if they had fasting total cholesterol ≥ 8 mmol/L, if they used anti-hypertensive or cholesterol-lowering medication, or if they had treated or non-treated diabetes. Subjects were tested for non-treated diabetes by an oral glucose-tolerance test (19). The power calculation was based on the primary outcome of the study, which was detecting a difference of at least 8.5 pmol/L in insulin concentration between the intervention groups.

### *Study design*

The study was a randomized fully controlled parallel dietary intervention trial (**Figure S1,** Supplemental Material). All subjects consumed a Western-type diet high in SFA (19% of total energy intake (en-%)) for a two-week run-in period, thereby standardizing the dietary conditions. After the run-in period, subjects were randomly allocated to one of the three intervention diets, which they had to consume for eight weeks.

### *Diets*

During these eight weeks, subjects continued on the Western-type diet high in SFA (SFA diet), received a Western-type diet in which part of the SFA was replaced by MUFA (MUFA diet, 20 en-% MUFA), or received a Mediterranean-type diet with a similar amount of MUFA (MED diet, 21 en-% MUFA) as the MUFA diet. The MED diet was higher in fatty fish, legumes, nuts, unrefined grain products, and red wine, and lower in dairy products and meat, compared with the other two intervention diets. During the study, 90% of the energy needs of the subjects was provided. The remaining 10% of the energy needs was chosen by the subjects from a list of low-fat and low-fiber products. All these choices were recorded in a food diary. Body weight was measured twice a week and energy intake was adjusted if the subject gained or lost weight. The Medical Ethical Committee of Wageningen University, Netherlands, approved the study, and all subjects gave written informed consent. This trial

was registered at clinicaltrials.gov as NCT00405197.

***Blood collection***

Overnight fasting venous blood samples were collected at baseline (at the end of the two-week run-in period), and after the eight-week intervention. All serum samples were kept at -80 °C until further analysis within one run.



***Serum metabolite measurements***

Our serum samples were processed by Nightingale Health's blood biomarker analysis service to obtain the metabolite profiles. This service employs a high-throughput NMR spectroscopy platform, using a single experimental setup that allows for the simultaneous quantification of 162 metabolic measures that represent a broad molecular signature of the systemic metabolite profile in serum. This platform has been used in multiple large-scale epidemiologic studies, of which an overview can be found in (20). Details of the methodology have been described previously (21, 22). This platform quantifies absolute concentration units of routine lipids, total lipid concentrations of 14 lipoprotein subclasses, fatty acid compositions, various glycolysis precursors, ketone bodies, and amino acids. All measured metabolites fall in the range of detection, and numbers on the analytical performance in terms of repeatability (CV%) can be found in the article of Holmes and colleagues (23).

***Statistical analysis***

We examined differences in baseline characteristics between the three diet groups by analysis of variance (ANOVA) testing, or Chi-square testing for categorical data. Statistical analysis of the metabolites were performed on log-transformed data. We tested the main effects of the diet × time interaction with an ANOVA, and we used linear mixed models to assess between diet effects. For the effect of the diets we included diet, time and the interaction between diet and time as fixed effects. We included subjects with a random intercept in the model. Significant metabolites were first selected using the False Discovery Rate (FDR) adjusted F-statistic (24) $p$-value < 0.05. Unadjusted $p$-values below 0.05 for the between diet effects were considered statistically significant within the metabolites that passed the F-test. Within diet effects were tested with a paired t-test, here FDR adjusted $p$-values below 0.05 were considered statistically significant. Using sparse partial least squares discriminant analysis (sPLS-DA) we attempted to separate the responses of the metabolites between the three

diets. The sPLS-DA model was made using the *caret* R library (25). This model was validated using ten times repeated ten-fold cross-validation. The final number of components for the sPLS-DA model selected by grid search was 4 for the diet-model. All analyses were done using R (version 3.4.2) (26). Heatmaps were made in Excel 2016, and the tree-structure figure was made using Cytoscape (version 3.2.1) (27).

## RESULTS

### *Subject characteristics*

Of the 60 subjects that entered this study, 57 completed the study. Mean daily intakes of energy and nutrients per diet have been published previously, as well as the baseline characteristics of these 57 subjects (3). For the metabolite profile analysis we had enough serum left for 47 out of the 57 subjects (Flowchart: **Figure 1**).

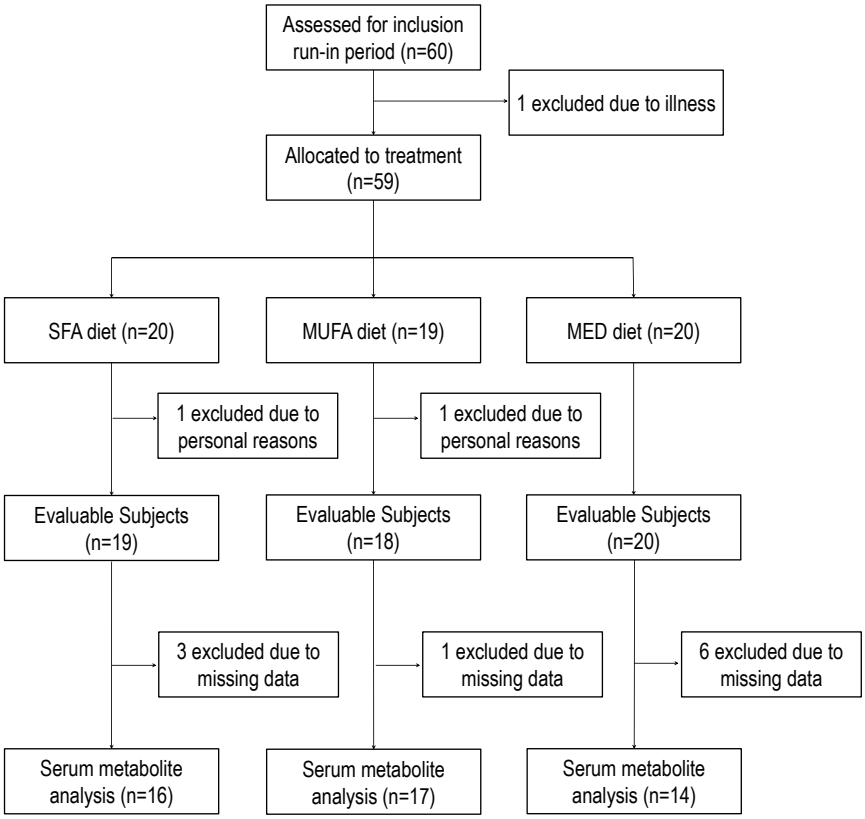



**Figure 1.** Flowchart of the subjects included for metabolite profile analysis in serum. Abbreviations: Mediterranean (MED), Monounsaturated Fatty Acid (MUFA), Saturated Fatty Acid (SFA).

In these subjects, 162 metabolites were determined in serum collected before and after the interventions. Baseline characteristics of the 47 subjects are summarized in **Table 1**. The baseline characteristics of the 10 subjects with missing data were not significantly different from the 47 included subjects (data not shown). Age differed significantly between the three intervention groups ($p$ =0.006). Age of the subjects in the SFA group was significantly lower compared to the other two diet groups.

**Table 1.** Baseline characteristics of the 47 subjects included in this study. Data is presented as mean ± standard deviation.

| | ***SFA diet*** (n=16) | ***MUFA diet*** (n=17) | ***MED diet*** (n=14) | *Differences at baseline p-value*[a] |
|---|---|---|---|---|
| Sex (m/f) | 8/8 | 8/9 | 4/10 | 0.444 |
| Age (years) | 51.4 ± 7.8$^{A}$ | 58.1 ± 5.2$^{B}$ | 57.4 ± 5.1$^{B}$ | 0.006 |
| Body weight (kg) | 82.2 ± 12.5 | 77.3 ± 11.9 | 84.4 ± 14.5 | 0.297 |
| BMI (kg/m$^2$) | 26.4 ± 2.9 | 27.2 ± 5.3 | 28.9 ± 6.5 | 0.425 |
| Waist circumference (cm) | | | | |
| Men | 103.1 ± 8.1 | 99.7 ± 7.8 | 101.5 ± 6.3 | 0.679 |
| Women | 89.0 ± 3.9 | 94.6 ± 17.4 | 98.5 ± 18.4 | 0.435 |
| Heart rate (bpm) | 67.8 ± 9.6 | 69.9 ± 10.1 | 67.2 ± 10.9 | 0.744 |
| Systolic blood pressure (mmHg) | 115.9 ± 12.5 | 119.7 ± 18.4 | 117.9 ± 9.5 | 0.744 |
| Diastolic blood pressure (mmHg) | 71.8 ± 10.0 | 75.9 ± 14.4 | 74.6 ± 9.5 | 0.597 |
| Total cholesterol (mmol/L) | 5.69 ± 1.18 | 5.75 ± 0.57 | 5.74 ± 0.81 | 0.979 |
| LDL cholesterol (mmol/L) | 4.05 ± 1.02 | 3.87 ± 0.62 | 3.90 ± 0.74 | 0.806 |
| HDL cholesterol (mmol/L) | 1.27 ± 0.27 | 1.42 ± 0.39 | 1.36 ± 0.52 | 0.553 |
| Triglycerides (mmol/L) | 1.06 ± 0.51 | 1.23 ± 0.46 | 1.28 ± 0.63 | 0.475 |

[a]Means were compared using ANOVA (or chi-square for categorical values) and corresponding $p$-values are shown. In case of an significant overall $p$-value: Bonferroni post-hoc test was performed and values with different superscript letters in the row are significantly different, $p$ <0.05.

### *Diet effect on metabolites*

No differences in baseline concentration of the 162 metabolites were observed between the three diets after correcting for multiple testing (data not shown). 52 of the 162 metabolic parameters were significantly changed between the three diet groups during the intervention (FDR $p$ < 0.05) (**Table S1**, Supplemental Material). Details on the number of significantly changed metabolites between the diets and within each diet group are summarized in **Figure 2**.

The main subgroup of metabolites that was affected by the diets were the lipids and lipoproteins. In **Figure 3** (and in more detail in **Figure S2**, Suppemental Material) the effects of consumption of the MUFA and MED diet are visualized. Briefly, the MUFA diet mainly decreased the LDL related

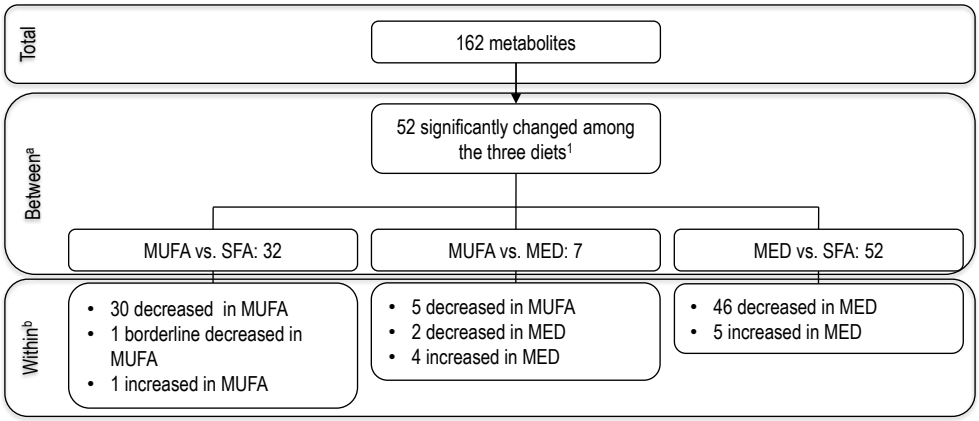



**Figure 2.** Significantly changed metabolites upon the three intervention diets. This flow diagram shows the number of metabolites of which the change in concentration was significantly different among the three diet groups. Main effects of the diet × time interaction were tested with an [1]ANOVA with an FDR correction (FDR $p$ <0.05) [2]Linear mixed model on the differential changes between the diet groups ($p$ < 0.05), on the metabolites that passed the F-test. [3]Results of the within diet group changes were tested with a paired t-test (FDR $p$ < 0.05). [4]FDR $p$-value = 0.052 in the within diet test. Abbreviations: Mediterranean (MED), Saturated Fatty Acid (SFA).

fractions and a subset of the cholesterol fractions including serum cholesterol, VLDL-cholesterol, free-cholesterol and remnant-cholesterol. The MED diet decreased exactly the same LDL and cholesterol fractions as the MUFA diet, however the MED diet additionally decreased multiple VLDL related fractions; mainly in the XL-, L-, and M- VLDL subclasses, plus total VLDL-TG concentration, and total TG concentration.

Changes in ApoB and the ApoB to ApoA1 ratio were also significantly different between the three diets, FDR $p$ = 0.022 and FDR $p$ = 0.025 respectively (Supplemental Material Table S1). ApoB decreased significantly in the MUFA diet group (-0.110 ± 0.090 g/L, $p$ <0.001), and the MED diet group (-0.151 ± 0.122 g/L, $p$ = 0.003), compared to the SFA diet group. The ApoB to ApoA1 ratio decreased significantly in the MUFA (-0.059 ± 0.46, $p$ <0.001) and MED diet group (-0.107 ± 0.081, $p$ = 0.003), compared to SFA diet group.

Glycolysis related metabolites, amino acids, proteins and glycoprotein acetyls were also determined. However, no differences between the diet groups were observed (Supporting Information Table S1) except for albumin (FDR $p$ = 0.048), which decreased within the MUFA diet (-0.002 ± 0.003 mmol/L, $p$ = 0.014) and the MED diet (-0.002 ± 0.003 mmol/L, $p$ = 0.048), compared to the SFA diet group.

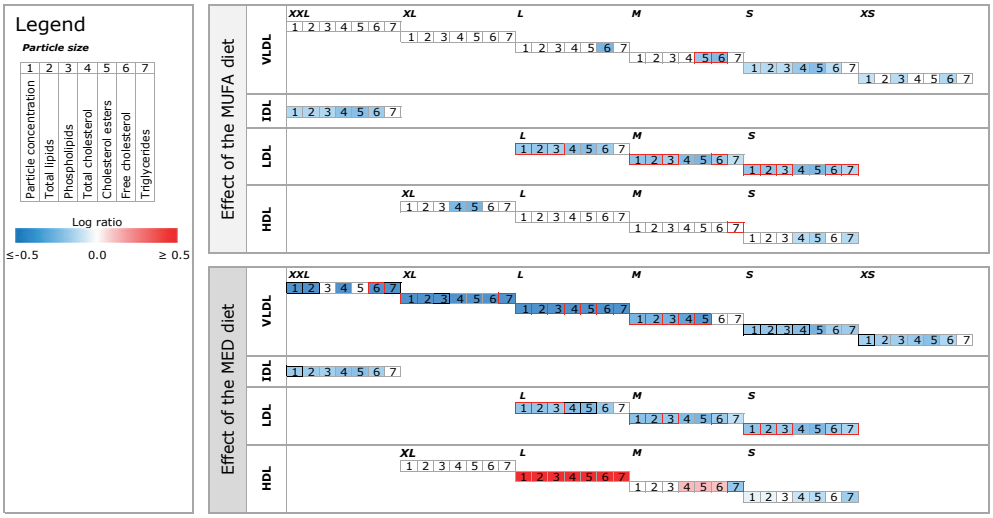



**Figure 3.** The effects of diets on lipoproteins and the subfractions. On the left of the figure the legend is presented with the log ratio scale and an explanation of the meaning of the numbers. On top of the figure the effect of the MUFA diet is visualized. At the bottom of the figure the effect of the MED diet is visualized. The color of the edge of a box represents the between treatment effect. If the edge is red, the ANOVA FDR $p < 0.05$, and between diet effect, tested with linear mixed models was $p < 0.05$. If the color is black the ANOVA FDR $p < 0.05$, and the between diet effect showed a trend ($0.05 < p \leq 0.06$). The color inside a box represents the changes within the MUFA diet (top) or MED diet (bottom), ranging from dark blue (log ratio (LR) ≤ -0.5) to dark red (LR ≥ 0.5), the inside is only colored if the paired t-test FDR $p < 0.05$.

### *Dietary exposure markers*

Dietary exposure markers were included in the metabolomics measurement in the serum samples (Supplemental Material Table S1). The following metabolites were significantly differently changed among the three diets: docosahexaenoic acid (DHA), DHA to total fatty acids (FA) ratio, omega-3 fatty acids (FAω3), FAω3 to FA ratio, conjugated linoleic acid (CLA), CLA to FA ratio, and MUFA to FA ratio, all FDR $p$ <0.001, except MUFA to FA ratio (FRD $p = 0.002$). Comparisons between the diets showed that DHA, and DHA to FA ratio were significantly increased in the MED diet group versus the SFA group (0.024 ± 0.035 mmol/L, FDR $p = 0.039$, and 0.363 ± 0.274%, FDR $p = 0.003$ respectively). Furthermore, FAω3 was significantly decreased in the MUFA group (-0.094 ± 0.048 mmol/L, FDR $p$ <0.001) and increased in the MED diet group (0.041 ± 0.077 mmol/L, FDR $p = 0.131$) compared to the SFA group, though within the MED diet group this increase was not significant. FAω3 to FA ratio decreased in the MUFA group (-0.583 ± 0.251%, FDR $p$ <0.001), but increased in the MED group (0.769 ± 0.632%, FDR $p = 0.003$) compared to the SFA group. CLA, and CLA to FA ratio decreased upon both the MUFA diet (-0.028 ± 0.013 mmol/L, FDR $p$ <0.001, and -0.222 ± 0.092%, FDR $p$ <0.001

respectively) and the MED diet (-0.022 ± 0.015 mmol/L, FDR $p$ = 0.003, -0.176 ± 0.130%, FDR $p$ = 0.006) versus the SFA diet group. Lastly, MUFA to FA ratio increased significantly in both the MUFA diet group (3.344 ± 2.124%, FDR $p$<0.001), and the MED diet group (2.200 ± 1.509%, FDR $p$ = 0.002).

To examine the differences in effect between the three diets we performed a sparse partial least squares discriminant analysis (sPLS-DA) model. The best fitting sPLS-DA model had 4 components, a Cohen's kappa of 0.77, an *eta* of 0.9, and an accuracy of 85.4% to place the right subject in the right diet group. The variables most important for the separation between the three intervention diets were: CLA, CLA/FA, DHA/FA, FAω3/FA and MUFA/FA; all exposure markers of the diets the subjects consumed. The changes in these five variables upon the three intervention diets are displayed in **Figure 4**.

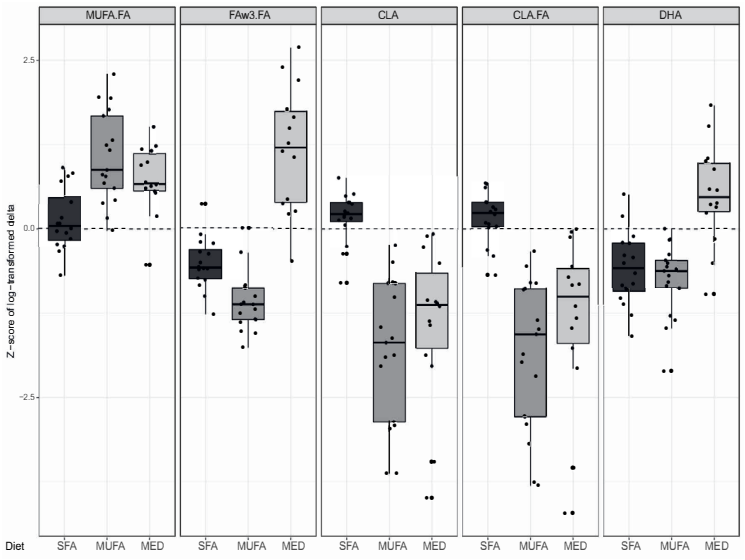
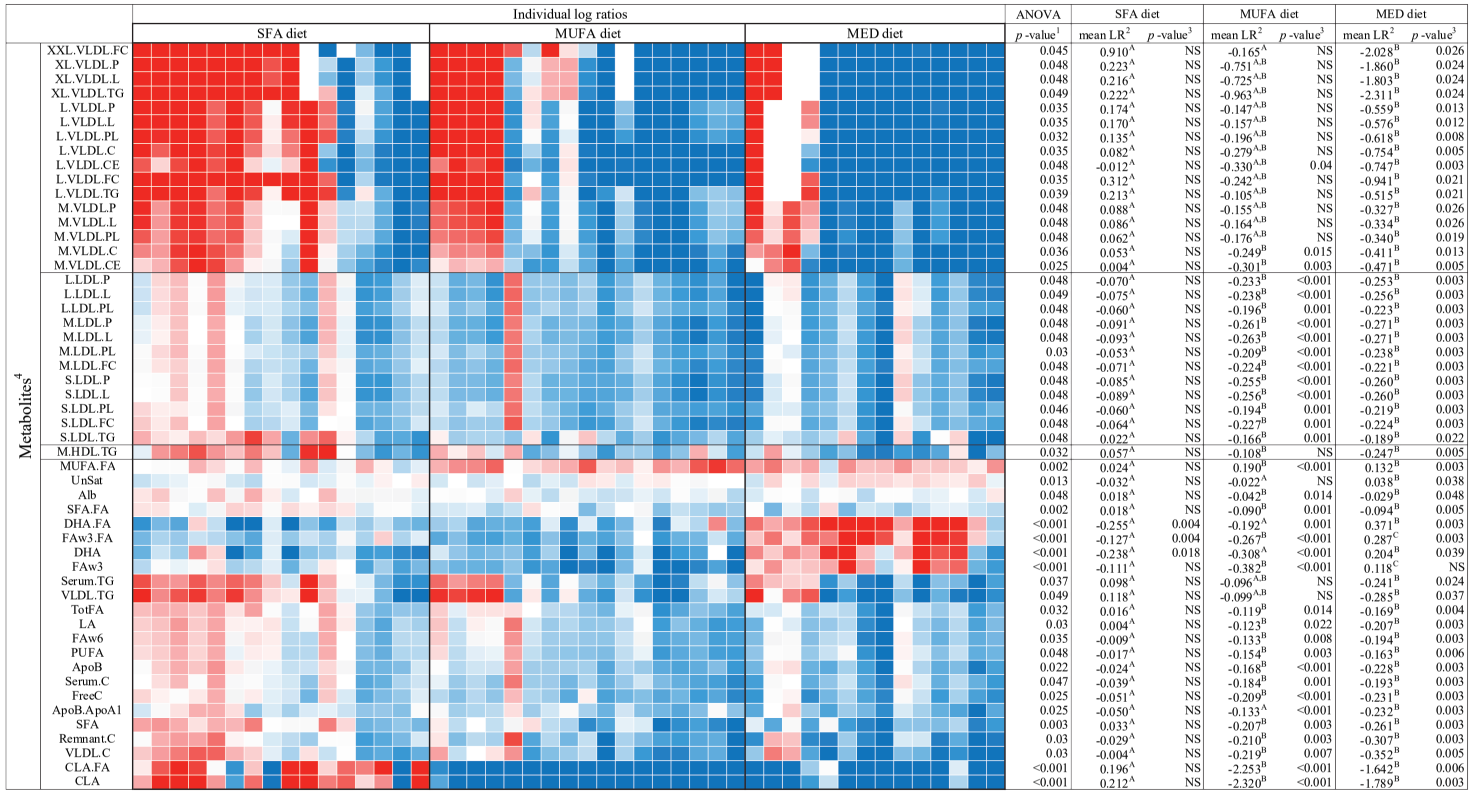



**Figure 4.** Boxplots of the changes upon the intervention in the 5 metabolites most important for the separation between the three intervention diets, as determined by sparse partial least squares discriminant analysis.

### *Individual changes*

To visualize the individual changes of the 52 metabolites that were significantly different among the three diets a heat map was created (**Figure 5**). The figure shows that for the dietary exposure biomarkers each individual within a diet group is affected in the same direction. Furthermore, the figure shows that the individual changes in the lipoproteins are robust within each lipoprotein subclass, however variations in response between subjects are present.




| | ANOVA | SFA diet | | MUFA diet | | MED diet | |
|---|---|---|---|---|---|---|---|
| | *p* -value[1] | mean LR[2] | *p* -value[3] | mean LR[2] | *p* -value[3] | mean LR[2] | *p* -value[3] |
| XXL.VLDL.FC | 0.045 | 0.910$^{A}$ | NS | -0.165$^{A}$ | NS | -2.028$^{B}$ | 0.026 |
| XL.VLDL.P | 0.048 | 0.223$^{A}$ | NS | -0.751$^{A,B}$ | NS | -1.860$^{B}$ | 0.024 |
| XL.VLDL.L | 0.048 | 0.216$^{A}$ | NS | -0.725$^{A,B}$ | NS | -1.803$^{B}$ | 0.024 |
| XL.VLDL.TG | 0.049 | 0.222$^{A}$ | NS | -0.963$^{A,B}$ | NS | -2.311$^{B}$ | 0.024 |
| L.VLDL.P | 0.035 | 0.174$^{A}$ | NS | -0.147$^{A,B}$ | NS | -0.559$^{B}$ | 0.013 |
| L.VLDL.L | 0.035 | 0.170$^{A}$ | NS | -0.157$^{A,B}$ | NS | -0.576$^{B}$ | 0.012 |
| L.VLDL.PL | 0.032 | 0.135$^{A}$ | NS | -0.196$^{A,B}$ | NS | -0.618$^{B}$ | 0.008 |
| L.VLDL.C | 0.035 | 0.082$^{A}$ | NS | -0.279$^{A,B}$ | NS | -0.754$^{B}$ | 0.005 |
| L.VLDL.CE | 0.048 | -0.012$^{A}$ | NS | -0.330$^{A,B}$ | 0.04 | -0.747$^{B}$ | 0.003 |
| L.VLDL.FC | 0.035 | 0.312$^{A}$ | NS | -0.242$^{A,B}$ | NS | -0.941$^{B}$ | 0.021 |
| L.VLDL.TG | 0.039 | 0.213$^{A}$ | NS | -0.105$^{A,B}$ | NS | -0.515$^{B}$ | 0.021 |
| M.VLDL.P | 0.048 | 0.088$^{A}$ | NS | -0.155$^{A,B}$ | NS | -0.327$^{B}$ | 0.026 |
| M.VLDL.L | 0.048 | 0.086$^{A}$ | NS | -0.164$^{A,B}$ | NS | -0.334$^{B}$ | 0.026 |
| M.VLDL.PL | 0.048 | 0.062$^{A}$ | NS | -0.176$^{A,B}$ | NS | -0.340$^{B}$ | 0.019 |
| M.VLDL.C | 0.036 | 0.053$^{A}$ | NS | -0.249$^{B}$ | 0.015 | -0.411$^{B}$ | 0.013 |
| M.VLDL.CE | 0.025 | 0.004$^{A}$ | NS | -0.301$^{B}$ | 0.003 | -0.471$^{B}$ | 0.005 |
| L.LDL.P | 0.048 | -0.070$^{A}$ | NS | -0.233$^{B}$ | <0.001 | -0.253$^{B}$ | 0.003 |
| L.LDL.L | 0.049 | -0.075$^{A}$ | NS | -0.238$^{B}$ | <0.001 | -0.256$^{B}$ | 0.003 |
| L.LDL.PL | 0.048 | -0.060$^{A}$ | NS | -0.196$^{B}$ | 0.001 | -0.223$^{B}$ | 0.003 |
| M.LDL.P | 0.048 | -0.091$^{A}$ | NS | -0.261$^{B}$ | <0.001 | -0.271$^{B}$ | 0.003 |
| M.LDL.L | 0.048 | -0.093$^{A}$ | NS | -0.263$^{B}$ | <0.001 | -0.271$^{B}$ | 0.003 |
| M.LDL.PL | 0.03 | -0.053$^{A}$ | NS | -0.209$^{B}$ | <0.001 | -0.238$^{B}$ | 0.003 |
| M.LDL.FC | 0.048 | -0.071$^{A}$ | NS | -0.224$^{B}$ | <0.001 | -0.221$^{B}$ | 0.003 |
| S.LDL.P | 0.048 | -0.085$^{A}$ | NS | -0.255$^{B}$ | <0.001 | -0.260$^{B}$ | 0.003 |
| S.LDL.L | 0.048 | -0.089$^{A}$ | NS | -0.256$^{B}$ | <0.001 | -0.260$^{B}$ | 0.003 |
| S.LDL.PL | 0.046 | -0.060$^{A}$ | NS | -0.194$^{B}$ | 0.001 | -0.219$^{B}$ | 0.003 |
| S.LDL.FC | 0.048 | -0.064$^{A}$ | NS | -0.227$^{B}$ | 0.001 | -0.224$^{B}$ | 0.003 |
| S.LDL.TG | 0.048 | 0.022$^{A}$ | NS | -0.166$^{B}$ | 0.001 | -0.189$^{B}$ | 0.022 |
| M.HDL.TG | 0.032 | 0.057$^{A}$ | NS | -0.108$^{B}$ | NS | -0.247$^{B}$ | 0.005 |
| MUFA.FA | 0.002 | 0.024$^{A}$ | NS | 0.190$^{B}$ | <0.001 | 0.132$^{B}$ | 0.003 |
| UnSat | 0.013 | -0.032$^{A}$ | NS | -0.022$^{A}$ | NS | 0.038$^{B}$ | 0.038 |
| Alb | 0.048 | 0.018$^{A}$ | NS | -0.042$^{B}$ | 0.014 | -0.029$^{B}$ | 0.048 |
| SFA.FA | 0.002 | 0.018$^{A}$ | NS | -0.090$^{B}$ | 0.001 | -0.094$^{B}$ | 0.005 |
| DHA.FA | <0.001 | -0.255$^{A}$ | 0.004 | -0.192$^{A}$ | 0.001 | 0.371$^{B}$ | 0.003 |
| FAw3.FA | <0.001 | -0.127$^{A}$ | 0.004 | -0.267$^{B}$ | <0.001 | 0.287$^{C}$ | 0.003 |
| DHA | <0.001 | -0.238$^{A}$ | 0.018 | -0.308$^{A}$ | <0.001 | 0.204$^{B}$ | 0.039 |
| FAw3 | <0.001 | -0.111$^{A}$ | NS | -0.382$^{B}$ | <0.001 | 0.118$^{C}$ | NS |
| Serum.TG | 0.037 | 0.098$^{A}$ | NS | -0.096$^{A,B}$ | NS | -0.241$^{B}$ | 0.024 |
| VLDL.TG | 0.049 | 0.118$^{A}$ | NS | -0.099$^{A,B}$ | NS | -0.285$^{B}$ | 0.037 |
| TotFA | 0.032 | 0.016$^{A}$ | NS | -0.119$^{B}$ | 0.014 | -0.169$^{B}$ | 0.004 |
| LA | 0.03 | 0.004$^{A}$ | NS | -0.123$^{B}$ | 0.022 | -0.207$^{B}$ | 0.003 |
| FAw6 | 0.035 | -0.009$^{A}$ | NS | -0.133$^{B}$ | 0.008 | -0.194$^{B}$ | 0.003 |
| PUFA | 0.048 | -0.017$^{A}$ | NS | -0.154$^{B}$ | 0.003 | -0.163$^{B}$ | 0.006 |
| ApoB | 0.022 | -0.024$^{A}$ | NS | -0.168$^{B}$ | <0.001 | -0.228$^{B}$ | 0.003 |
| Serum.C | 0.047 | -0.039$^{A}$ | NS | -0.184$^{B}$ | 0.001 | -0.193$^{B}$ | 0.003 |
| FreeC | 0.025 | -0.051$^{A}$ | NS | -0.209$^{B}$ | <0.001 | -0.231$^{B}$ | 0.003 |
| ApoB.ApoA1 | 0.025 | -0.050$^{A}$ | NS | -0.133$^{A}$ | <0.001 | -0.232$^{B}$ | 0.003 |
| SFA | 0.003 | 0.033$^{A}$ | NS | -0.207$^{B}$ | 0.003 | -0.261$^{B}$ | 0.003 |
| Remnant.C | 0.03 | -0.029$^{A}$ | NS | -0.210$^{B}$ | 0.003 | -0.307$^{B}$ | 0.003 |
| VLDL.C | 0.03 | -0.004$^{A}$ | NS | -0.219$^{B}$ | 0.007 | -0.352$^{B}$ | 0.005 |
| CLA.FA | <0.001 | 0.196$^{A}$ | NS | -2.253$^{B}$ | <0.001 | -1.642$^{B}$ | 0.006 |
| CLA | <0.001 | 0.212$^{A}$ | NS | -2.320$^{B}$ | <0.001 | -1.789$^{B}$ | 0.003 |

**Figure 5.** Heat map of the 52 significantly changed metabolic parameters between the three intervention diets, tested with an [1]ANOVA with an FDR correction (FDR $p < 0.05$). Differences between two diet groups ($p < 0.05$) tested with a linear mixed model, are indicated with a superscript letter in the corresponding row. Individual changes are indicated as individual log-ratios (LR) of values after the intervention versus before the intervention, in the SFA diet group (n = 16), MUFA diet group (n=17), and the MED diet group (n=14). These changes are presented on a color scale ranging from blue (decreased, LR ≤ -0.50) to red (increased, LR ≥ 0.50). Subjects are clustered within the diets (Euclidian distance and average linkage). [2]Mean log ratios per diet group [3]Corresponding FDR corrected p-values, as tested with a paired t-test.

## DISCUSSION

The present study in healthy men and women at risk of the metabolic syndrome, demonstrates that eight-week consumption of a MED diet resulted in an additional effect on serum metabolites compared to the effect of replacing SFA by MUFA alone. We observed that the MUFA and the MED diet decreased exactly the same LDL lipoprotein and cholesterol fractions. The MED diet additionally decreased several fractions of the larger VLDL lipoprotein subclasses, TG concentration in total VLDL, and total TG concentration.

This is the first fully controlled dietary intervention study examining not only the effect of a MUFA diet versus the effect of a MED diet on lipids and lipoprotein subclasses, but also the effect on the composition of the different lipoprotein subclasses. Both the MUFA and MED diets were responsible for decreasing the concentration of the large, medium, and small LDL subclasses. Also, within these LDL-subclasses we observed a decrease in various fractions, amongst which were the total lipids, and the phospholipids. As the MUFA fraction is the same in both diets, and different from the SFA diet, we extrapolate that the MUFA fraction is responsible for these effects on LDL. MUFA has previously been shown to decrease LDL-cholesterol (3, 28, 29), various LDL-subclass concentrations, and total LDL particle number (30). We here measured the effect of replacing SFA by MUFA on LDL lipoprotein composition in more detail, and found a reduction in multiple LDL fractions, such as a decrease in the phospholipid and lipid fractions of all three LDL subclasses, and a decrease in free cholesterol in medium and small-LDL subclass.

Apart from the effect of MUFA on LDL, we observed additional effects in the MED diet group. On top of the effects on LDL, the MED diet decreased larger VLDL subclasses, related subclass composition, total TG concentration in VLDL, and total TG concentration. In the PREDIMED trial a reduction in large VLDL lipoproteins and total serum-TG concentration was observed in the group that consumed a MED diet supplemented with nuts (30), which was similar to the finding in our MED diet group, which also contained nuts. In our study however, we measured the effect of the MED diet on VLDL lipoproteins in more detail, and we found a reduction in multiple VLDL subclasses and fractions. Moreover, all fractions in the large subclass were reduced, as was the lipid fraction in the extra-large, large, and medium VLDL subclass. As the MED-diet induced VLDL reductions were not observed in the MUFA diet, we assume that these effects were not caused by the MUFA component in the diet. Other components of

the MED diet are likely responsible for the observed effects. Consumption of omega-3 polyunsaturated fatty acids has been shown to decrease total TG and TG in VLDL (31-35). PUFAs are known to activate peroxisome proliferator-activated receptors (PPARs), which are involved in regulating the expression of genes causing an increase in β-oxidation, thereby reduce the bioavailability of lipids for production of VLDL in the liver (32, 36, 37). As n-3 PUFAs are present in fish and nuts, and both were components of our MED diet and not our MUFA diet, this might be a part of the explanation for the observed decrease in several VLDL fractions upon the MED diet. Another explanation could be that clearance of TG rich particles in the circulation is increased in the MED diet group (37).



Except for the triglycerides subfraction in M-HDL, we did not find significant differences between the MED diet compared to the other two diets on HDL related particles. However, we do observe a shift within the MED diet group from the smaller HDL particles to the larger HDL particles. This shift could have been caused by the consumption of alcohol in the MED diet, as alcohol has been associated with an increase in the larger HDL particles (38, 39). Furthermore, the shift could also have been caused by fatty fish, as a similar shift in HDL particles was observed in the 12-week intervention study by Lankinen (40) in the group with an increased consumption of amongst others, fatty fish. Moreover, Erkkilä *et al.* (41) also observed a shift towards the larger HDL particles after an eight-week intervention study with fatty fish intake four times a week.

***Risk for diseases***

Our study showed that both the MUFA and the MED diet decreased the concentration of the smallest LDL subclass, and the cholesterol and cholesterol ester content of this subclass. Especially the small LDL subclass is associated with an increased risk for CVD (42-44), indicating a potential favorable role of MUFA consumption on CVD risk. Moreover, the MUFA and MED diet both decreased LDL particle (P) concentration of all the LDL subclasses. A decrease in LDL-P concentration has been associated with a decrease in atherosclerotic risk, and it has been described to be a better predictor of CVD events compared to the conventionally used LDL-cholesterol concentrations (43). In addition, both the MUFA and MED diet significantly decreased ApoB concentration, and the ApoB/ApoA1 ratio. An increased ApoB/ApoA1 ratio is a well-known predictor for e.g. acute myocardial infarction and acute coronary events (45, 46). In summary, the lowering of small LDL concentration, LDL particle number, and the increase in the ApoB/ApoA1 ratio by both the MUFA and MED diet points towards a potential

beneficial effect of a high MUFA intake on risk for cardiovascular health.

Only the MED diet decreased total VLDL-TG concentration and serum TG concentration. An increased fasting serum TG concentration has been associated with coronary artery disease, increased mortality risk in coronary heart disease (CHD) patients, and an increased risk for developing both CHD and ischemic heart disease (47-50). Next to this, higher VLDL lipoprotein concentrations have been associated with ischemic heart disease risk (51-54), and higher levels of VLDL-TG have been observed in type 2 diabetes mellitus patients (55). Thus, the effects of the MED diet, e.g. the lowering of total VLDL-TG, and serum TG concentration, may indicate a potential additional beneficial effect on cardiovascular risk on top of the effect of the MUFA content. Furthermore, we observed that the concentrations of XL-, L- and M-VLDL particles, and triglycerides in XL- and L-VLDL particles were all decreased by the MED diet. These metabolites have been cross-sectionally and prospectively associated with the development of fatty liver (56, 57). This again indicates an additional favorable effect of the MED diet.

***Exposure markers***

The consistent changes observed in the metabolites: DHA, DHA to FA ratio, FAω3 to FA ratio, MUFA/FA, CLA, and CLA/FA ratio in the serum samples, confirm that these markers are suitable markers to measure dietary exposure. Indicating that they can possibly be used to assess whether an individual had a high consumption of olive oil (reflected by MUFA/FA), fish (reflected by DHA, DHA to FA ratio, and FAω3 to FA ratio), or butter (reflected by CLA, and CLA/FA ratio). Remarkably, we observed interpersonal differences in the diet-induced effect on other markers. In all three diet groups four to five individuals reacted differently compared to the rest of that diet group. As we found no differences at baseline between any of these measured serum metabolites, this points towards variation in response to the diets. It will be valuable to assess the consistency in such a response to diets, especially with respect to personalized dietary advise in the future (58).

***Limitations and strengths***

Even though our sample size was relatively small with 47 subjects, our dietary intervention had a profound effect on various metabolites as 32 percent of all metabolites was affected by the diets after applying an FDR correction. We hypothesize that this can mainly be explained by the high level

of dietary control in this study. In the present study, 90% of the energy needs was provided, and the remaining 10% was chosen by the subjects from a list of low-fat and low-fiber products. Other dietary intervention studies with a similar number of subjects (33, 41) provided only part of the intended diet, or only gave dietary advice (30, 40, 59, 60) and therefore possibly found smaller effects. Even though body weight was closely monitored, the subjects in the SFA group and the MED group did lose some weight. However, no difference was found in weight loss between the SFA, MUFA or MED diet group. Lastly, our study had a duration of 10 weeks in total (including the run-in period), to determine the long-term effects of adhering to a diet high in MUFA or a MED diet on changes in lipids, lipoprotein particles and CVD risk more long-term studies are needed.



***Summary and conclusion***

In summary, this 8 week fully controlled dietary intervention study showed that MUFA in the diet decreased LDL particle concentration in all three subclasses and several related fractions, including a decrease in the small LDL concentration. MUFA were also responsible for an increase in the ApoB/ApoA1 ratio. These are all favorable effects on risk factors for CVD. The MED diet additionally decreased total VLDL-TG, total serum-TG concentration, and particle concentration of three larger VLDL subclasses together with several related fractions, including triglycerides in the XL- and L-VLDL particles. Thus, additional favorable effects on other risk factors for CVD. In conclusion, we were able to disentangle the effect of the MUFA content in the MED diet from the effect of the other components in the MED diet. Our study clearly demonstrates that the MUFA component is responsible for reducing several LDL subclasses and fractions, and therefore causes a more anti-atherogenic lipid profile. Interestingly, consumption of the other components in the MED diet show additional health effects, by reducing several other risk factors for CVD.

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## CONFILCT OF INTEREST

There is no conflict of interest.

## AUTHOR CONTRIBUTIONS

*C.C.J.R.M.* and *R.W.J.H.* analyzed the data; *C.C.J.R.M.* wrote the manuscript, which was critically reviewed and improved by *E.J.M.F.* and *L.A.A.*; All authors read and approved the final manuscript.

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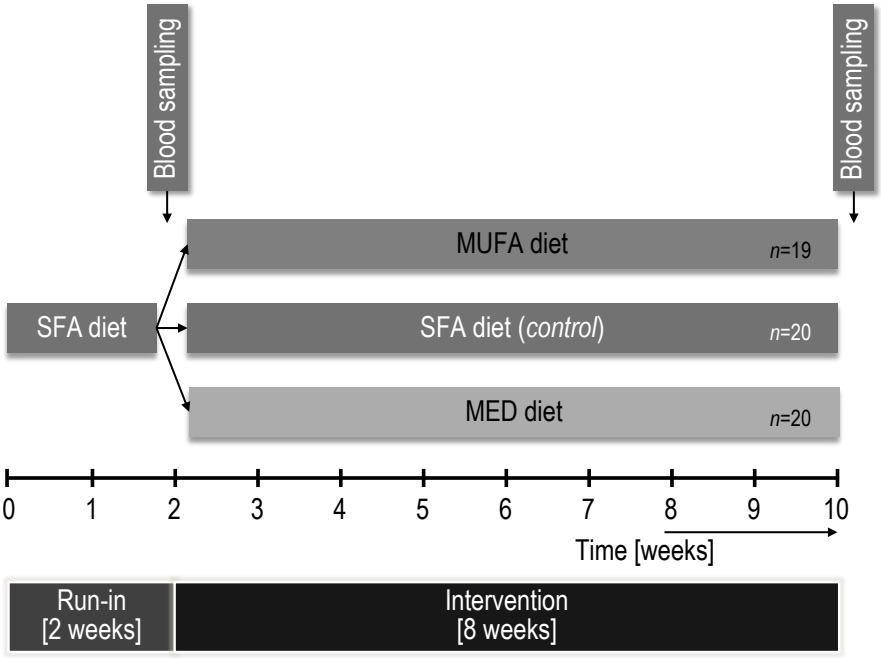
## LIST OF ABBREVIATIONS

| Abbreviation | Full description |
|---|---|
| .C | Total Cholesterol in that particular particle |
| .CE | Cholesterol Esters in that particular particle |
| .FC | Free Cholesterol in that particular particle |
| .L | Total Lipids in that particular particle |
| .P | Concentration of that particular particle |
| .PL | Phospholipids in that particular particle |
| .TG | Triglycerides in that particular particle |
| AcAce | Acetoacetate |
| Ace | Acetate |
| Ala | Alanine |
| Alb | Albumin |
| ApoA1 | Apolipoprotein A-I |
| ApoB | Apolipoprotein B |
| ApoB.ApoA1 | Ratio of apolipoprotein B to apolipoprotein A-I[1] |
| bOHBut | 3-Hydroxybutyrate |
| CHD | Coronary Heart Disease |
| Cit | Citrate |
| CLA | Conjugated Linoleic Acid |
| CLA.FA | Ratio of Conjugated Linoleic Acid to total Fatty Acids |
| Crea | Creatinine |
| CVD | Cardiovascular Diseases |
| DAG | Diacylglycerol |
| DAG.TG | Ratio of Diacylglycerol to Triglycerides |
| DHA | 22:6, Docosahexaenoic Acid |
| DHA.FA | Ratio of 22:6, Docosahexaenoic Acid to total Fatty Acids |
| EstC | Esterified Cholesterol |
| FALen | Estimated description of Fatty Acid chain Length, not actual carbon number |
| FAw3 | Omega-3 Fatty Acids |
| FAw3.FA | Ratio of omega-3 Fatty Acids to total Fatty Acids |
| FAw6 | Omega-6 Fatty Acids |
| FAw6.FA | Ratio of omega-6 Fatty Acids to total Fatty Acids |
| FDR | False Discovery Rate |
| FreeC | Free Cholesterol |
| Glc | Glucose |
| Gln | Glutamine |
| Gly | Glycine |
| Gp | Glycoprotein acetyls, mainly a1-acid glycop. |
| HDL.C | Total Cholesterol in all HDL particles |

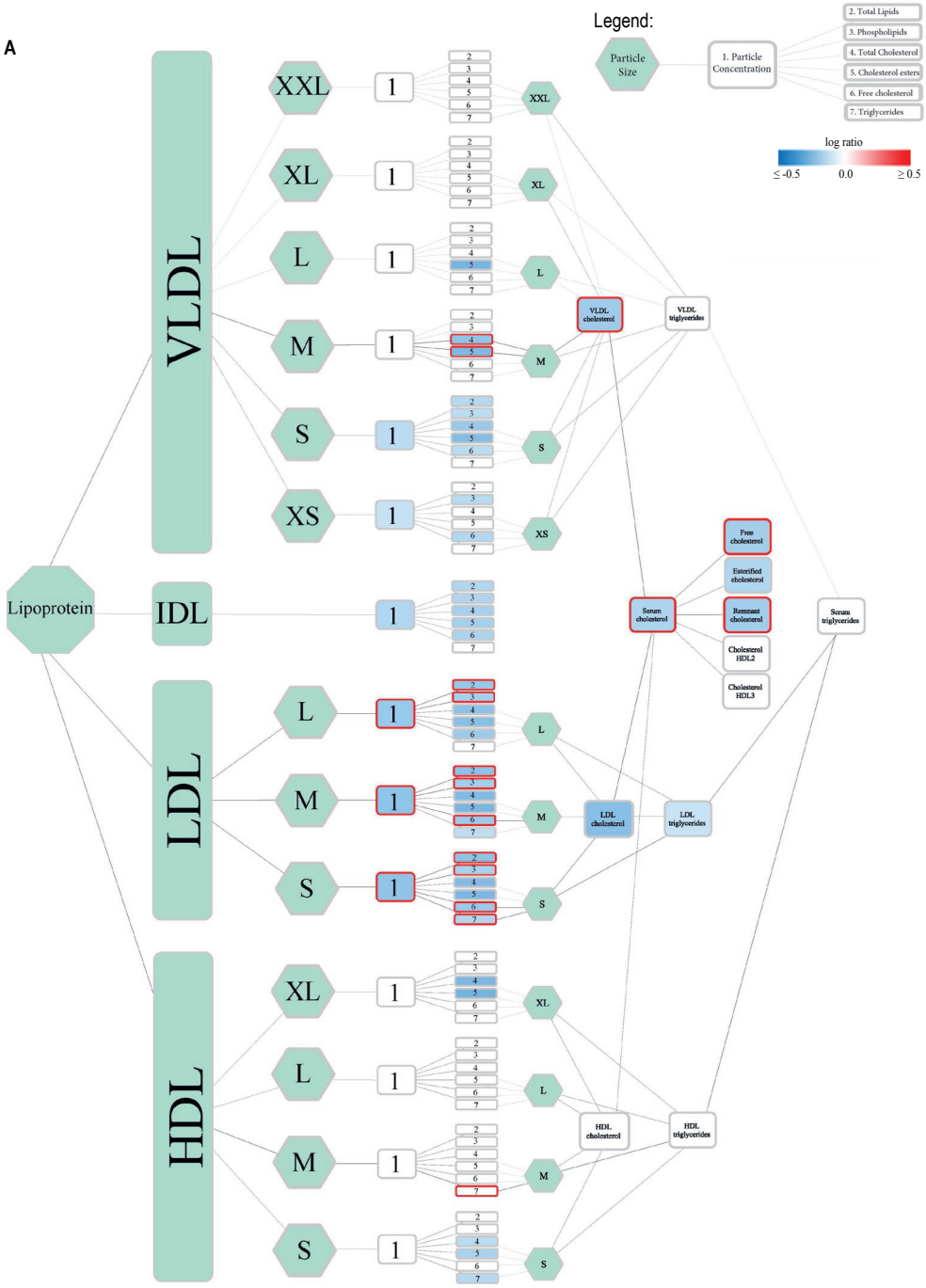
| Abbreviation | Full description |
|---|---|
| HDL.TG | Triglycerides all HDL particles |
| HDL2.C | Total Cholesterol in HDL2[1] |
| HDL3.C | Total Cholesterol in HDL3[1] |
| HDL-D | Mean Diameter for HDL particles |
| His | Histidine |
| IDL.P | IDL particles (Diameter >28.6 nm) |
| Ile | Isoleucine |
| L.HDL | Large HDL particles (Diameter >12.1 nm) |
| L.LDL | Large LDL particles (Diameter >25.5 nm) |
| L.VLDL | Large VLDL particles (Diameter >53.6 nm) |
| LA | 18:2, Linoleic Acid |
| LA.FA | Ratio of 18:2 Linoleic Acid to total Fatty Acids |
| Lac | Lactate |
| LDL | Low Density Lipoprotein |
| LDL.C | Total Cholesterol in all LDL particles |
| LDL.TG | Triglycerides in all LDL particles |
| LDL-D | Mean Diameter for LDL particles |
| Leu | Leucine |
| M.HDL | Medium HDL particles (Diameter >10.9 nm) |
| M.LDL | Medium LDL particles (Diameter >23 nm) |
| M.VLDL | Medium VLDL particles (Diameter >44.5 nm) |
| MED | Mediterranean |
| MUFA.FA | Ratio of Monounsaturated Fatty Acids to total Fatty Acids |
| PC | Phosphatidylcholine and other cholines |
| Phe | Phenylalanine |
| PPAR | Peroxisome Proliferator Activated Receptors |
| PUFA.FA | Ratio of Polyunsaturated Fatty Acids to total Fatty Acids |
| Pyr | Pyruvate |
| Remnant.C | Remnant Cholesterol (non-HDL, non-LDL -cholesterol) |
| S.HDL | Small HDL particles (Diameter >8.7 nm) |
| S.LDL | Small LDL particles (Diameter >18.7 nm) |
| S.VLDL | Small VLDL particles (Diameter >36.8 nm) |
| Serum.C | Serum total Cholesterol |
| Serum.TG | Serum total triglycerides |
| SFA | Saturated Fatty Acids |
| SFA.FA | Ratio of Saturated Fatty Acids to total Fatty Acids |
| SM | Sphingomyelins |
| sPLS-DA | sparse Partial Least Squares Discriminant Analysis |
| TG.PG | Ratio of Triglycerides to Phosphoglycerides |
| TotCho | Total Cholines |

| Abbreviation | Full description |
|---|---|
| TotFA | Total Fatty Acids |
| TotPG | Total phosphoglycerides |
| Tyr | Tyrosine |
| UnSat | Estimated degree of Unsaturation |
| Val | Valine |
| VLDL.C | Total Cholesterol in all VLDL particles |
| VLDL.TG | Triglycerides in all VLDL particles |
| VLDL-D | Mean Diameter for VLDL particles |
| XL.HDL | Very large HDL particles (Diameter >14.3 nm) |
| XL.VLDL | Very large VLDL particles (Diameter >64 nm) |
| XS.VLDL | Very small VLDL particles (Diameter >31.3 nm) |
| XXL.VLDL | Extremely large VLDL particles (Diameter >75 nm) |

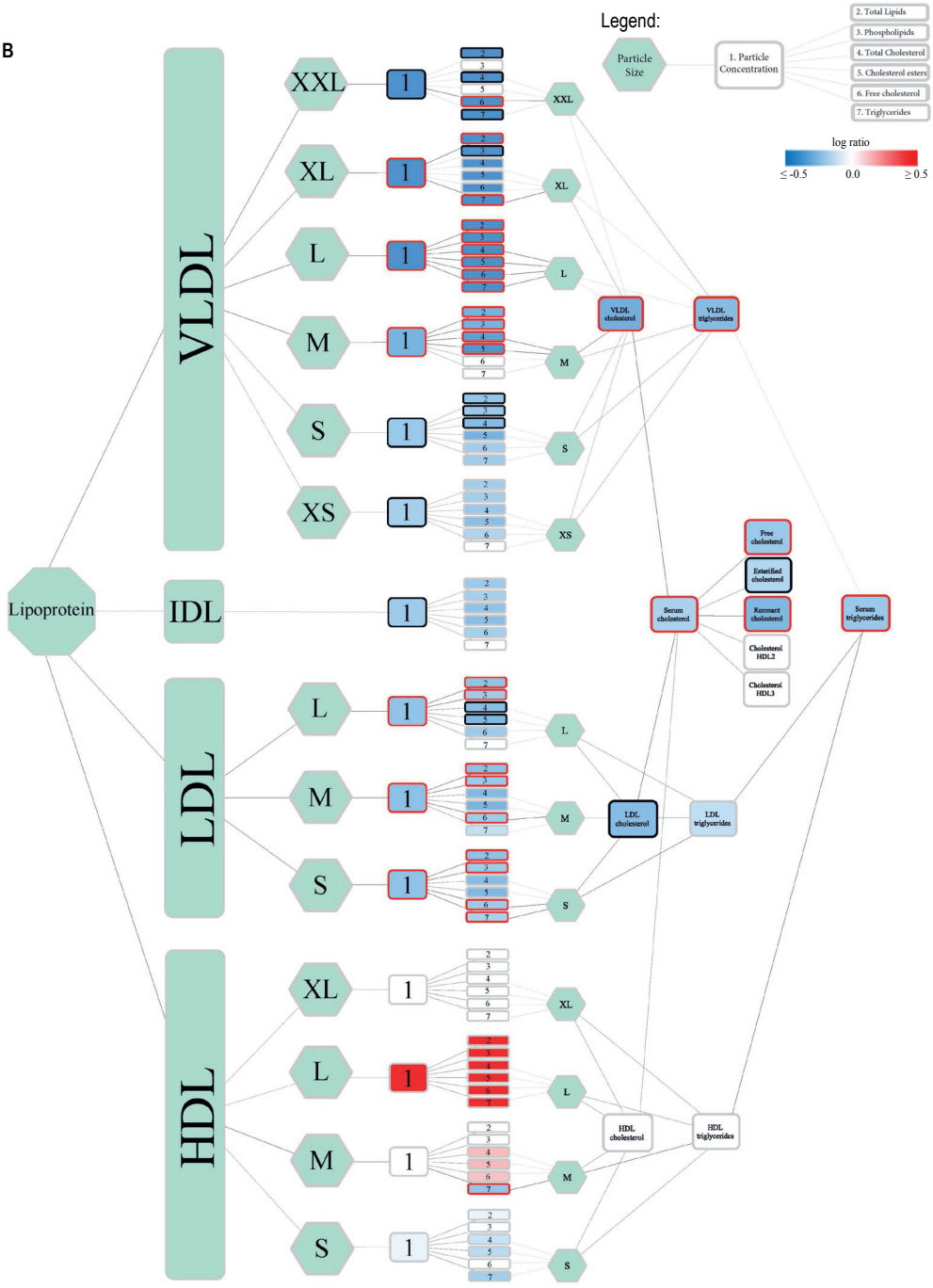
## SUPPLEMENTAL MATERIAL




**Supplemental Figure 1.** Study design. This study was a randomized fully controlled parallel dietary intervention trial. After a two week run-in period on a high saturated fatty acid (SFA) diet, subjects were randomly allocated to continue on the SFA diet, to continue on a high monounsaturated fatty acid (MUFA) diet, or to continue on a Mediterranean (MED)-type diet, for eight weeks. Before and after these eight weeks blood samples were drawn.




**Supplemental Figure 2.** *Continues on the next page.*




**Supplemental Figure 2.** The effects of diets on lipids and lipoproteins. Panel A. MUFA vs. SFA. Panel B. MED vs SFA. The color of the edge of the node represents the between treatment effet. If the edge is red, te ANOVA FDR $p < 0.05$, and between diet effect, tested with linear mixed models was $p < 0.05$. If the color is black the ANOVA FDR $p < 0.05$, and the between diet effect showed a trent ($0.05 < p \leq$ p 0.06). The color inside a node represents the changes within the MUFA diet (2A) or MED diet (2B), ranging from dark blue (log ratio (LR) ≤-0.5) to dark red (LR ≥ 0.5), the inside is only colored if the paired t-test FDR $p < 0.05$.

**Supplemental table 1.** Diet-induced changes from baseline (mean ± SD) of the 162 metabolic parameters. *Continues on the next pages.*

| | Metabolic parameter | | SFA | | MUFA | | MED | | Treatment effect between diets[1] |
|---|---|---|---|---|---|---|---|---|---|
| # | name | unit | Δ (mean ± SD) | FDR p[2] | Δ (mean ± SD) | FDR p[2] | Δ (mean ± SD) | FDR p[2] | FDR p |
| 1 | XXL.VLDL.P | nmol/l | 0.010 ± 0.041 | 0.517 | -0.008 ± 0.050 | 0.616 | -0.042 ± 0.065 | 0.040 | 0.056 |
| 2 | XXL.VLDL.L | mmol/l | 0.002 ± 0.009 | 0.517 | -0.002 ± 0.011 | 0.600 | -0.009 ± 0.014 | 0.039 | 0.056 |
| 3 | XXL.VLDL.PL | mmol/l | 0.000 ± 0.001 | 0.410 | 0.000 ± 0.001 | 0.813 | -0.001 ± 0.002 | 0.050 | 0.056 |
| 4 | XXL.VLDL.C | mmol/l | 0.000 ± 0.002 | 0.527 | -0.001 ± 0.002 | 0.258 | -0.002 ± 0.003 | 0.034 | 0.054 |
| 5 | XXL.VLDL.CE | mmol/l | 0.000 ± 0.001 | 0.571 | -0.001 ± 0.001 | 0.211 | -0.002 ± 0.002 | 0.052 | 0.078 |
| 6 | XXL.VLDL.FC | mmol/l | 0.000 ± 0.001$^{A}$ | 0.371 | 0.000 ± 0.001$^{A}$ | 0.819 | -0.001 ± 0.001$^{B}$ | 0.026 | 0.045 |
| 7 | XXL.VLDL.TG | mmol/l | 0.001 ± 0.006 | 0.517 | -0.001 ± 0.008 | 0.694 | -0.006 ± 0.010 | 0.043 | 0.056 |
| 8 | XL.VLDL.P | nmol/l | 0.065 ± 0.192$^{A}$ | 0.576 | -0.092 ± 0.349$^{A,B}$ | 0.178 | -0.293 ± 0.437$^{B}$ | 0.024 | 0.048 |
| 9 | XL.VLDL.L | mmol/l | 0.006 ± 0.019$^{A}$ | 0.576 | -0.009 ± 0.034$^{A,B}$ | 0.178 | -0.028 ± 0.043$^{B}$ | 0.024 | 0.048 |
| 10 | XL.VLDL.PL | mmol/l | 0.001 ± 0.004 | 0.581 | -0.002 ± 0.006 | 0.170 | -0.005 ± 0.007 | 0.026 | 0.056 |
| 11 | XL.VLDL.C | mmol/l | 0.001 ± 0.005 | 0.771 | -0.002 ± 0.007 | 0.131 | -0.006 ± 0.009 | 0.024 | 0.081 |
| 12 | XL.VLDL.CE | mmol/l | 0.000 ± 0.003 | 0.600 | -0.001 ± 0.004 | 0.108 | -0.004 ± 0.005 | 0.026 | 0.101 |
| 13 | XL.VLDL.FC | mmol/l | 0.000 ± 0.002 | 0.866 | -0.001 ± 0.003 | 0.165 | -0.002 ± 0.004 | 0.030 | 0.085 |
| 14 | XL.VLDL.TG | mmol/l | 0.005 ± 0.011$^{A}$ | 0.635 | -0.005 ± 0.022$^{A,B}$ | 0.170 | -0.018 ± 0.027$^{B}$ | 0.024 | 0.049 |
| 15 | L.VLDL.P | nmol/l | 0.364 ± 1.262$^{A}$ | 0.517 | -0.669 ± 2.101$^{A,B}$ | 0.331 | -1.773 ± 2.580$^{B}$ | 0.013 | 0.035 |
| 16 | L.VLDL.L | mmol/l | 0.021 ± 0.074$^{A}$ | 0.517 | -0.040 ± 0.121$^{A,B}$ | 0.316 | -0.104 ± 0.150$^{B}$ | 0.012 | 0.035 |
| 17 | L.VLDL.PL | mmol/l | 0.003 ± 0.014$^{A}$ | 0.577 | -0.008 ± 0.022$^{A,B}$ | 0.196 | -0.020 ± 0.027$^{B}$ | 0.008 | 0.032 |
| 18 | L.VLDL.C | mmol/l | 0.003 ± 0.020$^{A}$ | 0.766 | -0.012 ± 0.026$^{A,B}$ | 0.129 | -0.028 ± 0.035$^{B}$ | 0.005 | 0.035 |
| 19 | L.VLDL.CE | mmol/l | 0.000 ± 0.012$^{A}$ | 0.970 | -0.008 ± 0.013$^{A,B}$ | 0.040 | -0.017 ± 0.018$^{B}$ | 0.003 | 0.048 |
| 20 | L.VLDL.FC | mmol/l | 0.003 ± 0.009$^{A}$ | 0.496 | -0.004 ± 0.013$^{A,B}$ | 0.326 | -0.011 ± 0.017$^{B}$ | 0.021 | 0.035 |
| 21 | L.VLDL.TG | mmol/l | 0.014 ± 0.041$^{A}$ | 0.434 | -0.020 ± 0.074$^{A,B}$ | 0.495 | -0.057 ± 0.088$^{B}$ | 0.021 | 0.039 |
| 22 | M.VLDL.P | nmol/l | 0.834 ± 4.018$^{A}$ | 0.527 | -2.375 ± 5.531$^{A,B}$ | 0.155 | -5.271 ± 6.991$^{B}$ | 0.026 | 0.048 |

| | Metabolic parameter | | SFA | | MUFA | | MED | | Treatment effect between diets[1] |
|---|---|---|---|---|---|---|---|---|---|
| # | name | unit | Δ (mean ± SD) | FDR p[2] | Δ (mean ± SD) | FDR p[2] | Δ (mean ± SD) | FDR p[2] | FDR p |
| 23 | M.VLDL.L | mmol/l | 0.027 ± 0.136[A] | 0.527 | -0.082 ± 0.181[A,B] | 0.130 | -0.179 ± 0.232[B] | 0.026 | 0.048 |
| 24 | M.VLDL.PL | mmol/l | 0.004 ± 0.027[A] | 0.612 | -0.017 ± 0.033[A,B] | 0.085 | -0.036 ± 0.044[B] | 0.019 | 0.048 |
| 25 | M.VLDL.C | mmol/l | 0.005 ± 0.044[A] | 0.703 | -0.030 ± 0.041[B] | 0.015 | -0.057 ± 0.062[B] | 0.013 | 0.036 |
| 26 | M.VLDL.CE | mmol/l | 0.000 ± 0.027[A] | 0.975 | -0.021 ± 0.020[B] | 0.003 | -0.036 ± 0.034[B] | 0.005 | 0.025 |
| 27 | M.VLDL.FC | mmol/l | 0.004 ± 0.017 | 0.507 | -0.009 ± 0.022 | 0.175 | -0.021 ± 0.029 | 0.086 | 0.077 |
| 28 | M.VLDL.TG | mmol/l | 0.018 ± 0.068 | 0.410 | -0.035 ± 0.108 | 0.323 | -0.086 ± 0.128 | 0.051 | 0.060 |
| 29 | S.VLDL.P | nmol/l | 0.538 ± 5.891 | 0.844 | -3.531 ± 4.707 | 0.017 | -6.136 ± 7.115 | 0.015 | 0.056 |
| 30 | S.VLDL.L | mmol/l | 0.009 ± 0.116 | 0.880 | -0.072 ± 0.089 | 0.012 | -0.120 ± 0.136 | 0.013 | 0.056 |
| 31 | S.VLDL.PL | mmol/l | 0.001 ± 0.025 | 0.962 | -0.015 ± 0.018 | 0.010 | -0.025 ± 0.028 | 0.008 | 0.056 |
| 32 | S.VLDL.C | mmol/l | -0.001 ± 0.045 | 0.808 | -0.034 ± 0.032 | 0.003 | -0.046 ± 0.046 | 0.009 | 0.056 |
| 33 | S.VLDL.CE | mmol/l | -0.004 ± 0.030 | 0.527 | -0.025 ± 0.023 | 0.003 | -0.031 ± 0.029 | 0.006 | 0.064 |
| 34 | S.VLDL.FC | mmol/l | 0.002 ± 0.017 | 0.777 | -0.009 ± 0.012 | 0.020 | -0.014 ± 0.018 | 0.031 | 0.086 |
| 35 | S.VLDL.TG | mmol/l | 0.009 ± 0.049 | 0.616 | -0.023 ± 0.052 | 0.175 | -0.049 ± 0.068 | 0.037 | 0.081 |
| 36 | XS.VLDL.P | nmol/l | 0.131 ± 4.416 | 0.962 | -3.188 ± 5.329 | 0.048 | -5.236 ± 5.69 | 0.011 | 0.056 |
| 37 | XS.VLDL.L | mmol/l | 0.000 ± 0.056 | 0.846 | -0.041 ± 0.071 | 0.054 | -0.066 ± 0.074 | 0.012 | 0.066 |
| 38 | XS.VLDL.PL | mmol/l | -0.001 ± 0.020 | 0.657 | -0.017 ± 0.022 | 0.014 | -0.022 ± 0.023 | 0.010 | 0.068 |
| 39 | XS.VLDL.C | mmol/l | -0.003 ± 0.030 | 0.569 | -0.019 ± 0.046 | 0.161 | -0.031 ± 0.043 | 0.027 | 0.260 |
| 40 | XS.VLDL.CE | mmol/l | -0.002 ± 0.023 | 0.612 | -0.011 ± 0.035 | 0.267 | -0.023 ± 0.032 | 0.035 | 0.312 |
| 41 | XS.VLDL.FC | mmol/l | -0.001 ± 0.009 | 0.615 | -0.008 ± 0.013 | 0.029 | -0.009 ± 0.012 | 0.030 | 0.181 |
| 42 | XS.VLDL.TG | mmol/l | 0.005 ± 0.017 | 0.527 | -0.006 ± 0.014 | 0.170 | -0.012 ± 0.019 | 0.051 | 0.068 |
| 43 | IDL.P | nmol/l | -1.350 ± 12.829 | 0.528 | -13.325 ± 15.354 | 0.008 | -16.45 ± 16.076 | 0.006 | 0.056 |
| 44 | IDL.L | mmol/l | -0.015 ± 0.133 | 0.527 | -0.139 ± 0.160 | 0.008 | -0.169 ± 0.164 | 0.006 | 0.061 |

| # | Metabolic parameter name | unit | SFA Δ (mean ± SD) | FDR p[2] | MUFA Δ (mean ± SD) | FDR p[2] | MED Δ (mean ± SD) | FDR p[2] | Treatment effect between diets[1] FDR p |
|---|---|---|---|---|---|---|---|---|---|
| 45 | IDL.PL | mmol/l | -0.008 ± 0.035 | 0.410 | -0.037 ± 0.040 | 0.005 | -0.043 ± 0.041 | 0.006 | 0.081 |
| 46 | IDL.C | mmol/l | -0.015 ± 0.092 | 0.474 | -0.098 ± 0.117 | 0.009 | -0.119 ± 0.115 | 0.006 | 0.079 |
| 47 | IDL.CE | mmol/l | -0.010 ± 0.068 | 0.506 | -0.071 ± 0.086 | 0.011 | -0.09 ± 0.085 | 0.005 | 0.068 |
| 48 | IDL.FC | mmol/l | -0.005 ± 0.025 | 0.410 | -0.027 ± 0.032 | 0.008 | -0.028 ± 0.032 | 0.013 | 0.145 |
| 49 | IDL.TG | mmol/l | 0.005 ± 0.014 | 0.498 | -0.004 ± 0.017 | 0.356 | -0.007 ± 0.017 | 0.098 | 0.119 |
| 50 | L.LDL.P | nmol/l | -6.625 ± 24.01[A] | 0.365 | -32.13 ± 24.89[B] | <0.001 | -33.07 ± 26.67[B] | 0.003 | 0.048 |
| 51 | L.LDL.L | mmol/l | -0.051 ± 0.170[A] | 0.345 | -0.236 ± 0.182[B] | <0.001 | -0.24 ± 0.193[B] | 0.003 | 0.049 |
| 52 | L.LDL.PL | mmol/l | -0.010 ± 0.038[A] | 0.370 | -0.048 ± 0.040[B] | 0.001 | -0.053 ± 0.041[B] | 0.003 | 0.048 |
| 53 | L.LDL.C | mmol/l | -0.043 ± 0.129 | 0.321 | -0.180 ± 0.139 | <0.001 | -0.181 ± 0.144 | 0.003 | 0.056 |
| 54 | L.LDL.CE | mmol/l | -0.033 ± 0.100 | 0.321 | -0.139 ± 0.104 | <0.001 | -0.141 ± 0.109 | 0.003 | 0.056 |
| 55 | L.LDL.FC | mmol/l | -0.010 ± 0.030 | 0.321 | -0.042 ± 0.035 | 0.001 | -0.040 ± 0.035 | 0.004 | 0.068 |
| 56 | L.LDL.TG | mmol/l | 0.002 ± 0.012 | 0.615 | -0.006 ± 0.012 | 0.073 | -0.007 ± 0.014 | 0.062 | 0.091 |
| 57 | M.LDL.P | nmol/l | -7.66 ± 20.50[A] | 0.312 | -30.50 ± 21.17[B] | <0.001 | -30.11 ± 22.08[B] | 0.003 | 0.048 |
| 58 | M.LDL.L | mmol/l | -0.040 ± 0.104[A] | 0.312 | -0.157 ± 0.108[B] | <0.001 | -0.153 ± 0.112[B] | 0.003 | 0.048 |
| 59 | M.LDL.PL | mmol/l | -0.006 ± 0.022[A] | 0.387 | -0.032 ± 0.024[B] | <0.001 | -0.035 ± 0.024[B] | 0.003 | 0.030 |
| 60 | M.LDL.C | mmol/l | -0.033 ± 0.081 | 0.306 | -0.121 ± 0.084 | <0.001 | -0.115 ± 0.085 | 0.003 | 0.064 |
| 61 | M.LDL.CE | mmol/l | -0.027 ± 0.067 | 0.306 | -0.097 ± 0.066 | <0.001 | -0.092 ± 0.069 | 0.003 | 0.078 |
| 62 | M.LDL.FC | mmol/l | -0.006 ± 0.015[A] | 0.312 | -0.024 ± 0.018[B] | <0.001 | -0.023 ± 0.017[B] | 0.003 | 0.048 |
| 63 | M.LDL.TG | mmol/l | 0.000 ± 0.006 | 0.976 | -0.004 ± 0.006 | 0.009 | -0.004 ± 0.007 | 0.044 | 0.119 |
| 64 | S.LDL.P | nmol/l | -8.00 ± 22.17[A] | 0.320 | -34.56 ± 24.39[B] | <0.001 | -34.07 ± 24.65[B] | 0.003 | 0.048 |
| 65 | S.LDL.L | mmol/l | -0.024 ± 0.062[A] | 0.312 | -0.098 ± 0.071[B] | <0.001 | -0.095 ± 0.069[B] | 0.003 | 0.048 |
| 66 | S.LDL.PL | mmol/l | -0.005 ± 0.014[A] | 0.340 | -0.021 ± 0.018[B] | 0.001 | -0.023 ± 0.016[B] | 0.003 | 0.046 |

| | Metabolic parameter | | SFA | | MUFA | | MED | | Treatment effect between diets[1] |
|---|---|---|---|---|---|---|---|---|---|
| # | name | unit | Δ (mean ± SD) | FDR p[2] | Δ (mean ± SD) | FDR p[2] | Δ (mean ± SD) | FDR p[2] | FDR p |
| 67 | S.LDL.C | mmol/l | -0.019 ± 0.047 | 0.306 | -0.073 ± 0.053 | <0.001 | -0.068 ± 0.051 | 0.003 | 0.064 |
| 68 | S.LDL.CE | mmol/l | -0.016 ± 0.039 | 0.306 | -0.059 ± 0.040 | <0.001 | -0.054 ± 0.040 | 0.003 | 0.078 |
| 69 | S.LDL.FC | mmol/l | -0.003 ± 0.009[A] | 0.365 | -0.014 ± 0.013[B] | 0.001 | -0.014 ± 0.011[B] | 0.003 | 0.048 |
| 70 | S.LDL.TG | mmol/l | 0.000 ± 0.005[A] | 0.814 | -0.003 ± 0.004[B] | 0.001 | -0.004 ± 0.005[B] | 0.022 | 0.048 |
| 71 | XL.HDL.P | nmol/l | 7.73 ± 106.09 | 0.527 | -41.31 ± 63.43 | 0.213 | -0.68 ± 78.16 | 0.857 | 0.259 |
| 72 | XL.HDL.L | mmol/l | 0.006 ± 0.109 | 0.527 | -0.042 ± 0.061 | 0.203 | -0.003 ± 0.080 | 0.826 | 0.257 |
| 73 | XL.HDL.PL | mmol/l | 0.006 ± 0.053 | 0.517 | -0.020 ± 0.049 | 0.473 | 0.005 ± 0.046 | 0.863 | 0.388 |
| 74 | XL.HDL.C | mmol/l | -0.002 ± 0.061 | 0.577 | -0.022 ± 0.028 | 0.047 | -0.009 ± 0.037 | 0.567 | 0.218 |
| 75 | XL.HDL.CE | mmol/l | -0.001 ± 0.045 | 0.600 | -0.017 ± 0.022 | 0.029 | -0.006 ± 0.028 | 0.571 | 0.208 |
| 76 | XL.HDL.FC | mmol/l | -0.001 ± 0.016 | 0.527 | -0.005 ± 0.008 | 0.181 | -0.003 ± 0.011 | 0.567 | 0.237 |
| 77 | XL.HDL.TG | mmol/l | 0.002 ± 0.004 | 0.434 | -0.001 ± 0.005 | 0.616 | 0.000 ± 0.004 | 0.395 | 0.295 |
| 78 | L.HDL.P | nmol/l | 133.38 ± 150.59 | 0.114 | 82.06 ± 156.31 | 0.117 | 221.93 ± 210.94 | 0.024 | 0.165 |
| 79 | L.HDL.L | mmol/l | 0.084 ± 0.096 | 0.120 | 0.052 ± 0.100 | 0.117 | 0.143 ± 0.133 | 0.024 | 0.165 |
| 80 | L.HDL.PL | mmol/l | 0.036 ± 0.046 | 0.138 | 0.025 ± 0.050 | 0.130 | 0.064 ± 0.063 | 0.026 | 0.170 |
| 81 | L.HDL.C | mmol/l | 0.041 ± 0.052 | 0.165 | 0.025 ± 0.048 | 0.116 | 0.073 ± 0.069 | 0.026 | 0.236 |
| 82 | L.HDL.CE | mmol/l | 0.032 ± 0.039 | 0.165 | 0.020 ± 0.037 | 0.105 | 0.055 ± 0.053 | 0.026 | 0.237 |
| 83 | L.HDL.FC | mmol/l | 0.009 ± 0.013 | 0.206 | 0.005 ± 0.012 | 0.161 | 0.018 ± 0.016 | 0.032 | 0.257 |
| 84 | L.HDL.TG | mmol/l | 0.006 ± 0.007 | 0.060 | 0.002 ± 0.006 | 0.169 | 0.005 ± 0.006 | 0.024 | 0.209 |
| 85 | M.HDL.P | nmol/l | 119.38 ± 253.39 | 0.345 | 85.63 ± 242.27 | 0.223 | 60.71 ± 142.85 | 0.109 | 0.850 |
| 86 | M.HDL.L | mmol/l | 0.052 ± 0.109 | 0.340 | 0.038 ± 0.104 | 0.205 | 0.030 ± 0.063 | 0.085 | 0.883 |
| 87 | M.HDL.PL | mmol/l | 0.021 ± 0.047 | 0.345 | 0.013 ± 0.045 | 0.326 | 0.009 ± 0.028 | 0.199 | 0.769 |
| 88 | M.HDL.C | mmol/l | 0.029 ± 0.059 | 0.321 | 0.029 ± 0.058 | 0.086 | 0.029 ± 0.036 | 0.024 | 0.911 |

| # | Metabolic parameter name | unit | SFA Δ (mean ± SD) | FDR p[2] | MUFA Δ (mean ± SD) | FDR p[2] | MED Δ (mean ± SD) | FDR p[2] | Treatment effect between diets[1] FDR p |
|---|---|---|---|---|---|---|---|---|---|
| 89 | M.HDL.CE | mmol/l | 0.023 ± 0.047 | 0.321 | 0.024 ± 0.046 | 0.071 | 0.024 ± 0.029 | 0.021 | 0.903 |
| 90 | M.HDL.FC | mmol/l | 0.006 ± 0.012 | 0.340 | 0.005 ± 0.013 | 0.192 | 0.005 ± 0.008 | 0.043 | 0.898 |
| 91 | M.HDL.TG | mmol/l | 0.002 ± 0.009[A] | 0.614 | -0.004 ± 0.006[B] | 0.052 | -0.008 ± 0.008[B] | 0.005 | 0.032 |
| 92 | S.HDL.P | nmol/l | -53.75 ± 353.72 | 0.675 | -181.25 ± 376.58 | 0.089 | -176.43 ± 207.98 | 0.016 | 0.510 |
| 93 | S.HDL.L | mmol/l | -0.013 ± 0.079 | 0.648 | -0.043 ± 0.084 | 0.075 | -0.036 ± 0.045 | 0.021 | 0.534 |
| 94 | S.HDL.PL | mmol/l | 0.018 ± 0.040 | 0.345 | 0.020 ± 0.050 | 0.180 | 0.011 ± 0.026 | 0.171 | 0.898 |
| 95 | S.HDL.C | mmol/l | -0.030 ± 0.044 | 0.145 | -0.056 ± 0.047 | 0.001 | -0.042 ± 0.036 | 0.004 | 0.378 |
| 96 | S.HDL.CE | mmol/l | -0.033 ± 0.039 | 0.067 | -0.057 ± 0.042 | <0.001 | -0.044 ± 0.034 | 0.003 | 0.378 |
| 97 | S.HDL.FC | mmol/l | 0.002 ± 0.008 | 0.564 | 0.001 ± 0.010 | 0.887 | 0.002 ± 0.005 | 0.209 | 0.831 |
| 98 | S.HDL.TG | mmol/l | -0.001 ± 0.008 | 0.814 | -0.006 ± 0.007 | 0.003 | -0.008 ± 0.008 | 0.006 | 0.068 |
| 99 | VLDL.D | nm | 0.206 ± 0.505 | 0.371 | -0.025 ± 0.906 | 0.896 | -0.300 ± 0.911 | 0.283 | 0.287 |
| 100 | LDL.D | nm | 0.050 ± 0.073 | 0.162 | 0.050 ± 0.115 | 0.121 | 0.021 ± 0.097 | 0.483 | 0.685 |
| 101 | HDL.D | nm | 0.063 ± 0.117 | 0.306 | 0.047 ± 0.089 | 0.093 | 0.108 ± 0.113 | 0.010 | 0.318 |
| 102 | Serum.C | mmol/l | -0.069 ± 0.452[A] | 0.517 | -0.597 ± 0.529[B] | 0.001 | -0.601 ± 0.503[B] | 0.003 | 0.047 |
| 103 | VLDL.C | mmol/l | 0.003 ± 0.130[A] | 0.967 | -0.098 ± 0.106[B] | 0.007 | -0.169 ± 0.159[B] | 0.005 | 0.030 |
| 104 | Remnant.C | mmol/l | -0.010 ± 0.209[A] | 0.666 | -0.197 ± 0.195[B] | 0.003 | -0.288 ± 0.243[B] | 0.003 | 0.030 |
| 105 | LDL.C | mmol/l | -0.093 ± 0.256 | 0.312 | -0.375 ± 0.275 | <0.001 | -0.365 ± 0.275 | 0.003 | 0.056 |
| 106 | HDL.C | mmol/l | 0.038 ± 0.099 | 0.370 | -0.024 ± 0.126 | 0.380 | 0.053 ± 0.124 | 0.109 | 0.118 |
| 107 | HDL2.C | mmol/l | 0.033 ± 0.090 | 0.370 | -0.012 ± 0.111 | 0.658 | 0.051 ± 0.115 | 0.092 | 0.173 |
| 108 | HDL3.C | mmol/l | 0.004 ± 0.015 | 0.517 | -0.010 ± 0.021 | 0.089 | 0.001 ± 0.012 | 0.823 | 0.068 |
| 109 | EstC | mmol/l | -0.033 ± 0.337 | 0.569 | -0.399 ± 0.416 | 0.003 | -0.390 ± 0.362 | 0.005 | 0.056 |
| 110 | FreeC | mmol/l | -0.035 ± 0.125[A] | 0.387 | -0.195 ± 0.136[B] | <0.001 | -0.211 ± 0.151[B] | 0.003 | 0.025 |

| # | Metabolic parameter name | unit | SFA Δ (mean ± SD) | SFA FDR p[2] | MUFA Δ (mean ± SD) | MUFA FDR p[2] | MED Δ (mean ± SD) | MED FDR p[2] | Treatment effect between diets[1] FDR p |
|---|---|---|---|---|---|---|---|---|---|
| 111 | Serum.TG | mmol/l | 0.069 ± 0.222[A] | 0.410 | -0.111 ± 0.271[A,B] | 0.188 | -0.257 ± 0.354[B] | 0.024 | 0.037 |
| 112 | VLDL.TG | mmol/l | 0.052 ± 0.180[A] | 0.410 | -0.087 ± 0.264[A,B] | 0.323 | -0.224 ± 0.322[B] | 0.037 | 0.049 |
| 113 | LDL.TG | mmol/l | 0.003 ± 0.022 | 0.747 | -0.014 ± 0.020 | 0.014 | -0.014 ± 0.026 | 0.038 | 0.068 |
| 114 | HDL.TG | mmol/l | 0.009 ± 0.023 | 0.410 | -0.008 ± 0.017 | 0.134 | -0.012 ± 0.022 | 0.089 | 0.056 |
| 115 | DAG | mmol/l | 0.001 ± 0.009 | 0.655 | -0.005 ± 0.009 | 0.116 | -0.004 ± 0.011 | 0.988 | 0.193 |
| 116 | DAG.TG | Ratio | 0.001 ± 0.009 | 0.684 | -0.004 ± 0.009 | 0.131 | -0.001 ± 0.008 | 0.567 | 0.185 |
| 117 | TotPG | mmol/l | 0.036 ± 0.189 | 0.747 | -0.128 ± 0.213 | 0.047 | -0.109 ± 0.173 | 0.048 | 0.107 |
| 118 | TG.PG | Ratio | 0.015 ± 0.101 | 0.697 | -0.017 ± 0.141 | 0.961 | -0.084 ± 0.173 | 0.216 | 0.318 |
| 119 | PC | mmol/l | 0.021 ± 0.168 | 0.890 | -0.149 ± 0.203 | 0.015 | -0.123 ± 0.160 | 0.024 | 0.068 |
| 120 | SM | mmol/l | 0.004 ± 0.056 | 0.967 | -0.033 ± 0.056 | 0.076 | -0.048 ± 0.049 | 0.009 | 0.089 |
| 121 | TotCho | mmol/l | 0.036 ± 0.193 | 0.755 | -0.141 ± 0.189 | 0.029 | -0.137 ± 0.175 | 0.024 | 0.068 |
| 122 | ApoA1 | g/l | 0.031 ± 0.072 | 0.387 | -0.032 ± 0.103 | 0.202 | 0.003 ± 0.075 | 0.870 | 0.145 |
| 123 | ApoB | g/l | -0.008 ± 0.103[A] | 0.648 | -0.110 ± 0.090[B] | 0.001 | -0.151 ± 0.122[B] | 0.003 | 0.022 |
| 124 | ApoB.ApoA1 | Ratio | -0.023 ± 0.062[A] | 0.365 | -0.059 ± 0.046[A] | <0.001 | -0.107 ± 0.081[B] | 0.003 | 0.025 |
| 125 | TotFA | mmol/l | 0.187 ± 1.171[A] | 0.784 | -0.988 ± 1.250[B] | 0.014 | -1.346 ± 1.319[B] | 0.004 | 0.032 |
| 126 | FALen | | 0.013 ± 0.242 | 0.905 | 0.038 ± 0.314 | 0.804 | 0.050 ± 0.365 | 0.648 | 0.944 |
| 127 | UnSat | | -0.026 ± 0.037[A] | 0.145 | -0.017 ± 0.042[A] | 0.141 | 0.032 ± 0.047[B] | 0.038 | 0.013 |
| 128 | DHA | mmol/l | -0.017 ± 0.018[A] | 0.018 | -0.028 ± 0.023[A] | <0.001 | 0.024 ± 0.035[B] | 0.039 | 0.000 |
| 129 | LA | mmol/l | 0.037 ± 0.328[A] | 0.962 | -0.283 ± 0.399[B] | 0.022 | -0.461 ± 0.411[B] | 0.003 | 0.030 |
| 130 | CLA | mmol/l | 0.003 ± 0.008[A] | 0.365 | -0.028 ± 0.013[B] | <0.001 | -0.022 ± 0.015[B] | 0.003 | 0.000 |
| 131 | FAw3 | mmol/l | -0.023 ± 0.029[A] | 0.067 | -0.094 ± 0.048[B] | <0.001 | 0.041 ± 0.077[C] | 0.131 | 0.000 |
| 132 | FAw6 | mmol/l | 0.014 ± 0.389[A] | 0.880 | -0.374 ± 0.430[B] | 0.008 | -0.528 ± 0.478[B] | 0.003 | 0.035 |

| # | Metabolic parameter name | unit | SFA Δ (mean ± SD) | FDR p[2] | MUFA Δ (mean ± SD) | FDR p[2] | MED Δ (mean ± SD) | FDR p[2] | Treatment effect between diets[1] FDR p |
|---|---|---|---|---|---|---|---|---|---|
| 133 | PUFA | mmol/l | -0.010 ± 0.411[A] | 0.747 | -0.469 ± 0.464[B] | 0.003 | -0.487 ± 0.500[B] | 0.006 | 0.048 |
| 134 | MUFA | mmol/l | 0.082 ± 0.347 | 0.589 | 0.133 ± 0.339 | 0.129 | -0.109 ± 0.405 | 0.530 | 0.291 |
| 135 | SFA | mmol/l | 0.115 ± 0.508[A] | 0.618 | -0.638 ± 0.636[B] | 0.003 | -0.743 ± 0.551[B] | 0.003 | 0.003 |
| 136 | DHA.FA | % | -0.189 ± 0.145[A] | 0.004 | -0.153 ± 0.151[A] | 0.001 | 0.363 ± 0.274[B] | 0.003 | 0.000 |
| 137 | LA.FA | % | -0.225 ± 1.062 | 0.581 | -0.031 ± 1.577 | 0.813 | -0.700 ± 1.275 | 0.074 | 0.463 |
| 138 | CLA.FA | % | 0.025 ± 0.068[A] | 0.370 | -0.222 ± 0.092[B] | <0.001 | -0.176 ± 0.130[B] | 0.006 | 0.000 |
| 139 | FAw3.FA | % | -0.281 ± 0.209[A] | 0.004 | -0.583 ± 0.251[B] | <0.001 | 0.769 ± 0.632[C] | 0.003 | 0.000 |
| 140 | FAw6.FA | % | -0.575 ± 1.453 | 0.371 | -0.344 ± 1.515 | 0.369 | -0.614 ± 1.415 | 0.152 | 0.898 |
| 141 | PUFA.FA | % | -0.875 ± 1.521 | 0.301 | -0.919 ± 1.524 | 0.045 | 0.171 ± 1.421 | 0.735 | 0.145 |
| 142 | MUFA.FA | % | 0.388 ± 1.321[A] | 0.517 | 3.344 ± 2.124[B] | <0.001 | 2.200 ± 1.508[B] | 0.003 | 0.002 |
| 143 | SFA.FA | % | 0.475 ± 1.282[A] | 0.410 | -2.438 ± 1.993[B] | 0.001 | -2.371 ± 2.181[B] | 0.005 | 0.002 |
| 144 | Glc | mmol/l | 0.000 ± 0.381 | 0.976 | 0.143 ± 0.342 | 0.232 | -0.056 ± 0.416 | 0.666 | 0.498 |
| 145 | Lac | mmol/l | 0.103 ± 0.420 | 0.443 | -0.074 ± 0.414 | 0.461 | -0.350 ± 0.711 | 0.116 | 0.102 |
| 146 | Pyr | mmol/l | 0.013 ± 0.027 | 0.345 | 0.003 ± 0.029 | 0.406 | -0.009 ± 0.039 | 0.667 | 0.318 |
| 147 | Cit | mmol/l | 0.001 ± 0.018 | 0.967 | 0.002 ± 0.020 | 0.712 | 0.005 ± 0.027 | 0.567 | 0.898 |
| 148 | Ala | mmol/l | 0.005 ± 0.048 | 0.713 | -0.003 ± 0.047 | 0.887 | -0.014 ± 0.049 | 0.422 | 0.657 |
| 149 | Gln | mmol/l | 0.097 ± 0.047 | 0.000 | 0.098 ± 0.046 | <0.001 | 0.093 ± 0.054 | 0.003 | 0.909 |
| 150 | Gly | mmol/l | -0.007 ± 0.034 | 0.635 | 0.002 ± 0.012 | 0.875 | -0.016 ± 0.027 | 0.063 | 0.291 |
| 151 | His | mmol/l | -0.005 ± 0.012 | 0.498 | -0.009 ± 0.011 | 0.011 | -0.004 ± 0.011 | 0.283 | 0.509 |
| 152 | Ile | mmol/l | 0.002 ± 0.009 | 0.513 | 0.002 ± 0.010 | 0.509 | -0.003 ± 0.007 | 0.236 | 0.295 |
| 153 | Leu | mmol/l | 0.002 ± 0.010 | 0.527 | 0.004 ± 0.013 | 0.392 | -0.001 ± 0.007 | 0.609 | 0.558 |
| 154 | Val | mmol/l | 0.008 ± 0.023 | 0.387 | 0.009 ± 0.021 | 0.243 | 0.016 ± 0.014 | 0.006 | 0.640 |

| # | Metabolic parameter name | unit | SFA Δ (mean ± SD) | SFA FDR p[2] | MUFA Δ (mean ± SD) | MUFA FDR p[2] | MED Δ (mean ± SD) | MED FDR p[2] | Treatment effect between diets[1] FDR p |
|---|---|---|---|---|---|---|---|---|---|
| 155 | Phe | mmol/l | 0.003 ± 0.008 | 0.442 | 0.004 ± 0.010 | 0.195 | -0.002 ± 0.009 | 0.668 | 0.378 |
| 156 | Tyr | mmol/l | 0.000 ± 0.009 | 0.898 | 0.001 ± 0.008 | 0.950 | -0.004 ± 0.008 | 0.058 | 0.318 |
| 157 | Ace | mmol/l | 0.010 ± 0.008 | 0.007 | -0.001 ± 0.038 | 0.012 | 0.013 ± 0.017 | 0.023 | 0.750 |
| 158 | AcAce | mmol/l | -0.003 ± 0.012 | 0.527 | -0.007 ± 0.014 | 0.199 | -0.002 ± 0.009 | 0.567 | 0.806 |
| 159 | bOHBut | mmol/l | -0.023 ± 0.045 | 0.340 | -0.029 ± 0.044 | 0.076 | -0.019 ± 0.045 | 0.093 | 0.944 |
| 160 | Crea | mmol/l | 0.002 ± 0.007 | 0.527 | 0.005 ± 0.008 | 0.031 | 0.001 ± 0.008 | 0.648 | 0.371 |
| 161 | Alb | signal area | 0.001 ± 0.004[A] | 0.527 | -0.002 ± 0.003[B] | 0.014 | -0.002 ± 0.003[B] | 0.048 | 0.048 |
| 162 | Gp | mmol/l | 0.190 ± 0.146 | 0.004 | 0.137 ± 0.173 | 0.005 | 0.061 ± 0.129 | 0.073 | 0.109 |

Data are presented as mean ± standard deviation

[1]Differences between the changes from baseline were tested with an ANOVA and their corresponding FDR corrected *p*-values are shown. In case of a significant overall FDR *p*-value <0.05, comparisons between diets were made using linear mixed models: different superscript letters in the row indicate significant differences ($p$ <0.05). [2]FDR corrected *p*-values of the within diet effect, using paired t-tests.

# 4

# Effect of two energy restricted diets differing in nutrient quality on microRNA expression in human subcutaneous adipose tissue, a parallel intervention trial.

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IN PREPARATION

## ABSTRACT

**Scope:** MicroRNAs are small non-coding endogenous RNA molecules that post-transcriptionally regulate gene expression. They are key players in obesity-related metabolic dysfunction. However, the effects of both energy restriction (ER) and changes in nutrient quality on microRNA expression in human subcutaneous adipose tissue (SAT) remain to be identified.

**Methods and results:** A 12-week parallel 25% ER dietary intervention trial was conducted on abdominally obese subjects, randomized to: 1) a 25% ER low-quality diet, 2) a 25% ER high-quality diet, and 3) a non-ER control group. Before and after the intervention period, genome-wide SAT microRNA profiles were assessed in 88 subjects. The low-quality diet increased the expression of miR-450-5p, let-7f-1-3p and miR-1263, and the high-quality diet increased miR-6819-5p expression. Both ER diets reduced the expression of miR-7977, miR-4443, and miR-143-5p (all $p$-value < 0.05). We observed that expression of several microRNA targeted genes was changed when SAT microRNA and transcriptome data were integrated.

**Conclusion:** Both 25% ER and differences in nutrient quality affect SAT microRNA expression. Our explorative study showed that microRNAs can likely be affected by diet. The exact functional significance of the nutrition-induced effects on miRNA expression in human SAT needs to be further elucidated.

## INTRODUCTION

Since 1980, the worldwide prevalence of overweight and obesity has increased to an extent that nearly a third of the world population is now classified as overweight or obese (1). Obesity is a major health challenge, because it increases the risk of developing diseases such as type 2 diabetes mellitus and cardiovascular diseases, and thereby negatively affects both life expectancy and quality of life (2). Obesity is often a consequence of chronic overnutrition, resulting in the storage of excess energy in the form of fat, primarily in the adipose tissue. As such, the adipose tissue is a key organ affected early during the development of obesity. The expanding storage of energy in the adipose tissue can result in altered energy and lipid metabolism, adipocyte hypertrophy, insulin resistance, a state of inflammation, and elevated free fatty acids (FFA) in the circulation (3, 4). An effective way to improve these disturbances in metabolic functioning in the adipose tissue is by weight loss due to dietary energy restriction. With regard to nutrients, there is some evidence that saturated fatty acids (5), and fructose can have adverse effects on adipose tissue health (6), whereas nutrients such as unsaturated fatty acids and isoflavones might increase adipose tissue health (7-10). In addition, high-protein diets have a more beneficial effect on ectopic fat storage (11). Consequently, both energy restriction and nutrient quality might have distinct effects on molecular mechanisms related to adipose tissue health and obesity.



One of the key players involved in obesity-related metabolic dysfunction are microRNAs (12-17). MicroRNAs (miRNAs) are small non-coding endogenous RNA molecules that post-transcriptionally regulate gene expression (18, 19). As such, miRNAs have received growing attention in the field of obesity research (20-26). A large number of miRNAs have been found to be expressed in the subcutaneous adipose tissue (SAT) of human subjects (27, 28), some of which have shown to be associated with several parameters of obesity, the metabolic syndrome and diabetes (22, 28-31). The effect on miRNA changes following weight-reduction regimes in humans is not well studied. Recently, differential miRNA expression in SAT tissue after a hypocaloric diet in combination with exercise-induced weight loss was observed (32), as well as after surgery-induced weight loss (33-35). However, the results of these studies showed little overlap, and the effects of solely diet-induced weight loss without exercise on miRNA expression in SAT remain to be explored. In addition, the effect of nutrient quality within a weight-loss diet on miRNA expression in the SAT in humans is unknown. In several mice models and cell culture studies high-fat diets, saturated fatty acids, and polyunsaturated fatty

acids have shown to modulate the expression and function of several miRNAs, as summarised by Quintanilha *et al.* (36) and A McGregor *et al.* (37). Hence, we hypothesized that different types of nutrients can differentially change miRNA expression in human SAT. Therefore, we examined not only the effects of energy restriction on miRNA expression in human SAT, but also examined the effect changing nutrient quality. Additionally, as miRNAs are capable of altering gene expression (33, 38), our secondary aim was to integrate the diet-induced changes in microRNA expression with the diet-induced changes in whole genome expression profiles in the SAT of the same subjects. Finally, we integrated the diet-induced changes in miRNA expression with the changes observed in clinical and anthropometric measures, such as body weight and subcutaneous and visceral adipose tissue mass.

## MATERIALS AND METHODS

### *Subjects and study design*

In total, 110 men and women participated in this parallel-designed randomized 12-week intervention study. An extensive description of the study design, recruitment, methods, and results of the primary outcome measures have been described elsewhere (39). Briefly, the study population consisted of healthy abdominally obese men and women, aged 40-70 years. Abdominal obesity was defined as having a BMI >27 kg/m$^2$, or a waist circumference of >88 cm for women, or >102 cm for men. Subjects were randomized over three groups; a low-quality (n=40) or a high-quality (n=40) 25% ER diet group, or a non-ER control group (n=30). The intervention of the 25% ER diet groups was based on dietary advice in the form of meal plans, and on the provision of key food products, while subjects in the control group were instructed to maintain their habitual diet, without any additional support. The 25% ER diets were equally energy-restricted and matched for alcohol, sodium, and total fat intake, but the high-quality diet was enriched with soy protein, fibre, monounsaturated fatty acids (MUFAs), and n-3 polyunsaturated fatty acids (PUFAs). In the low-quality diet these components were substituted by SFA, animal protein, and fructose. The study was approved by the Medical Ethics Committee of Wageningen University, and registered at ClinicalTrials.gov, identifier: NCT02194504.

### *Adipose tissue biopsies*

Before and after the 12-week intervention, subcutaneous adipose tissue (SAT) biopsies were taken caudally from the umbilicus under local anaesthesia (1% lidocaine) by trained nurses using a small liposuction cannula. Sampling was conducted after an overnight fast. Samples were rinsed with

phosphate-buffered saline to eliminate blood and were immediately snap frozen in liquid nitrogen and stored at -80 ºC until further analyses.

***RNA isolation***

RNA, including miRNA, was isolated from frozen SAT samples using TRIzol®/chloroform extraction (Thermo Fisher Scientific, Waltham, USA) and purified using the Qiagen Mini column kit according to manufacturer's protocol (Qiagen, Hilden, Germany). RNA yield was quantified for all samples on a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Subsequently, RNA integrity was measured for 149 of the 176 samples on an Agilent 2100 BioAnalyzer with RNA 6000 Nanochips (Agilent Technologies, Santa Clara, USA). The measured 149 samples had an RNA integrity (RIN) score of 6.2 or higher, with an average RIN-score of 8.0. RNA quality of the 27 samples of which no gene expression array was available, was assumed to be sufficient.



***MiRNA microarray***

MiRNAs were analysed using the Affymetrix miRNA 4.1 microarray platform (Affymetrix, Santa Clara, CA), covering all entries in Sanger miRbase v.20, including 2578 mature and 2025 pre-miRNAs, as well as 1996 small nucleolar (sno)- and small cajal body-specific (sca)-RNAs. Therefore, when indicating all small RNA types, the term 'small nuclear RNA (snRNA)' will be used, while the term 'miRNAs' will be used when indicating only the mature and pre- miRNAs. For snRNA analysis, 240 µg of total RNA was labelled with the FlashTag Biotin HSR RNA Labelling kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Microarray plates were washed, stained, and scanned on the GeneTitan Instrument (Affymetrix). Raw data are published and publicly available in in the Gene Expression Omnibus (GEO, http://ncbi.nlm.nih.gov/geo/, Barrett and Edgar (40)), under accession number GSEXXXX[1]. Quality of the snRNA measurements was checked via the Transcriptomic Analysis Console Software (TACS version 4.0.2, Affymetrix®, Santa Clara, USA). TACS was also used to compute the normalized expression estimates of the probesets by the robust multi-array averaging (RMA) algorithm (41), and detection above background (DABG) *p*-values (42). Only probesets with DABG detection *p*-value ≤ 0.05 in at least 46 microarray samples were considered for downstream analysis. In case multiple probesets were present for the same snRNA, the probeset with the highest expression value was selected.

1 Correct reference number will be added, once the paper has been accepted for publication.

***Statistical analysis: snRNA data set***

Significant differences in snRNA expression were assessed using linear models implemented in the Bioconductor library *limma* (43), and an intensity-based moderated t-statistic (44). For all comparisons snRNAs were defined as significantly different when the moderated *p*-value was <0.05. Analysis of changes in snRNA expression were conducted by comparing the change (12 wk versus 0 wk) in gene expression among the three different groups (F-test) as well as by analysing the change within an intervention group (paired t-test). Because of the explorative nature of the study, unadjusted moderated *p*-values <0.05 were considered as statistically significant. To observe whether changes in snRNAs among the three diet groups could be separated, sparse partial least squares discriminant analysis (sPLS-DA) was performed. The sPLS-DA model was made using the *caret* (45) and *spls* (46) R libraries. This model was validated using two times repeated five-fold cross-validation. All analyses were done using R v4.0.2 (47), and heatmaps were made using Excel 2016.

***Transcriptomics data set***

The mRNA expression data obtained in the Belly fat study by Schutte et al (39) was used in the current analysis. These data are available under accession number GSEXXXX[2]. Affymetrix gene chip Human 2.1 ST arrays (Affymetrix, Santa Clara, CA) were used to measure whole genome gene expression. Further pre-processing and quality control were performed by Schutte *et al.* (39) as previously described. Significant differences in gene expression were assessed using LIMMA (43). For all comparisons genes were defined as significantly different when the moderated p-value was <0.05. Analysis of changes in gene expression were conducted by comparing the change (12 wk versus 0 wk) in gene expression among the three different groups (F-test) as well as by analysing the change within an intervention group (paired t-test). This gene expression dataset was used for the integration with the miRNA data.

***Integration of miRNA with mRNA data: Network Analysis***

To integrate the miRNA data with mRNA data Cytoscape version 3.7.1 was used. Cytoscape is a widely adopted network visualization and analysis tool (48). For the integration, significant differentially changed mature miRNAs among the three diet groups (F-test, *p*-value <0.05) were loaded into Cytoscape. Using the CyTargetLinker 4.0.0+ app in Cytoscape (49), miRNA–target gene interactions

2 Correct reference number will be added, once the paper has been accepted for publication

from the validated database miRTarBase v7.0 (50), and the prediction database TargetScan v7.2 (51) were added. Subsequently, only the genes that were differentially changed among the three diet groups (F-test, *p*-value <0.05) were selected. This created a network of differentially expressed miRNAs among the different diets and their changed target genes. Subsequently, pathway analysis was done on the significantly changed target genes for each of the ER diets using EnrichR (Pathways: KEGG 2019 Human) (52, 53).

***Statistical analysis: baseline characteristics and diet effects on fasting parameters***

Differences in baseline characteristics were examined among the three diet groups by ANOVA testing, or Chi-square testing for categorical data. Data are reported as means ± SD, where appropriate. Analyses of changes in fasting parameters were conducted using ANCOVA (with values after treatment as dependent variable, adjusted for baseline values), after which LSD post hoc testing was applied. Again, a *p*-value < 0.05 was considered statistically significant. These analyses were conducted using IBM SPSS Statistics v25.



## RESULTS

In total, 110 subjects were randomly allocated to one of the three intervention groups. Adipose tissue biopsies from 88 out of the 100 subjects that completed the trial were available for miRNA microarray analysis (Flowchart: **Figure S1**).

***Effects of the dietary interventions on fasting parameters***

Baseline characteristics and the effects of the dietary interventions on fasting parameters are presented for the subsample of 88 subjects in **Table S1**. Effects observed in this subsample did not deviate from the findings found in the total study population (39). In short, twelve weeks of ER resulted in significant weight loss of 6.2 ± 3.9 kg in the low-quality and 8.5 ± 3.1 kg in the high-quality diet group, which was significantly different from the control diet group, and significantly different between the two ER groups, despite equal ER. Furthermore, both ER diets caused a significant reduction in waist circumference, subcutaneous and visceral abdominal adipose tissue mass, intrahepatic lipids, and fasting plasma glucose and insulin. Only the high quality diet significantly reduced fasting serum total cholesterol concentration.

***Effects of nutrient quality and energy restriction on fasting snRNA expression in SAT***

Next, the effects of the dietary interventions on fasting snRNA expression in SAT were examined. Of the 6609 snRNAs present on the array, 1391 passed the DABG filtering criteria. Principle component analysis on fasting snRNA expression changes indicated that the samples from the three diet groups were uniformly distributed (**Figure S2**, Supplemental Material). Subsequent sPLS-DA analysis lead to a best fitting model consisting of 5 components, and a regularization parameter *eta* of 0.8. The model with the best cross-validation performance had an accuracy of 41.1%, and a Cohen's kappa of 0.11 to place the right subject in the right diet group, indicating that the model could place a subject in the right diet group with slightly higher accuracy than by chance. In this model, the top 3 variables most important for the separation for the low-quality and the control diet were: CDBox 14q11-7, and mature miRNAs -450b-5p, and -7975. For the separation of the high-quality diet group HAcaBox ACA41 and stemloops mir-4286, and mir-548q were most important.

Univariate analysis on the 1391 snRNAs that passed the filtering criteria, resulted in 69 significantly different snRNAs among the three intervention groups (*p*-value <0.05, **Figure 1**).

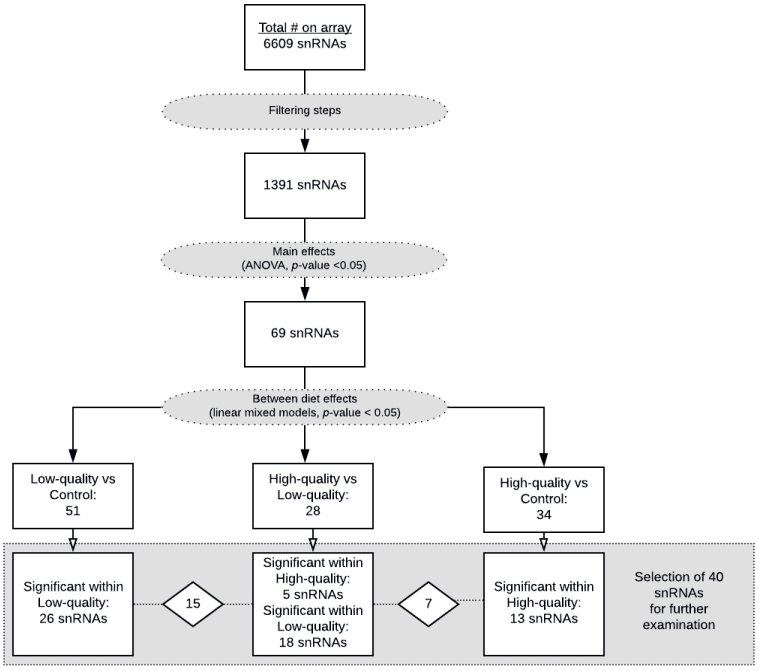



**Figure 1.** Flowchart of the effects of nutrient quality and energy restriction on snRNA expression. Main effects of the diet × time interaction were tested with an ANOVA, and linear mixed models were used to assess between diet effects. Within-diet group changes were tested with a paired t-test (Significance was set at *p*-value <0.05).

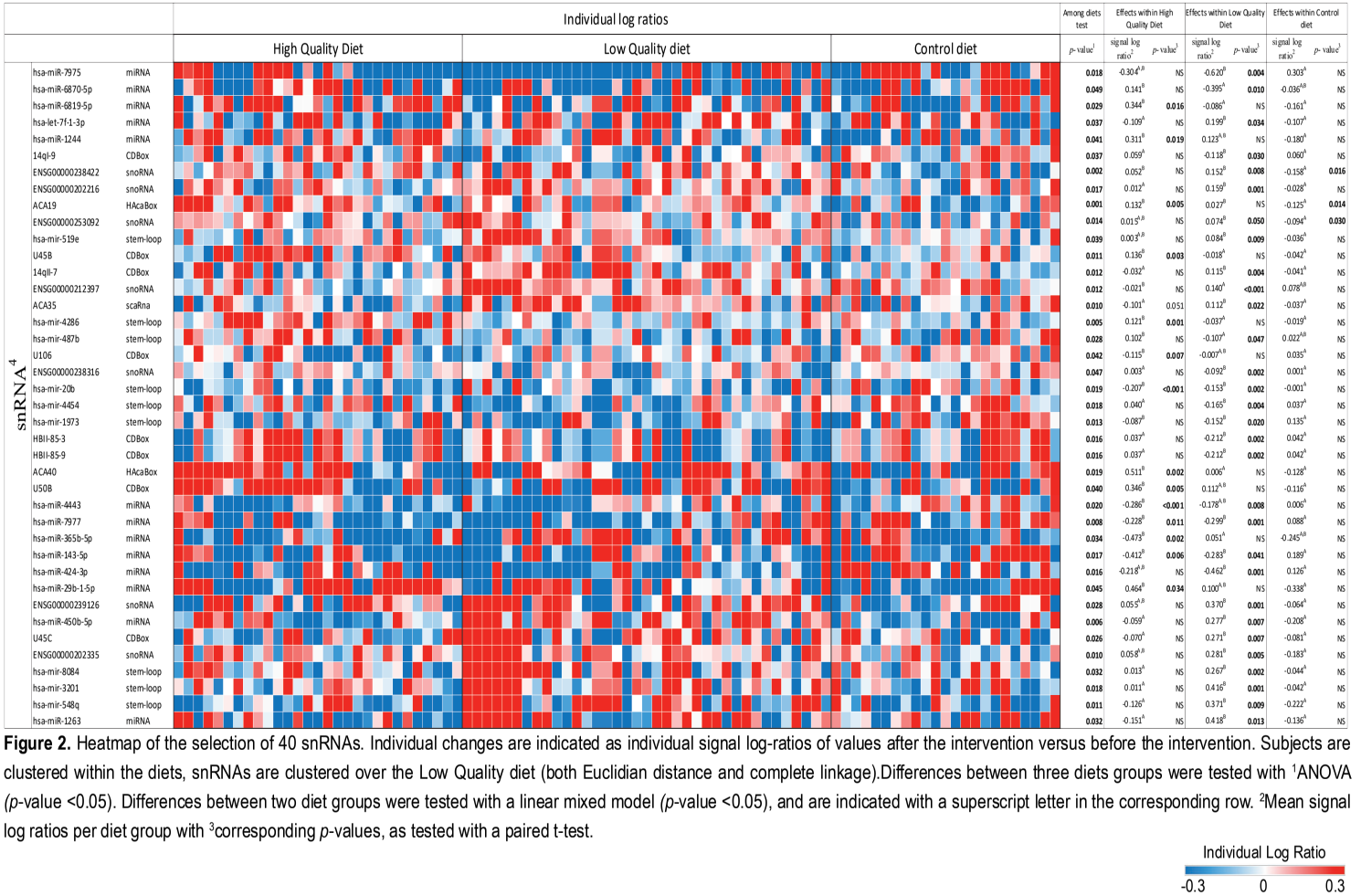
Post hoc analyses showed that of these 69 snRNAs, 51 were differentially changed in the low-quality diet versus the control diet group, 34 were differentially changed in the high-quality versus the control diet group, and 28 were differentially changed between the high-quality and the low-quality diet groups. Of the snRNAs that changed significantly according to the post hoc tests, 29 snRNAs changed significantly within the low-quality, and 11 snRNAs changed significantly within the high-quality diet group. These 40 snRNAs were selected for further examination and individual diet-induced changes are shown in **Figure 2**. Illustrated in this figure is the high interindividual variation in snRNA expression response upon the interventions.

***Network analysis of miRNAs and their mRNA targets***

Next, the miRNA data was integrated with the available data on whole genome gene expression changes. For this integration analyses, changes in expression of the 13 mature miRNAs out of the 40 snRNAs were used. In total, 2199 miRNA-gene interactions and 1900 genes were found for the 13 mature miRNAs. Subsequently, only those targeted genes of which the expression was significantly changed among the three diets (ANOVA, *p*-value <0.05) were selected leaving 131 miRNA-gene interactions and 104 genes. The resulting networks for the high-quality and the low-quality diet groups can be found in **figure 3A** and **3B** respectively. MiR-7977, of which the expression was decreased by both ER diets had the most known or predicted significantly changed gene targets. However, more of the target genes were affected by the high-quality compared to the low-quality diet. The same was observed for miR-143-5p, as also for this miRNA more target genes were affected by the high-quality diet compared to the low-quality diet. In the high-quality diet group, miR-6819-5p was significantly increased and its target genes were almost all significantly downregulated, while in the low-quality diet group with no change in miR-6819-5p, the targeted genes mostly increased their expression. In the low-quality diet group, miR-450b-5p was significantly upregulated and most of its predicted target genes were significantly increased, while these same target genes did not change within the high-quality diet group. Overall, when comparing the ER diet groups, there were more predicted or known gene targets significantly changed within the high-quality compared to the low-quality diet group.

***Pathway analyses of significantly increased and repressed genes***

To examine in which pathways the significantly changed target genes of the 13 miRNAs were involved, KEGG pathway analysis using EnrichR was performed. For each of the ER diets, the significantly




| | | Among diets test | Effects within High Quality Diet | | Effects within Low Quality Diet | | Effects within Control diet | |
|---|---|---|---|---|---|---|---|---|
| | | *p*- value[1] | signal log ratio[2] | *p*- value[3] | signal log ratio[2] | *p*- value[3] | signal log ratio[2] | *p*- value[3] |
| hsa-miR-7975 | miRNA | **0.018** | -0.304[A,B] | NS | -0.620[B] | **0.004** | 0.303[A] | NS |
| hsa-miR-6870-5p | miRNA | **0.049** | 0.141[B] | NS | -0.395[A] | **0.010** | -0.036[A,B] | NS |
| hsa-miR-6819-5p | miRNA | **0.029** | 0.344[B] | **0.016** | -0.086[A] | NS | -0.161[A] | NS |
| hsa-let-7f-1-3p | miRNA | **0.037** | -0.109[A] | NS | 0.199[B] | **0.034** | -0.107[A] | NS |
| hsa-miR-1244 | miRNA | **0.041** | 0.311[B] | **0.019** | 0.123[A,B] | NS | -0.180[A] | NS |
| 14qI-9 | CDBox | **0.037** | 0.059[A] | NS | -0.118[B] | **0.030** | 0.060[A] | NS |
| ENSG00000238422 | snoRNA | **0.002** | 0.052[B] | NS | 0.152[B] | **0.008** | -0.158[A] | **0.016** |
| ENSG00000202216 | snoRNA | **0.017** | 0.012[A] | NS | 0.159[B] | **0.001** | -0.028[A] | NS |
| ACA19 | HAcaBox | **0.001** | 0.132[B] | **0.005** | 0.027[B] | NS | -0.125[A] | **0.014** |
| ENSG00000253092 | snoRNA | **0.014** | 0.015[A,B] | NS | 0.074[B] | **0.050** | -0.094[A] | **0.030** |
| hsa-mir-519e | stem-loop | **0.039** | 0.003[A,B] | NS | 0.084[B] | **0.009** | -0.036[A] | NS |
| U45B | CDBox | **0.011** | 0.136[B] | **0.003** | -0.018[A] | NS | -0.042[A] | NS |
| 14qII-7 | CDBox | **0.012** | -0.032[A] | NS | 0.115[B] | **0.004** | -0.041[A] | NS |
| ENSG00000212397 | snoRNA | **0.012** | -0.021[B] | NS | 0.140[A] | **<0.001** | 0.078[A,B] | NS |
| ACA35 | scaRna | **0.010** | -0.101[A] | 0.051 | 0.112[B] | **0.022** | -0.037[A] | NS |
| hsa-mir-4286 | stem-loop | **0.005** | 0.121[B] | **0.001** | -0.037[A] | NS | -0.019[A] | NS |
| hsa-mir-487b | stem-loop | **0.028** | 0.102[B] | NS | -0.107[A] | **0.047** | 0.022[A,B] | NS |
| U106 | CDBox | **0.042** | -0.115[B] | **0.007** | -0.007[A,B] | NS | 0.035[A] | NS |
| ENSG00000238316 | snoRNA | **0.047** | 0.003[A] | NS | -0.092[B] | **0.002** | 0.001[A] | NS |
| hsa-mir-20b | stem-loop | **0.019** | -0.207[B] | **<0.001** | -0.153[B] | **0.002** | -0.001[A] | NS |
| hsa-mir-4454 | stem-loop | **0.018** | 0.040[A] | NS | -0.165[B] | **0.004** | 0.037[A] | NS |
| hsa-mir-1973 | stem-loop | **0.013** | -0.087[B] | NS | -0.152[B] | **0.020** | 0.135[A] | NS |
| HBII-85-3 | CDBox | **0.016** | 0.037[A] | NS | -0.212[B] | **0.002** | 0.042[A] | NS |
| HBII-85-9 | CDBox | **0.016** | 0.037[A] | NS | -0.212[B] | **0.002** | 0.042[A] | NS |
| ACA40 | HAcaBox | **0.019** | 0.511[B] | **0.002** | 0.006[A] | NS | -0.128[A] | NS |
| U50B | CDBox | **0.040** | 0.346[B] | **0.005** | 0.112[A,B] | NS | -0.116[A] | NS |
| hsa-miR-4443 | miRNA | **0.020** | -0.286[B] | **<0.001** | -0.178[A,B] | **0.008** | 0.006[A] | NS |
| hsa-miR-7977 | miRNA | **0.008** | -0.228[B] | **0.011** | -0.299[B] | **0.001** | 0.088[A] | NS |
| hsa-miR-365b-5p | miRNA | **0.034** | -0.473[B] | **0.002** | 0.051[A] | NS | -0.245[A,B] | NS |
| hsa-miR-143-5p | miRNA | **0.017** | -0.412[B] | **0.006** | -0.283[B] | **0.041** | 0.189[A] | NS |
| hsa-miR-424-3p | miRNA | **0.016** | -0.218[A,B] | NS | -0.462[B] | **0.001** | 0.126[A] | NS |
| hsa-miR-29b-1-5p | miRNA | **0.045** | 0.464[B] | **0.034** | 0.100[A,B] | NS | -0.338[A] | NS |
| ENSG00000239126 | snoRNA | **0.028** | 0.055[A,B] | NS | 0.370[B] | **0.001** | -0.064[A] | NS |
| hsa-miR-450b-5p | miRNA | **0.006** | -0.059[A] | NS | 0.277[B] | **0.007** | -0.208[A] | NS |
| U45C | CDBox | **0.026** | -0.070[A] | NS | 0.271[B] | **0.007** | -0.081[A] | NS |
| ENSG00000202335 | snoRNA | **0.010** | 0.058[A,B] | NS | 0.281[B] | **0.005** | -0.183[A] | NS |
| hsa-mir-8084 | stem-loop | **0.032** | 0.013[A] | NS | 0.267[B] | **0.002** | -0.044[A] | NS |
| hsa-mir-3201 | stem-loop | **0.018** | 0.011[A] | NS | 0.416[B] | **0.001** | -0.042[A] | NS |
| hsa-mir-548q | stem-loop | **0.011** | -0.126[A] | NS | 0.371[B] | **0.009** | -0.222[A] | NS |
| hsa-miR-1263 | miRNA | **0.032** | -0.151[A] | NS | 0.418[B] | **0.013** | -0.136[A] | NS |

**Figure 2.** Heatmap of the selection of 40 snRNAs. Individual changes are indicated as individual signal log-ratios of values after the intervention versus before the intervention. Subjects are clustered within the diets, snRNAs are clustered over the Low Quality diet (both Euclidian distance and complete linkage).Differences between three diets groups were tested with [1]ANOVA (*p*-value <0.05). Differences between two diet groups were tested with a linear mixed model (*p*-value <0.05), and are indicated with a superscript letter in the corresponding row. [2]Mean signal log ratios per diet group with [3]corresponding *p*-values, as tested with a paired t-test.

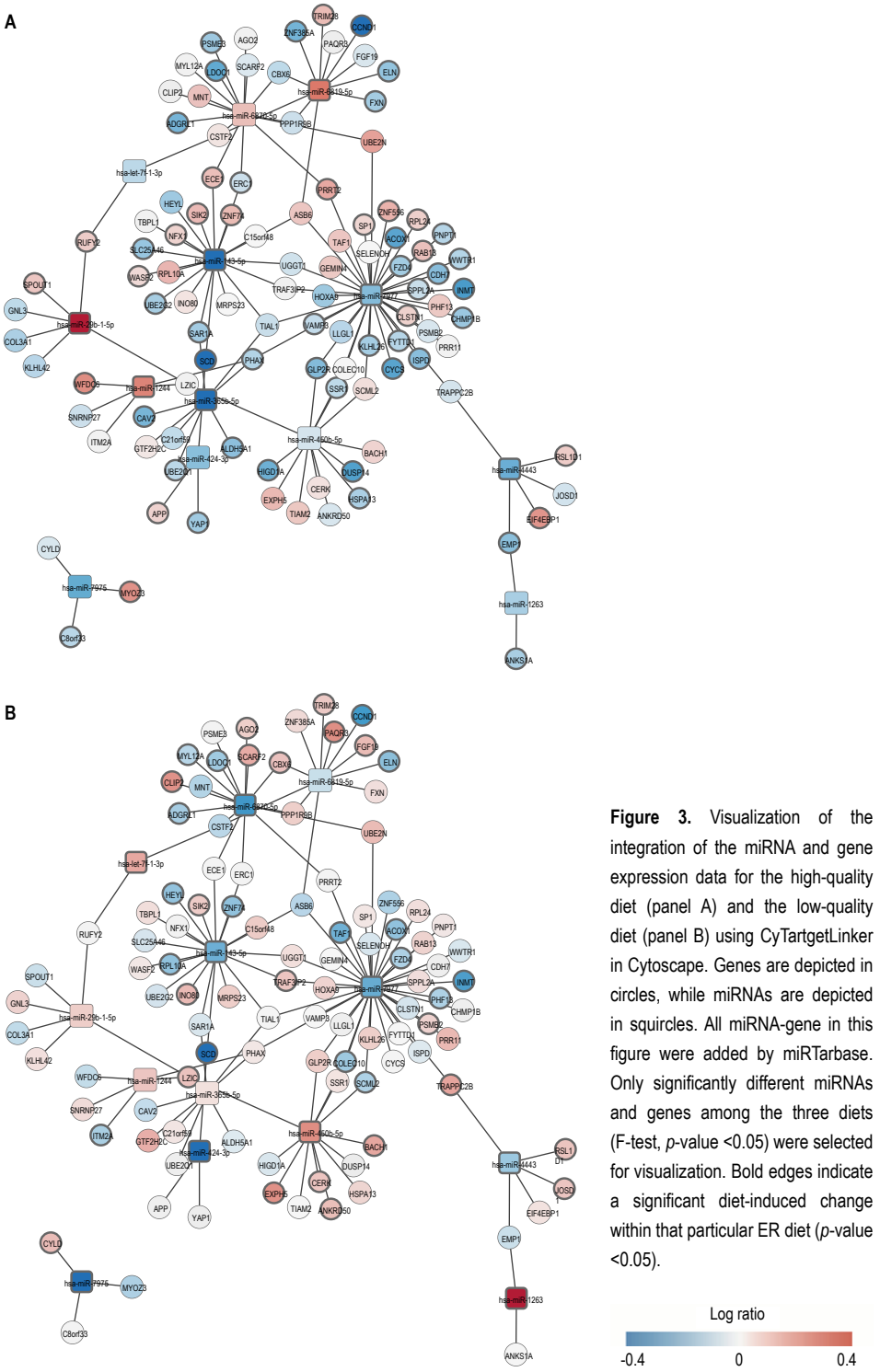



**Figure 3.** Visualization of the integration of the miRNA and gene expression data for the high-quality diet (panel A) and the low-quality diet (panel B) using CyTartgetLinker in Cytoscape. Genes are depicted in circles, while miRNAs are depicted in squircles. All miRNA-gene in this figure were added by miRTarbase. Only significantly different miRNAs and genes among the three diets (F-test, *p*-value <0.05) were selected for visualization. Bold edges indicate a significant diet-induced change within that particular ER diet (*p*-value <0.05).

changed gene targets ($p$-value <0.05, as indicated by bold borders in Figure 3A and B) were selected. This led to a selection of 19 increased and 38 decreased genes for the high-quality, and 20 increased and 17 decreased genes for the low-quality diet group. KEGG pathway analysis of the increased genes, revealed one pathway for the high-quality and two pathways for the low-quality diet group ($p$-value < 0.05, **Figure S3A**, Supplemental Material). KEGG pathway analyses of the repressed genes revealed 34 pathways for the high-quality and 26 pathways for the low-quality diet group ($p$-value < 0.05), of which both the top 10s are displayed in **Figure S3B**. In the high-quality as well as the low-quality diet group, a decrease in target genes involved in unsaturated fatty acids synthesis and the peroxisome proliferator-activated receptors (PPAR) signalling pathway were observed, indicating a suppression of genes in these pathways targeted by miRNAs in both ER groups. Additionally, a suppression of the 5' AMP-activated protein kinase (AMPK) signalling pathway and fatty acid degradation was observed in the low-quality diet group.

***Association of snRNAs with several anthropometric and biochemical parameters***

To explore whether the expression of snRNAs in the SAT correlated with cardiometabolic markers and anthropometric measures, correlation analyses were performed at baseline for the whole group, as well as for the diet-induced changes per intervention group. For the baseline measurements, 24 out of the 40 snRNAs showed significant correlations with one or more anthropometric or biochemical parameter ($p$-value <0.05) (**Figure S4**, Suppemental Material). Most of these correlations were modest ($-0.285 \leq \rho \leq 0.356$), except for the correlation between HAcaBox ACA40 and subcutaneous adipose tissue mass ($\rho = -0.458$ , $p$-value <0.001). The diet-induced changes per intervention group (**Figure S5**, Suppemental Material) were inconclusive, and showed a random pattern across the parameters that was not in line with what was observed at baseline for the whole group.

## DISCUSSION

We have characterized the effects of two 25% energy-restricted diets differing in nutrient quality on microRNA expression profiles in an important target organ for weight loss interventions; the subcutaneous adipose tissue. Both ER-diets had shared effects on miRNAs expression, but also low-quality and high-quality diet specific effects were observed.

***Effect energy restriction on miRNA expression***

Both ER-diets reduced the expression of miR-7977, miR-4443, as well as miR-143-5p, therefore these changes are likely caused by energy restriction. Effects on these specific miRNAs have not been reported in other diet-induced weight loss studies examining miRNA expression in human SAT (32, 54). However, these studies either also increased physical activity alongside the dietary intervention (32), or applied a lower caloric restriction (54). Expression of circulating miR-7977 has been shown to be associated with a high level of insulin (55), and it was found that miR-7977 expression was increased by insulin in *in vitro* epithelial cells. In line with these results, our findings show that the ER-induced reduction in miR-7977 in the SAT was paralleled by a decrease in circulating insulin. However, no significant associations between either the baseline or the diet-induced changes in insulin and miR-7977 were found. In human colon cancer cells, insulin has shown to upregulate the expression of miR-4443 (56), whose expression was reduced by both ER diets. Interestingly, we observed a relatively weak but significant positive correlation between insulin and miR-4443 before the intervention in the whole group, but none of the ER diet-induced improvements in insulin correlated with a change in miR-4443. The latter could also be due to the lower sample size. Another miRNA downregulated by both ER diets was miR-143-5p. Expression of miR-143-5p in adipose tissue was previously observed to be lower in individuals after weight loss due to bariatric surgery compared to before surgery (34). Additionally, in an animal model miR-143-5p activated the AMPK signalling pathway (57) involved in glucose and fatty acid metabolism (58). Accordingly, the low-quality diet reduced the expression of miR-143-5p target genes in the AMPK signalling pathway. However, no such effect was observed upon the high-quality diet. In summary, we observed ER-induced effects that may partly be related to ER-induced changes in insulin concentrations in the circulation.

Apart from the current study, two other human studies have examined the effect of diet-induced weight loss on miRNA expression in subcutaneous adipose tissue (32, 54). Kristensen *et al*. (32) reported a downregulation of miR-20b-5p, and an upregulation of miR-29a-3p and miR-29a-5p in the SAT of 19 women with severe obesity after a 15-week weight loss intervention, findings we could not confirm in our study. These differences could be due to the fact that the subjects in the study of Kristensen followed a hypocaloric diet in combination with exercise, while our subjects were instructed to maintain their habitual level of physical exercise. Additionally, the study populations were different, as the

subjects in the study of Kristensen *et al.* had a BMI of 47 kg/m$^2$, and lost on average 17 kg, while our study population had a BMI around 31 kg/m$^2$ and lost on average between 6.2 and 8.5 kg. Giardina *et al.* (54) reported no effects on microRNA expression in SAT after 6 months of ER in combination with a low-glycaemic index diet, a high-glycaemic index diet, or a low-fat diet. The exact height of the ER applied in these subjects was not indicated, but based on the amount of weight lost (between 5 and 10 kg on average over 6 months), we expect the ER to be lower than the ER of 25% applied in our study.

Next to the studies on diet-induced weight loss, the literature describes several studies examining the effects of bariatric surgery-induced weight loss (33, 34, 59). Surgery-induced weight loss was found to affect the expression of several miRNAs in adipose tissue, including miR200c-3p, miR-24-2-5p, miR-128-3p (33), for which we observed trends towards a significant change in expression in the current study. A decrease in expression of miR200c-3p and miR-24-2-5p was observed in the high-quality diet group, but the decrease was not significantly different from the low-quality and control diet groups. Additionally, miR-128-3p was decreased within both ER groups, but this was not significant different from the control diet group. It must be noted that the study populations in these types of studies have a BMI of around 40 kg/m$^2$ or higher, and lose on average around 48 kg, which is enormous compared to the 6.2-8.5 kg lost in our study. The more subtle diet-induced weight loss might explain the trend we found in our study. This was shown by Ortega and colleagues, who did observe effects of surgery-induced weight loss, but failed to report any effects on circulating miRNA expression after an energy deficit of 500-1000 kcal/day for 14 weeks (60).

***Effect nutrient quality on miRNA expression changes***

Several miRNAs were changed by the low-quality diet specifically. The expression of miR-450b-5p and miR-1263 were increased by the low-quality diet, however in terms of their role in weight loss, diet, or possible function in the adipose tissue, to the best of our knowledge, no literature is available. Another miRNA increased by low-quality diet only was let-7f-1-3p, and although the let-7 family is thought to play a significant role in glucose metabolism (61), no specific role for let-7f-1-3p has been identified yet. MiR-6819-5p was the only mature miRNA increased by the high-quality diet. MiR-6819-5p is reported to be involved in cancer related diseases (62, 63), but no literature on its role in adipose tissue could be found. The high-quality diet contained higher amounts of PUFA compared to the low-quality diet, 7.7 en% and 4.1 en% respectively (39). Several animal models, cell cultures (64-67) and

one human study (68) reported that miRNA expression was altered by PUFAs. However, we found no overlap between their observations and the high-quality diet induced miRNA expression changes in our study. Of note, also little overlap was reported among the previous studies themselves (64-68), which might be caused by the use of different types of models, and tissues, e.g. inflammatory cells and liver. Similar to the findings on PUFA, we found no overlap between our findings and findings of studies that examined the effects of the other nutrients present in our intervention diets (69-72). However, in these studies mostly murine models were used (69, 70, 72), and one study examined the acute effects of a high intake of saturated fat on microRNA expression in human PBMCs (71), but not adipose tissue. In summary, both the low-quality and the high-quality diet induced the expression of specific miRNAs, but these findings could not be linked to or explained by previous findings. This was partly because of the lack of knowledge on these miRNAs and partly due to the use of different models, such as animal and cell culture models. Therefore, the functional significance of the observed effects on miRNA expression induced by changes in nutrient quality needs to be further investigated.



***Strengths and Limitations***

The current study is unique in that it allowed to investigate and compare the effects of both ER and changes in nutrient quality by a whole diet approach, in a real-life fashion. Another strength of the study was the examination of the effects on miRNA expression, a molecular regulatory layer that has yet to be explored within the nutrition field, and the integration of these data with gene expression data in the same study population. However, the study also had several limitations. It is known that gender influences miRNA expression in WAT (31), but due to the sample size, we were unable to perform separate analyses. However, as the ratio of men versus women was similar in each of the intervention arms, we do not expect that this would have affected our outcome measure. We did not verify our microRNA array results by ddPCR, but the microRNA array has been validated for snRNA measurements, and previous studies show an acceptable agreement of 70% between the two methods (73). Also, the miRNA-mRNA integration is dependent on the accuracy and limitations of the target prediction algorithms available, and currently there are no universally accepted criteria for validating miRNA targets yet (37, 74). Lastly, although it is interesting to examine the associations between miRNA and their predicted gene targets to explore the possible regulatory mechanisms of the miRNAs, most of the action of miRNAs takes place at the translational level, which is not examined using the here applied approach.

***Conclusion***

The present intervention study is the first to report that miRNA expression in human SAT is influenced by diet-induced weight loss in combination with changes in nutrient quality. We observed common induced ER effects that could partly be related to changes in insulin concentrations in the circulation. Apart from the shared ER effects, both the low-quality and the high-quality diet induced the expression of several specific miRNAs. Our explorative study showed that microRNAs can likely be affected by diet. The exact functional significance of the nutrition-induced effects on miRNA expression in human SAT needs to be further elucidated.

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## CONFILCT OF INTEREST

There is no conflict of interest.

## AUTHOR CONTRIBUTIONS

*S.S.* and *L.A.A.* designed and developed the study. *C.C.J.R.M., R.W.J.H., G.J.E.J.H.* analysed data. *C.C.J.R.M.* and *S.L.M.S.* integrated the microRNA and mRNA data. *C.C.J.R.M.* wrote the paper, which was critically reviewed and improved by *L.A.A. L.A.A.* had primary responsibility for final content. All authors read and approved the final manuscript.

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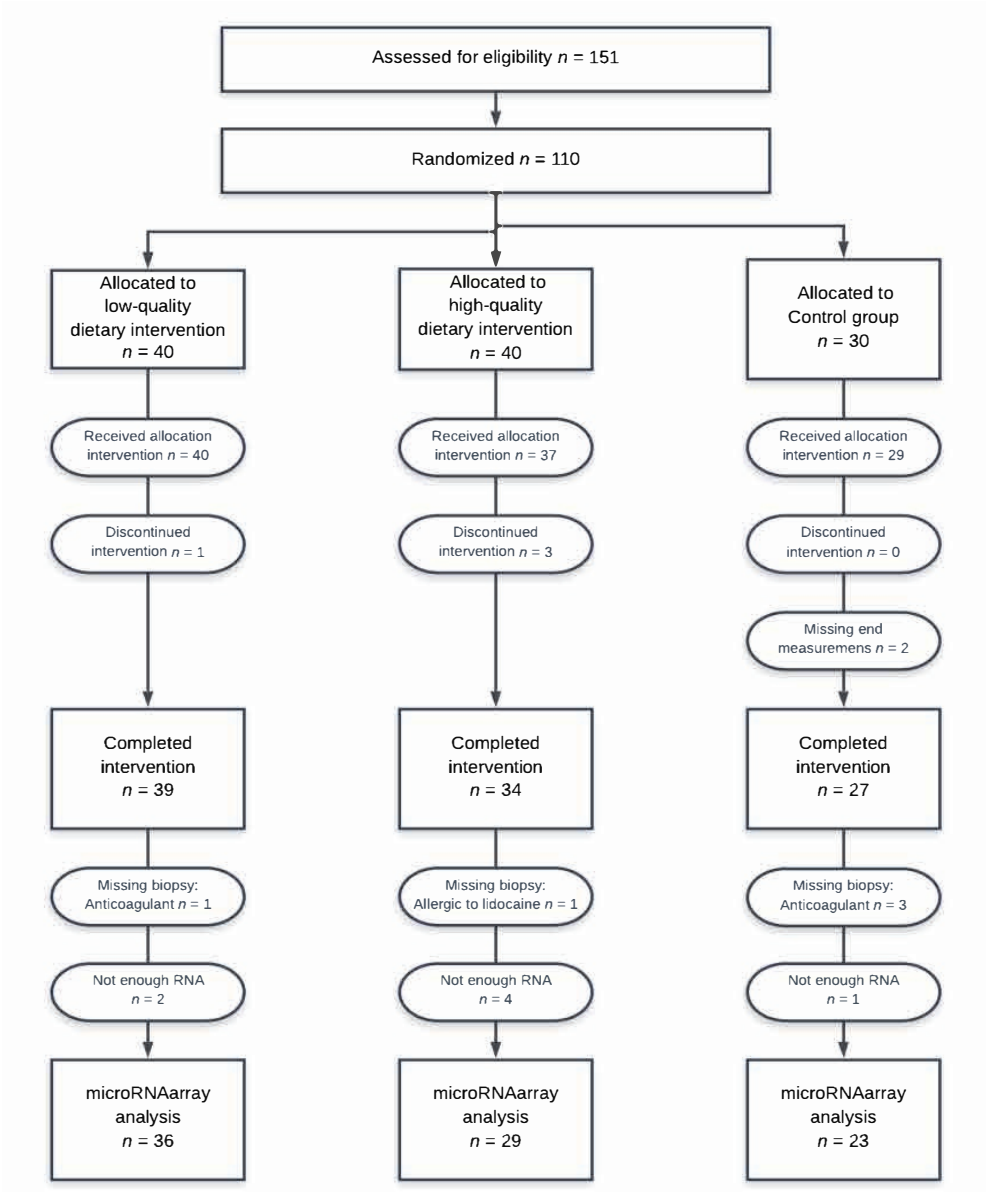
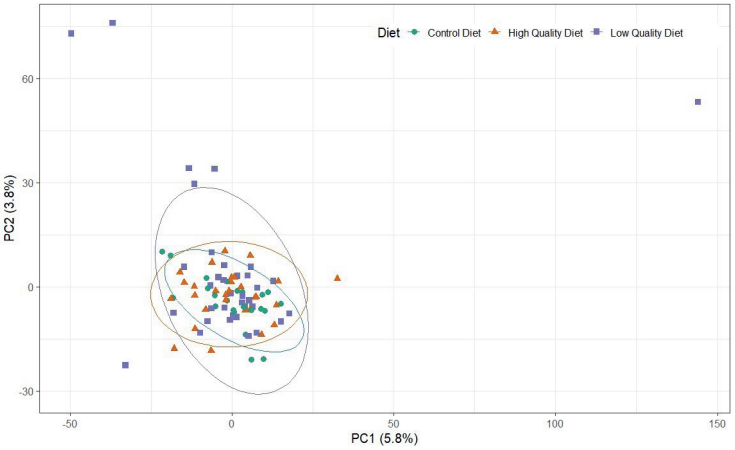
## LIST OF ABBREVIATIONS

| Abbreviation | Full description |
|---|---|
| AMPK | 5' AMP-activated protein Kinase |
| CON | Control group |
| ER | Energy Restriction |
| FFA | Free Fatty Acids |
| GEO | Gene Expression Omnibus |
| miRNA | microRNA |
| PPAR | Peroxisome Proliferator Activated Receptors |
| RIN | RNA integrity |
| SAT | Subcutaneous Adipose Tissue |
| scaRNA | small cajal body-specific RNA |
| snoRNA | small nucleolar RNA |
| snRNA | small nuclear RNA |

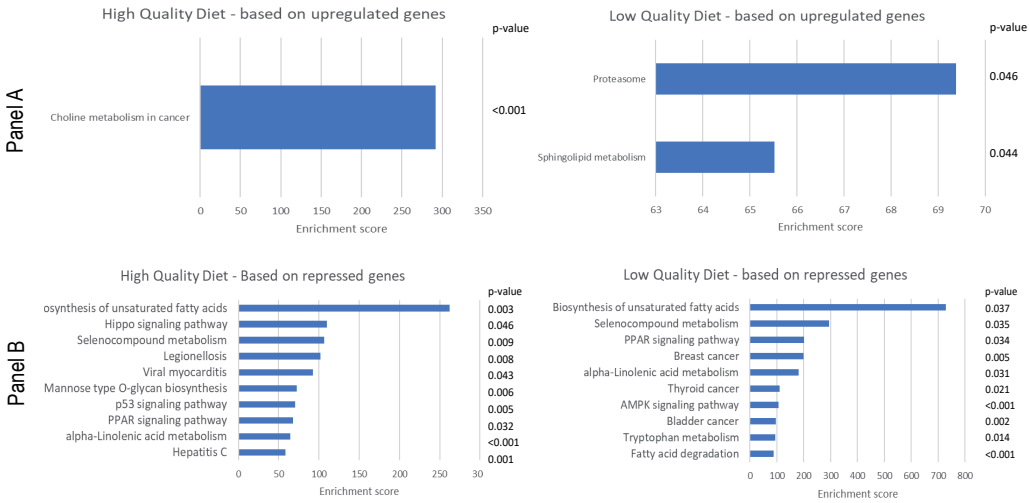
## SUPPLEMENTAL MATERIAL



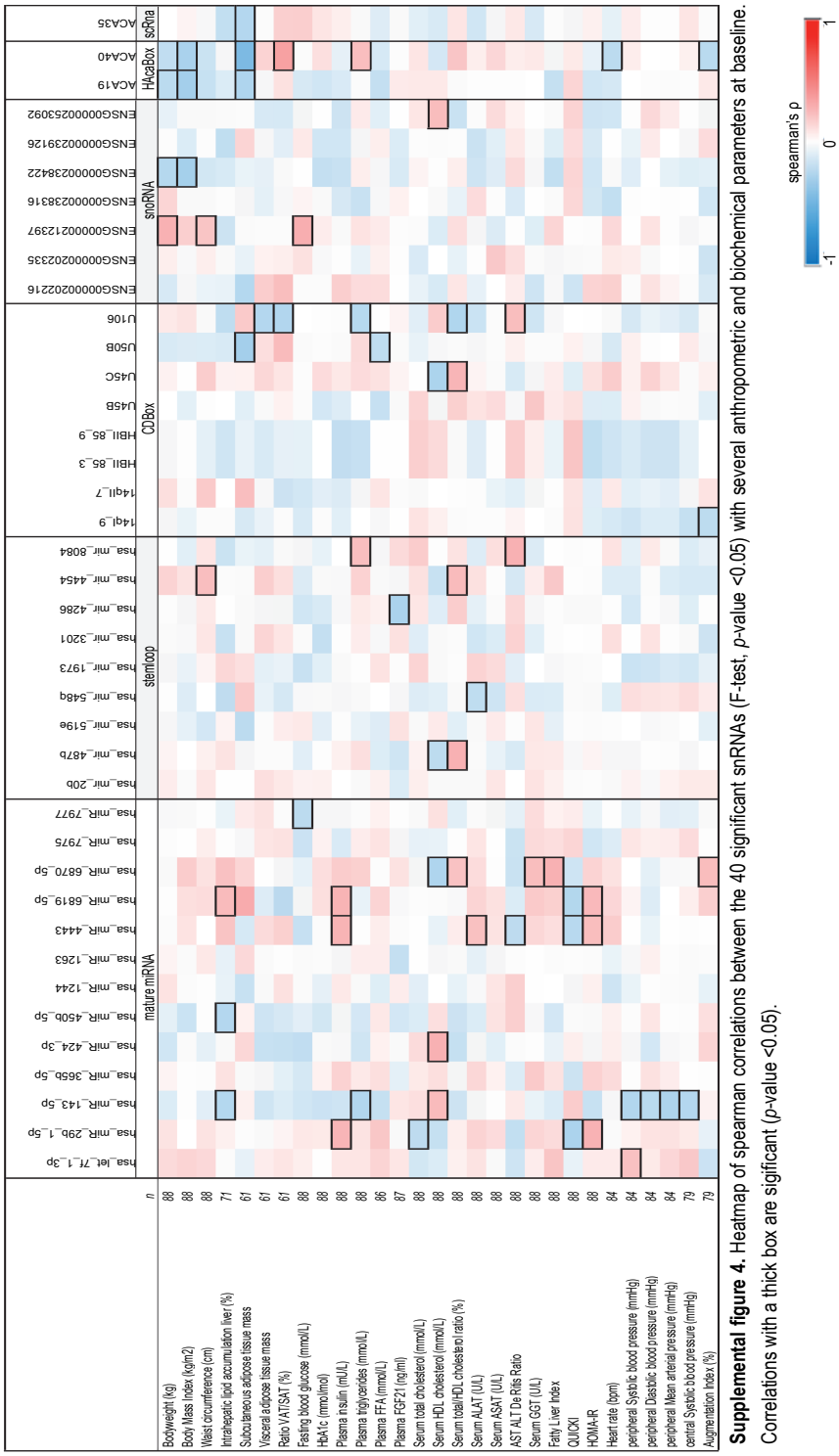

**Supplemental Figure 1.** Flowchart participants.



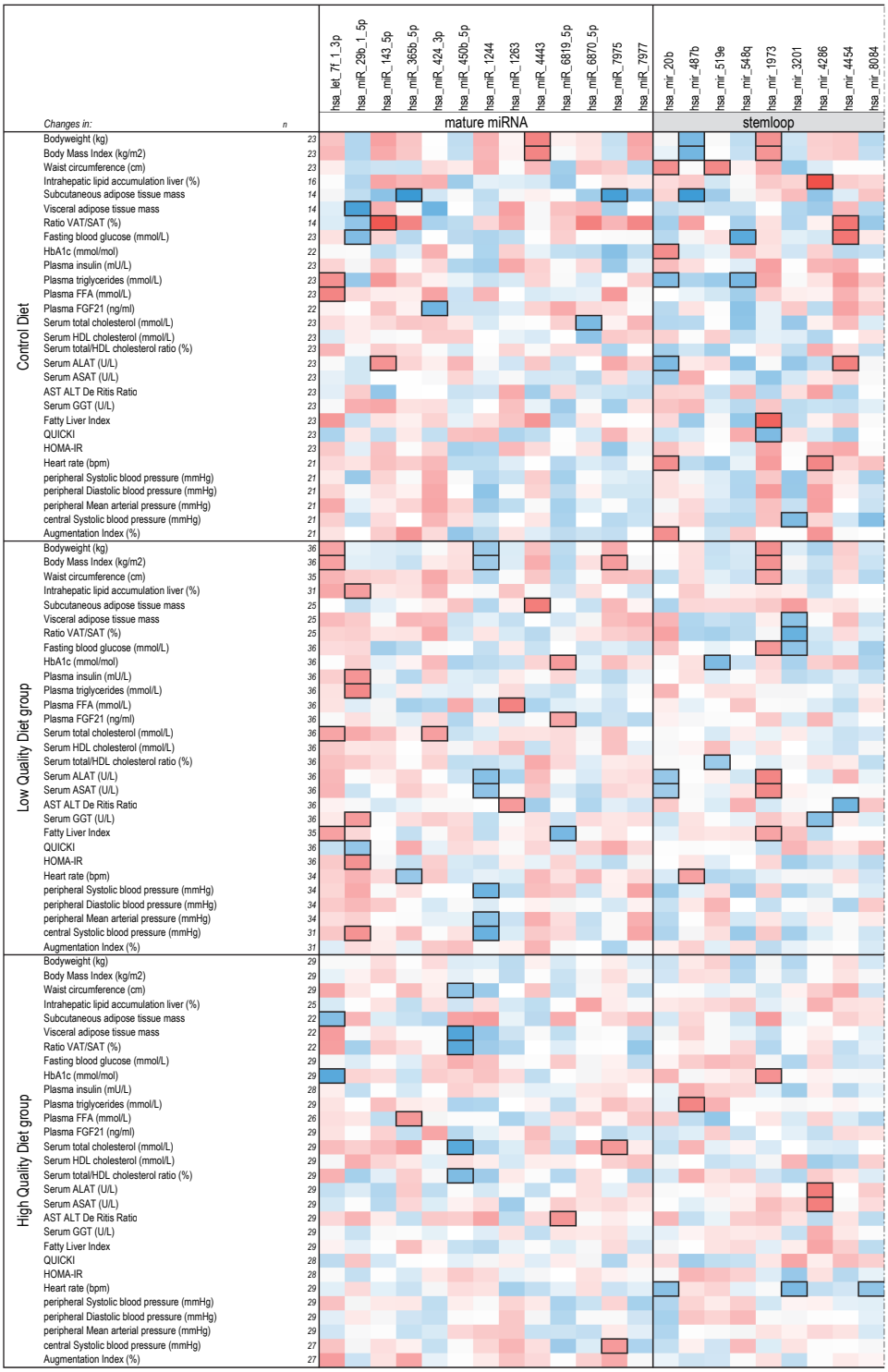

**Supplemental Figure 2.** Principle component analysis (PCA) to visualise the diet-induced changes in miRNA expression in the subcutaneous adipose tissue of abdominally obese men and women. The drawn ellipses represent the 95% confidence intervals.



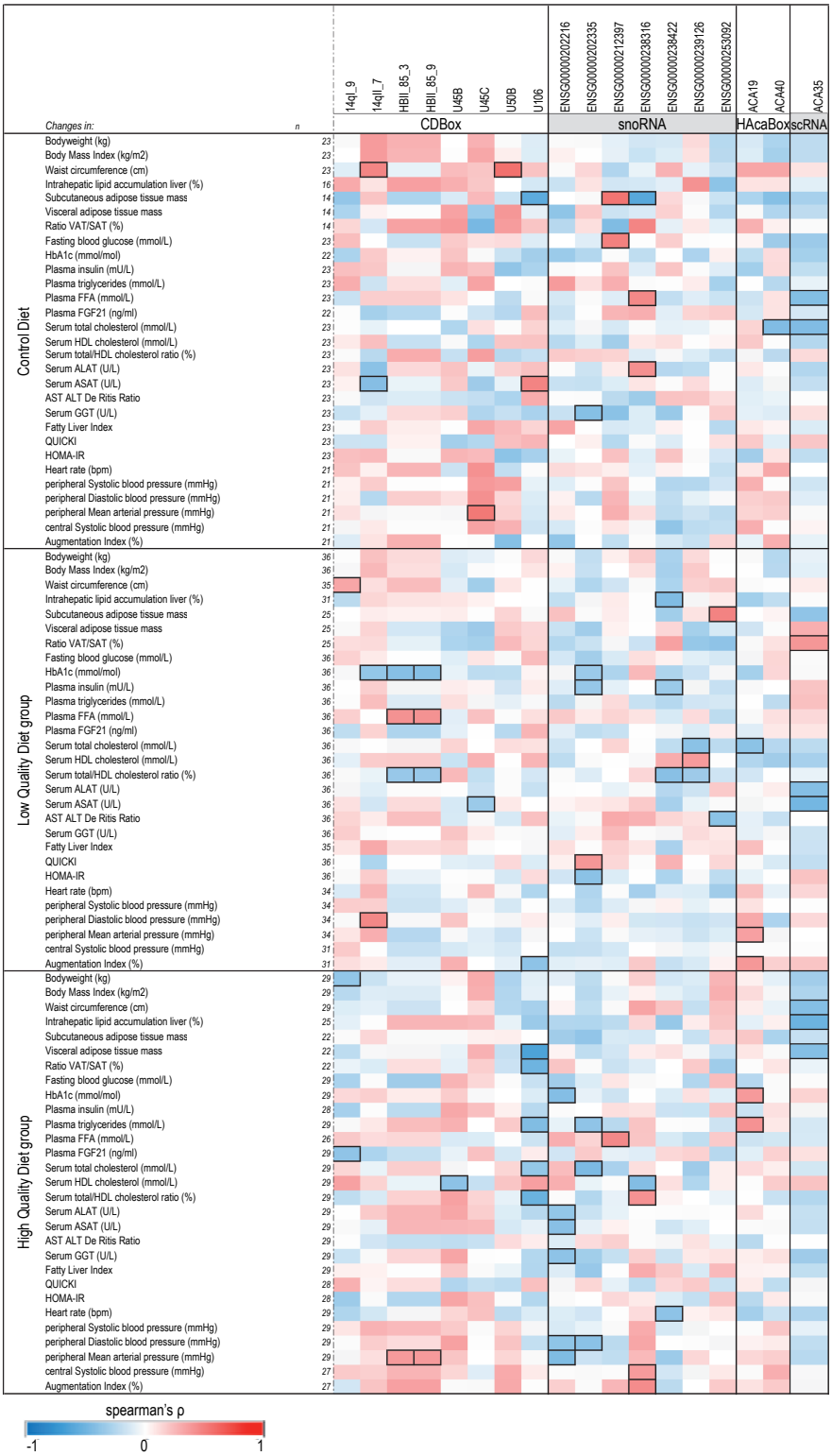

**Supplemental Figure 3.** KEGG pathway analyses using EnrichR based on significantly increased genes (Panel A), and the significantly decreased genes (panel B). ANOVA p-value <0.05. Results for the high quality diet are presented at te top, for the low quality diet at the bottom.




**Supplemental figure 4.** Heatmap of spearman correlations between the 40 significant snRNAs (F-test, *p*-value <0.05) with several anthropometric and biochemical parameters at baseline. Correlations with a thick box are sigificant (*p*-value <0.05).




**Supplemental figure 5.** *Continues on the next page.*




**Supplemental figure 5.** Spearman correlations between the diet-induced changes in the 40 significant snRNAs (F-test, *p*-value <0.05) with diet-induced changes in several anthropometric and biochemical parameters. Correlations in bold with a thick box are significant (*p*-value ≤ 0.001), correlations with a thick box only are significant (*p*-value <0.05), italic numbers indicate borderline significance (0.05 ≤ *p*-value < 0.06).

**Supplemental table 1.** Baseline characteristics and effects of the dietary interventions on fasting parameters. *Continues on the next page.*

| | n | Baseline | n | Change | Within group p-value | difference between groups p-value |
|---|---|---|---|---|---|---|
| **Age, years** | | | | | | |
| Low-quality diet group | 36 | 59 ± 8 | | | | |
| High-quality diet group | 29 | 59 ± 8 | | | | |
| Control group | 23 | 59 ± 8 | | | | |
| **Gender§, n males, %** | | | | | | |
| Low-quality diet group | 36 | 15, 42% | | | | |
| High-quality diet group | 29 | 13, 45% | | | | |
| Control group | 23 | 10, 44% | | | | |
| **Body weight, kg** | | | | | | |
| Low-quality diet group | 36 | 93.5 ± 14.4 | 36 | -6.2 ± 3.9$^{A}$ | <0.001 | <0.001 |
| High-quality diet group | 29 | 94.7 ± 13.3 | 29 | -8.5 ± 3.1$^{B}$ | <0.001 | |
| Control group | 23 | 90.7 ± 12.5 | 23 | 0.9 ± 1.7$^{C}$ | 0.015 | |
| **BMI, kg/m²** | | | | | | |
| Low-quality diet group | 36 | 31.4 ± 3.3 | 36 | -2.1 ± 1.3$^{A}$ | <0.001 | <0.001 |
| High-quality diet group | 29 | 31.9 ± 3.5 | 29 | -2.8 ± 1.0$^{B}$ | <0.001 | |
| Control group | 23 | 30.8 ± 3.9 | 23 | 0.3 ± 0.6$^{C}$ | 0.012 | |
| **Waist circumference, cm** | | | | | | |
| Low-quality diet group | 36 | 108.6 ± 10.0 | 35 | -2.9 ± 5.5$^{A}$ | 0.004 | <0.001 |
| High-quality diet group | 29 | 109.0 ± 9.8 | 29 | -4.4 ± 6.0$^{A}$ | 0.001 | |
| Control group | 23 | 105.5 ± 7.8 | 23 | 4.4 ± 5.4$^{B}$ | 0.001 | |
| **Abdominal SAT, cm²*** | | | | | | |
| Low-quality diet group | 25 | 333.9 ± 96.8 | 25 | -43.9 ± 33.4$^{A}$ | <0.001 | <0.001 |
| High-quality diet group | 22 | 352.2 ± 105.9 | 22 | -54.9 ± 59.8$^{A}$ | <0.001 | |
| Control group | 14 | 355.7 ± 121.9 | 14 | 4.9 ± 38.2$^{B}$ | 0.416 | |
| **Abdominal VAT, cm²*** | | | | | | |
| Low-quality diet group | 25 | 150.9 ± 80.2 | 25 | -44.5 ± 49.3$^{A}$ | <0.001 | <0.001 |
| High-quality diet group | 22 | 179.8 ± 99.6 | 22 | -66.7 ± 52.2$^{A}$ | <0.001 | |
| Control group | 14 | 111.6 ± 25.2 | 14 | 1.7 ± 18.1$^{B}$ | 0.803 | |
| **VAT/SAT ratio*** | | | | | | |
| Low-quality diet group | 25 | 51.7 ± 36.6 | 25 | -9.9 ± 17.4$^{A}$ | 0.001 | 0.035 |
| High-quality diet group | 22 | 63.5 ± 59.4 | 22 | -18.8 ± 28.9$^{A}$ | 0.001 | |
| Control group | 14 | 35.0 ± 16.3 | 14 | -0.2 ± 6.1$^{B}$ | 0.825 | |
| **Intrahepatic lipids, % of water peak*** | | | | | | |
| Low-quality diet group | 30 | 7.5 ± 6.7 | 31 | -3.5 ± 5.3$^{A}$ | <0.001 | <0.001 |
| High-quality diet group | 25 | 6.0 ± 7.8 | 25 | -3.6 ± 5.4$^{B}$ | 0.004 | |
| Control group | 16 | 6.2 ± 6.1 | 16 | 1.4 ± 2.1$^{C}$ | 0.076 | |

| | n | Baseline | n | Change | Within group p-value | difference between groups p-value |
|---|---|---|---|---|---|---|
| **Plasma glucose, mmol/L** | | | | | | |
| Low-quality diet group | 36 | 5.6 ± 0.7 | 36 | -0.3 ± 0.6$^{A}$ | 0.013 | 0.003 |
| High-quality diet group | 29 | 5.7 ± 0.4 | 29 | -0.3 ± 0.4$^{A}$ | 0.001 | |
| Control group | 23 | 5.6 ± 0.4 | 23 | 0.0 ± 0.4$^{B}$ | 0.955 | |
| **Plasma insulin, mU/L** | | | | | | |
| Low-quality diet group | 36 | 14.7 ± 10.6 | 36 | -3.7 ± 7.6$^{A}$ | 0.006 | 0.007 |
| High-quality diet group | 29 | 13.1 ± 7.7 | 28 | -3.8 ± 5.1$^{A}$ | 0.001 | |
| Control group | 23 | 13.1 ± 5.2 | 23 | -0.0 ± 6.5$^{B}$ | 0.994 | |
| **hbA1c** | | | | | | |
| Low-quality diet group | 36 | 36.3 ± 2.2 | 36 | -0.7 ± 2.0 | 0.044 | 0.055 |
| High-quality diet group | 29 | 36.9 ± 2.7 | 29 | -1.4 ± 2.0 | 0.001 | |
| Control group | 23 | 35.4 ± 2.5 | 22 | 0.2 ± 1.3 | 0.505 | |
| **Serum total cholesterol, mmol/L** | | | | | | |
| Low-quality diet group | 36 | 5.8 ± 1.1 | 36 | -0.1 ± 0.8$^{A}$ | 0.324 | 0.013 |
| High-quality diet group | 29 | 5.5 ± 0.8 | 29 | -05 ± 0.6$^{B}$ | <0.001 | |
| Control group | 23 | 5.6 ± 0.9 | 23 | -0.0 ±0.5$^{A}$ | 0.965 | |
| **Serum HDL cholesterol, mmol/L** | | | | | | |
| Low-quality diet group | 36 | 1.3 ± 0.4 | 36 | -0.0 ± 0.2 | 0.095 | 0.469 |
| High-quality diet group | 29 | 1.4 ± 0.4 | 29 | -0.0 ± 0.2 | 0.158 | |
| Control group | 23 | 1.4 ± 0.4 | 23 | -0.0 ± 0.3 | 0.797 | |
| **Plasma triglycerides, mmol/L** | | | | | | |
| Low-quality diet group | 36 | 1.8 ± 0.8 | 36 | -0.2 ± 0.6$^{A}$ | 0.031 | 0.026 |
| High-quality diet group | 29 | 1.7 ± 0.6 | 29 | -0.4 ± 0.6$^{B}$ | 0.001 | |
| Control group | 23 | 1.7 ± 0.7 | 23 | -0.1 ± 0.5$^{A}$ | 0.458 | |
| **Plasma free fatty acids, mmol/L** | | | | | | |
| Low-quality diet group | 36 | 0.42 ± 0.22 | 36 | -0.02 ± 0.20 | 0.633 | 0.527 |
| High-quality diet group | 27 | 0.52 ± 0.17 | 26 | 0.03 ± 0.36 | 0.686 | |
| Control group | 23 | 0.42 ± 0.19 | 23 | 0.04 ± 0.25 | 0.392 | |

Data are presented as mean ±SD. Values noted with an * are log2 transformed for statistical analysis to improve normality. Changes within groups are analyzed using paired t-test, changes between groups are analyzed using general linear model for univariate analysis with baseline values as covariates (ANCOVA). Post hoc analysis were conducted using LSD, different superscript letters (A,B,C) indicate significant post hoc differences ($p$<0.05) between the diet groups. § no differences in gender were found between the groups ($p$ = 0.967), as tested by a chi square test. Abbreviations: BMI: body mass index, SAT: subcutaneous adipose tissue mass (assessed by MRI), VAT: visceral adipose tissue mass (assessed by MRI), IHL: intrahepatic lipid (assessed by $^{1}$H-MRS), HbA1c: glycosylated hemoglobin, HDL: high-density lipoprotein.

# 5

## Fasting induces ANGPTL4 and reduces LPL activity in human adipose tissue

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## ABSTRACT

**Objective:** Studies in mice have shown that the decrease in lipoprotein lipase (LPL) activity in adipose tissue upon fasting is mediated by induction of the inhibitor ANGPTL4. Here, we aimed to validate this concept in humans by determining the effect of a prolonged fast on ANGPTL4 and LPL gene and protein expression in human subcutaneous adipose tissue.

**Methods:** To that end, twenty-three volunteers ate a standardized meal at 18.00h and fasted until 20.00h the next day. Blood was drawn and periumbilical adipose tissue biopsies were collected 2h and 26h after the meal.

**Results:** Consistent with previous mouse data, LPL activity in human adipose tissue was significantly decreased by fasting (-60%), concurrent with increased *ANGPTL4* mRNA (+90%) and decreased *ANGPTL8* mRNA (-94%). ANGPTL4 protein levels in adipose tissue were also significantly increased by fasting (+46%), whereas LPL mRNA and protein levels remained unchanged. In agreement with the adipose tissue data, plasma ANGPTL4 levels increased upon fasting (+100%), whereas plasma ANGPTL8 decreased (-79%). Insulin, levels of which significantly decreased upon fasting, downregulated ANGPTL4 mRNA and protein in primary human adipocytes. By contrast, cortisol, levels of which significantly increased upon fasting, upregulated ANGPTL4 mRNA and protein in primary human adipocytes, as did fatty acids.

**Conclusion:** ANGPTL4 levels in human adipose tissue are increased by fasting, likely via increased plasma cortisol and free fatty acids and decreased plasma insulin, resulting in decreased LPL activity.

This clinical trial was registered with identifier NCT03757767.

## INTRODUCTION

Elevated plasma triglyceride levels are associated with elevated risk of atherosclerotic cardiovascular disease (1-4). Triglycerides are mainly transported in the blood as part of intestine-derived chylomicrons and liver-derived very-low density lipoproteins (VLDLs). The triglycerides in these lipoprotein particles are cleared from the bloodstream through the action of lipoprotein lipase (LPL) (5, 6). Adipocytes and myocytes produce and secrete large amounts of LPL, which is subsequently transported to the luminal side of the capillary endothelium by glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (GPIHBP1) (7-9). As a result, mutations in GPIHBP1 or LPL can lead to severe hypertriglyceridemia. In line with the physiological fluctuations in lipid requirement in various tissues, the activity of LPL is highly variable. For example, LPL activity in adipose tissue is decreased by fasting to reduce lipid storage (10-15). Besides regulation via changes in LPL gene transcription, LPL activity is primarily controlled at the post-translational level (12, 13, 16, 17). Key factors involved in post-translational regulation of LPL include fatty acids, which inhibit LPL via product inhibition (18), and the apolipoproteins C1, C2, C3 and A5. In addition, LPL is regulated by three members of the Angiopoietin-like protein family (ANGPTL): ANGPTL3, ANGPTL4 and ANGPTL8 (19).



The current literature places ANGPTL3, ANGPTL4 and ANGPTL8 at the centre of the physiological partitioning of circulating triglycerides among various metabolic tissues (19, 20). ANGPTL3 is secreted by the liver as a complex with ANGPTL8 and regulates postprandial LPL activity in adipose tissue and muscle in an endocrine fashion (21, 22). Whereas the production of ANGPTL3 is relatively insensitive to feeding and fasting, the synthesis of ANGPTL8 is highly induced by feeding, which is mediated by insulin (23). After feeding, the combined action of ANGPTL3/ANGPTL8 reduces the clearance of plasma triglycerides in brown adipose tissue, heart, and muscle, thereby rerouting plasma triglycerides to white adipose tissue and ensuring the replenishment of triglyceride stores (21, 22, 24, 25). By contrast, ANGPTL4 has emerged as the dominant regulator of LPL activity in the fasted state. Befitting its original name fasting-induced adipose factor (FIAF), *Angptl4* was cloned as a fasting-induced gene in murine adipose tissue and liver (26). Subsequent studies demonstrated that ANGPTL4 inhibits LPL activity and raises plasma triglyceride levels in mice (27-29). Olivecrona found that *Angptl4* mRNA in rat adipose tissue turns over rapidly and that changes in *Angptl4* mRNA expression are inversely correlated to LPL activity, both during the fed-to-fasted and fasted-to-fed transitions (30). Consistent with a predominant role of ANGPTL4 during fasting, transgenic ANGPTL4

overexpression markedly reduces plasma triglyceride clearance in mice in the fasted but not in the fed state, leading to a reduced uptake of TG-derived fatty acids by numerous tissues such as adipose tissue (31). Conversely, ANGPTL4 deficiency in mice is associated with enhanced clearance of plasma triglycerides and uptake of TG-derived fatty acids into adipose tissue in the fasted state (32). Furthermore, the fasting-induced decrease in adipose tissue LPL activity was abolished in *Angptl4-/-* mice, indicating that ANGPTL4 mediates the repression of LPL activity during fasting (14). ANGPTL4 inhibits LPL activity by promoting LPL unfolding via direct protein-protein interactions (33). In mouse adipose tissue, this action of ANGPTL4 triggers LPL cleavage and subsequent degradation (34, 35). The existence of a mechanism regulating LPL degradation/turnover during fasting and requiring the induction of a gene separate from *Lpl* was already suggested prior to the cloning of ANGPTL4 (13, 16).

The predominant role of ANGPTL4 in LPL regulation during fasting is likely at least partly related to the upregulation of ANGPTL4 mRNA and protein levels in mouse adipose tissue by fasting (14, 26, 32, 34, 35). In addition, recent evidence suggests that the inhibitory effect of ANGPTL4 on LPL is counteracted by ANGPTL8, levels of which decrease in adipose tissue during fasting (36). At the whole body level, the upregulation of ANGPTL4 during fasting ensures that triglycerides are directed to non-adipose tissues to be used as fuel rather than being stored. The importance of ANGPTL4 in the regulation of human plasma triglyceride metabolism is supported by human genetic studies, which have shown that carriers of the E40K mutation and other inactivating variants have reduced plasma triglyceride concentrations and reduced risk of coronary artery disease (37, 38). The crucial role of ANGPTL4 in governing plasma lipid levels in mice and humans has made ANGPTL4 an attractive therapeutic target for correcting dyslipidaemia and associated cardiovascular disorders.

While there is overwhelming support for the role of ANGPTL4 as a fasting-induced inhibitor of LPL activity in rodent adipose tissue, evidence on ANGPTL4 in human adipose tissue is lacking. We have previously shown that human plasma ANGPTL4 levels increase with caloric restriction and during extended fasting (39) and that tissue ANGPTL4 and LPL protein levels negatively correlate in a cross-sectional analysis of human adipose tissue samples (40). However, whether fasting influences ANGPTL4 protein levels and LPL activity in human adipose tissue remains unclear. Accordingly, the primary objective of this study is to determine the effect of a prolonged fast on ANGPTL4 gene and protein expression in human subcutaneous adipose tissue. An additional aim is to study the effect of a

prolonged fast on LPL gene expression, LPL protein expression, and on LPL activity in subcutaneous adipose tissue. To characterize the mechanisms for the regulation of ANGPTL4 by fasting in human adipose tissue, we performed in vitro studies using primary human adipocytes.

## MATERIALS AND METHODS

### *FASTING study*

The FASTING study was approved by the Medical Ethics Committee of Wageningen University and registered at ClinicalTrials.gov, identifier: NCT03757767. In short, 24 healthy volunteers aged 40-70 years (median age 55 years) with a BMI of 22-30 kg/m$^2$ (median BMI 25 kg/m$^2$) were asked to consume a standardized meal until full (ad libitum), consisting of 22 energy% protein, 24 energy% fat, 51 energy% carbohydrate and 476 kJ per 100 gram. Two hours after consumption of the standardized meal, blood samples and a subcutaneous adipose tissue biopsy were taken. Twenty-four hours later, a second subcutaneous adipose tissue biopsy was taken and again blood samples were drawn. After consumption of the standardized meal until after the second measurements, subjects were only allowed to drink water. The subcutaneous adipose tissue samples were obtained by needle biopsy from the periumbilical area under local anaesthesia. The samples were rinsed to eliminate blood and were immediately frozen in liquid nitrogen. All samples were stored in aliquots at -80°C.



### *Isolation and differentiation of human stromal vascular fraction*

Anonymous samples of subcutaneous and visceral adipose tissue were collected from the abdominal region of patients undergoing elective cosmetic surgery at the Amsterdam Plastic Surgery, Amsterdam, The Netherlands (subcutaneous) or bariatric surgery for weight management at the Department of Bariatric Surgery, Rijnstate Hospital/Vitalys Clinic, Arnhem, The Netherlands (visceral). All study subjects gave written informed consent for the use of the tissue.

Material was collected in DMEM supplemented with 1% PS and 1% bovine serum albumin (BSA; Sigma-Aldrich). Upon arrival in the lab, the material was minced with scissors immediately and digested in collagenase-containing medium (DMEM with 3.2 mM CaCl2, 1.5 mg/ml collagenase type II (C6885, Sigma-Aldrich), 10% FBS, 0.5% BSA, and 15 mM HEPES) for 45-60 min at 37°C, with occasional vortexing. Cells were filtered through a 100-µm cell strainer (Falcon) to remove remaining cell clumps and lymph nodes. The cell suspension subsequently was centrifuged at 1600 rpm for 10 min and the

pellet was resuspended in erythrocyte lysis buffer (155 mM $NH_4Cl$, 12 mM $NaHCO_3$, 0.1 mM EDTA). Upon incubation for 2 min at room temperature, cells were centrifuged at 1200 rpm for 5 min and the pelleted cells were resuspended in Growth medium (DMEM + 10% FBS + 1% P/S) and plated.

Upon confluence, the stromal vascular fraction (SVF) from subcutaneous origin were differentiated according to the standard protocol for 3T3-L1 cells with addition of 1 µM rosiglitazone (41). Briefly, confluent SVFs were plated in 1:1 surface ratio, and differentiation was induced 2 days afterwards by switching to a differentiation induction cocktail (DMEM containing 10% FBS, 1% P/S, 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, 7 µg/ml insulin and 1 µM rosiglitazone) for 3 days. Subsequently, cells were maintained in Growth medium with addition of 7 µg/mL insulin for 3-6 days and switched to Growth medium only for 3 days, after which experiments have been performed. Average rate of differentiation was at least 80% as determined by eye.

SVFs from visceral origin were differentiated according to the 3D protocol described by Emont *et al.* (42) due to the very low differentiation rate of visceral preadipocytes when differentiated in a 2D well format. Briefly, pre-adipocytes were seeded at a concentration of 300,000 cells/500 µL collagen gel in a 24-well plate format. Pre-adipocytes were resuspended in Growth medium to a concentration of 6x106 cells/mL, of which, per well, 50 µL were mixed with 100 µL 5x DMEM (Biozol, #1-25K34-I), 50 µL FBS and 50 µL 0.1N NaOH and 250 µL collagen solution (Corning, #354249), in this order, to create the 3D gel. After each step the solution was carefully mixed by pipetting up and down. Collagen solution was previously diluted to 8 mg/mL with 0.02N Acetic acid. The 3D gel was allowed to polymerize for 10-20 min in the incubator after which 0.5 mL of Growth medium were added per well. Differentiation was induced the next day according to the protocol described for the differentiation of subcutaneous pre-adipocytes. All cells were maintained in a humidified incubator at 37 ºC with 5% $CO_2$.

***Cell culture treatments***

Treatments of primary cells were done within 2 days after reaching differentiation. Cells were maintained in DMEM containing 10% FBS and 1% P/S until treatment with insulin (500 nM), cortisol (1 µM), dexamethasone (1 µM), or a mixture of oleate and palmitate (2:1, 300 µM total) for 24h. In a separate experiment, primary cells were incubated with 40 µg/mL cycloheximide for indicated

durations. All compounds were from Sigma-Aldrich.

***RNA isolation & Quantitative real-time PCR***

Total RNA from subcutaneous adipose tissue from the FASTING study was isolated using TRIzol reagent (Thermo Fisher Scientific, the Netherlands) and purified using the Qiagen RNeasy Mini kit (Qiagen, the Netherlands). Total RNA from in vitro studies was isolated homogenizing in TRIzol (Thermo Fisher Scientific) either with a Qiagen Tissue Lyser II (Qiagen, Venlo, The Netherlands) (visceral) or by pipetting up and down (subcutaneous). Reverse transcription was performed using the iScript™ cDNA Synthesis Kit (Biorad, the Netherlands) according to the manufacturer's protocol using 400-750 ng RNA for in vitro studies and 350 ng from human adipose tissue. Quantitative PCR amplifications were done on a CFX 384 Bio-Rad thermal cycler (Bio-Rad, the Netherlands) using SensiMix PCR reagents (Bioline, GC Biotech, the Netherlands). Gene expression values were normalized to one of housekeeping genes. Primer sequences of genes are provided in **Supplemental Table 1**.

***Western blots***

Protein lysates were made of subcutaneous adipose tissue from participants included in the FASTING study. The material was lysed in RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium; deoxycholate, 0.1% SDS; Thermo Fisher Scientific, the Netherlands) supplemented with protease and phosphatase inhibitors (Roche, The Netherlands) to make 20% protein lysates. After a 30-minute incubation on ice, the lysates were spun down at 13.000 rpm at 4 °C for 15 min in order to get rid of non-dissolved material and fat. Following the transfer of the infranatant to a clean tube, this procedure was repeated twice to get rid of excess fat. Protein concentrations of lysates were determined with BCA reagent (Thermo Fisher Scientific, the Netherlands). Next, lysates were mixed with 4x LSB loading buffer and denatured at 95 °C for 5 minutes. For each participant, 10 µg of protein was loaded per lane on 26- wells Criterion 8-16% TGX gels (Bio-Rad, the Netherlands) and separated by SDS gel electrophoresis. Separated proteins were transferred to a PVDF membrane by means of a Transblot Turbo System (Bio-Rad, the Netherlands). Primary antibodies (goat anti-human LPL antibody (Santa Cruz Biotechnology, #Y-20) and rabbit anti-human ANGPTL4 antibody (1187) (43) were used at a ratio of 1:1000 (#Y-20) or 1:5000 (1187). Rabbit anti-human GAPDH was used at 1:2000 (Cell signalling, #2118). All primary antibodies were incubated overnight at 4 °C. Corresponding secondary antibodies (HRP-conjugated) (Sigma-Aldrich, the Netherlands) were used at a 1:5000

dilution. All incubations were done in Tris-buffered saline, pH 7.5, with 0.1% Tween-20 (TBS-T) and 5% dry milk, whereas all washing steps were done in TBS-T without dry milk. Blots were visualized using the ChemiDoc MP system and Clarity ECL substrate (Bio-Rad, the Netherlands). Quantification of bands was performed using ImageLab software (Bio-Rad, the Netherlands).

***Quantification of plasma parameters***

Blood samples were collected in EDTA-coated tubes and centrifuged at 4°C for 15 min at 10000 g. Plasma was collected and stored at -80°C. Measurements of plasma levels of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate were performed using kits from WAKO Diagnostics (Cat: 3055 and 417-73501/413-73601, WAKO Diagnostics, Germany) according to the manufacturer's protocol. Glucose, insulin, and triglycerides as well as total-, HDL-, and LDL-cholesterol were determined in lithium heparin plasma samples by hospital Gelderse Vallei, Ede, The Netherlands. Plasma ANGPTL4 concentrations were determined using the ELISA kit from R&D systems (Cat: DY3485, R&D systems, the Netherlands) according to the manufacturer's protocol. Plasma Cortisol concentrations were determined using the ELISA kit from R&D systems (Cat: KGE008B R&D systems, the Netherlands) according to the manufacturer's protocol. Plasma ANGPTL8 concentrations were determined by a sandwich ELISA assay using two monoclonal antibodies: a capture antibody to the N-terminal domain and detection antibody to the C-terminal domain (Hobbs, manuscript in preparation).

***LPL activity measurements***

Frozen subcutaneous adipose tissues biopsies were homogenized in 9 volumes of buffer at pH 8.2 containing 0.025 M ammonia, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail tablets (Complete Mini, Roche Diagnosis, Germany) using a Polytron PT 3000 Homogenizer (Kinematica). The homogenates were centrifuged for 15 min at 10,000 rpm, 4°C. Aliquots of the supernatants were used for determination of LPL activity as previously described using a phospholipid-stabilized emulsion of soy bean triacylglycerols and 3H-oleic acid-labelled triolein with the same composition as Intralipid 10% (Fresenius Kabi, Uppsala, Sweden) (13). The incubation was at 25°C for 100 or 120 min. One milliunit of enzyme activity corresponds to 1 nmol of fatty acids released per min. Enzyme activity is expressed per g wet tissue weight. Protein contents in homogenates of adipose tissue were measured using Markwell's modified Lowry method (44).

### *Statistical analyses*

Differences in plasma parameters, LPL activity, and subcutaneous adipose tissue gene expression between the fed and fasted state were evaluated using a paired Student's t-test. Individuals are always represented by the same colour in the various fed-fasted line graphs. Differences in gene expression in the primary adipocytes were evaluated by unpaired Student's t-test. P-values < 0.05 were considered statistically significant.

## RESULTS

Between October and December 2018, 38 individuals were assessed for eligibility of which 14 were excluded from participation (**Figure 1**). The remaining 24 participants were invited to the research facilities for the FASTING study. One participant dropped out of the study, due to personal reasons that were not related to the study. The remaining 23 participants completed the study.

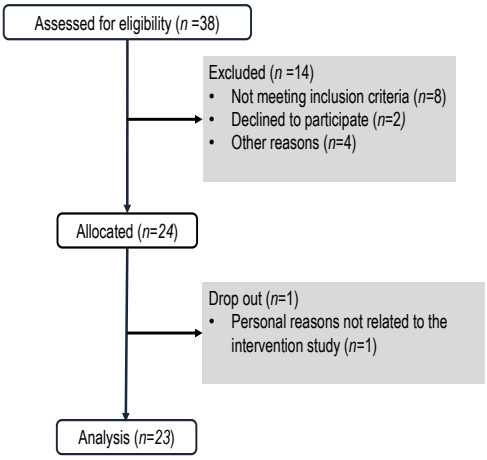


**Figure 1.** Flow chart of the FASTING study.

The main objective of this study was to determine the effect of a prolonged fast on ANGPTL4 gene and protein expression in human subcutaneous adipose tissue, and to link these effects with changes in LPL expression and activity. To that end, 23 healthy middle-aged men and women underwent a 24h fast. Participants received a standardized meal at 18.00h, followed by blood sampling and collection of an adipose tissue biopsy at 20.00h, representing the fed state. At 20.00h the next day, a second blood sample and adipose tissue biopsy were collected. Accordingly, the two blood samples and adipose tissue biopsies were taken at the same time, thereby avoiding the potential influence of circadian rhythmicity. The participant characteristics are listed in **Table 1**.

**Table 1.** Participants characteristics.

| | **Participants (n = 24)** |
|---|---|
| Gender, n males (%) | 8 (33%) |
| Age, years | 43–71 |
| Weight, kg | 76.5 ± 10.5 |
| BMI, kg/m$^2$ | 25.3 ± 2.4 |
| Plasma cholesterol, mM | 5.76 ± 0.96 |
| HDL cholesterol, mM | 1.68 ± 0.32 |
| LDL cholesterol, mM | 3.02 ± 0.84 |

Fasting significantly increased plasma levels of non-esterified fatty acids and β-hydroxybutyrate, and significantly decreased plasma triglycerides (**Figure 2**). The direction of the change is consistent with the known stimulatory effect of fasting on adipose tissue lipolysis and hepatic ketogenesis, and the inhibitory effect on plasma triglyceride secretion. Plasma glucose levels showed a more mixed response with most individuals showing a decrease, while 6 individuals showed an increase (Figure 2). Overall, these parameters confirm compliance to the fasting protocol.

In agreement with data from rats and mice (14), fasting led to a marked decrease in adipose tissue LPL activity (-60%, $p$<0.001) (**Figure 3A**), which was consistently observed in all participants. To determine the potential cause of the decrease in LPL activity, we measured the mRNA levels of *LPL, ANGPTL4* and *ANGPTL8* by qPCR in a subsection of the participants. *LPL* mRNA was slightly lower after the 24h fast but the difference did not reach statistical significance (**Figure 3B**). In most but not all individuals, adipose *ANGPTL4* mRNA levels went up by fasting, with a mean increase of nearly two-fold (+90%, $p$<0.001). By contrast, *ANGPTL8* mRNA levels went down drastically by fasting (-94%, $p$<0.001) (Figure 3B).

To determine if the increase in *ANGPTL4* mRNA by fasting was accompanied by an increase in ANGPTL4 protein, we assessed ANGPTL4 protein levels in the subcutaneous adipose tissue biopsies using Western blot. As shown previously (43), only full length ANGPTL4 was detectable in human adipose tissue, which is in contrast to human liver where we observed substantial N-terminal ANGPTL4 cleavage product (**Supplemental figure 1A**). In most but not all individuals, the level of full length ANGPTL4 protein increased upon fasting (+46%, $p$<0.05). ). Conversely, the mean LPL protein level was lower after fasting, although this decrease did not reach statistical significance (**Figure 4A,B**). To determine if changes in ANGPTL4 mRNA and protein levels were associated with

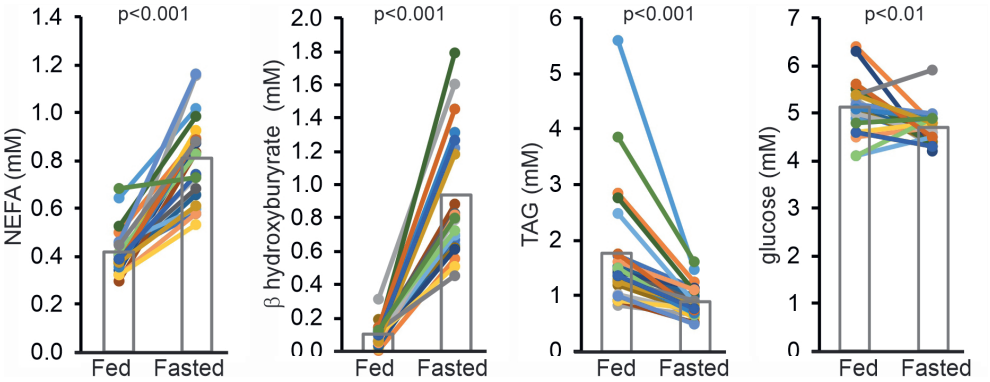


**Figure 2.** Influence of fasting on plasma metabolites. Plasma concentrations of non-esterified fatty acids (NEFA), β-hydroxybutyrate, triacylglycerol (TAG) and glucose after 2h (Fed) and 26h (Fasted) of fasting. Each line represents one individual. Individuals are depicted in the same colour in all figures. Bars represent group means (N = 23).

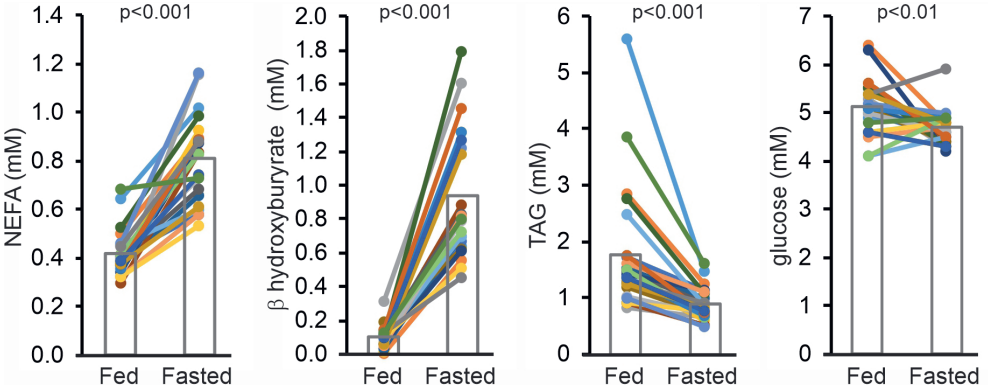


**Figure 3.** Fasting reduces LPL activity and increases *ANGPTL4* mRNA expression in human adipose tissue biopsies. A) LPL activity measured in subcutaneous adipose tissue samples collected after 2h (Fed) and 26h (Fasted) of fasting. Every line represents one individual and bars represent group means (N = 23). B) Relative mRNA levels for *LPL, ANGPTL4* and *ANGPTL8* in subcutaneous adipose tissue biopsies collected after 2h (Fed) and 26h (Fasted) of fasting, as determined by qPCR. Each line represents one individual and bars represent group means (N = 16). Individuals are depicted in the same colour in all figures. The lower number of samples is due to limited availability of biopsy material. Statistical differences were assessed using the paired Student's t-test.

changes in plasma ANGPTL4 levels, we measured the plasma ANGPTL4 concentration before and after fasting using ELISA. In agreement with previous data (39), plasma ANGPTL4 concentrations went up in all participants (+100%, $p<0.001$) (**Figure 4C**). As the ANGPTL4 ELISA only measures full length and C-terminal ANGPTL4 (45), we also determined plasma levels of N-terminal ANGPTL4 by Western blot. Fasting modestly yet significantly induced plasma N-terminal ANGPTL4 levels (+15%, $p<0.05$) (**Figure 4D**). By contrast, plasma ANGPTL8 concentrations were decreased by fasting in all

participants (-79%, $p$<0.001) (**Figure 4E**).

The expression of ANGPTL4 in adipose tissue of mice is known to be under control of various stimuli and transcriptional regulators. For instance, ANGPTL4 expression was previously found to be repressed by insulin (46). In our study, fasting drastically reduced plasma levels of insulin (-95%, $p$<0.001) (**Figure 5A**), suggesting the increase in adipose ANGPTL4 levels during fasting might be related to the decline in plasma insulin. To investigate whether insulin lowers ANGPTL4 expression in vivo, we extracted data from a transcriptomics dataset of adipose tissue biopsies from human subjects before and after 3 hours intravenously maintained euglycemic hyperinsulinemia (47).

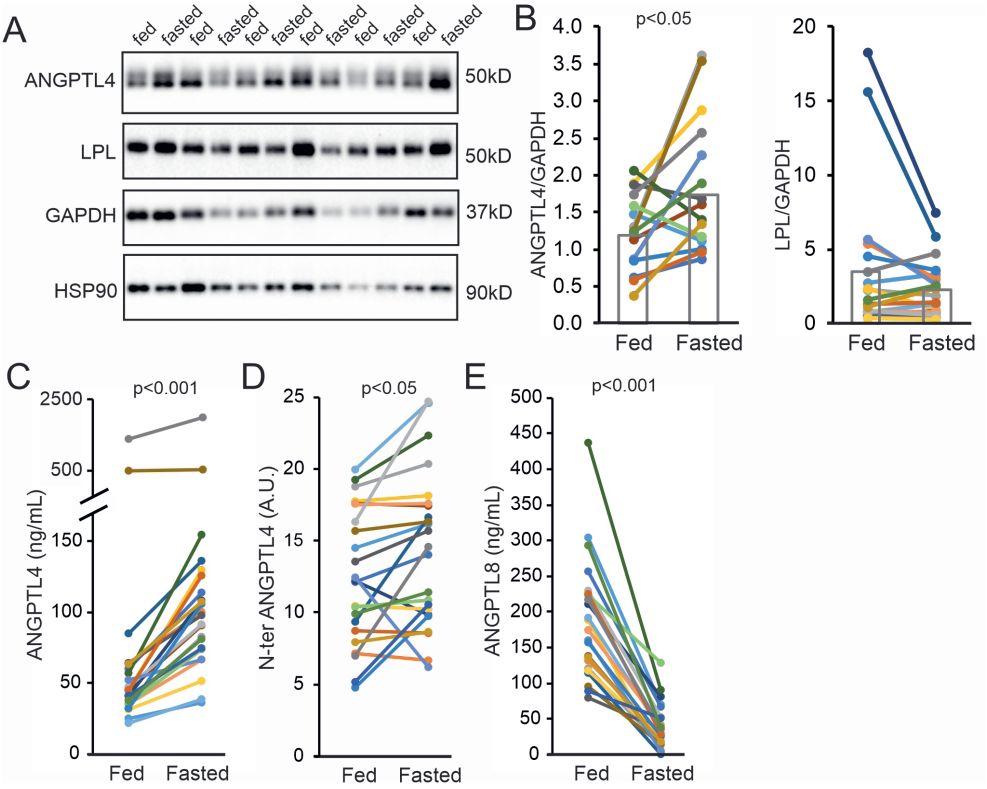


**Figure 4.** Fasting increases ANGPTL4 protein in human adipose tissue and plasma. A) Representative Western blots for ANGPTL4, LPL, GAPDH and HSP90 in subcutaneous adipose tissue samples of 6 individuals collected after 2h (Fed) and 26h (Fasted) of fasting. B) Quantitative analysis of ANGPTL4 and LPL protein levels in subcutaneous adipose tissue normalized to GAPDH. Each line represents one individual and bars representing group means (N = 19 resp. 20. C) Plasma levels of ANGPTL4 after 2h (Fed) and 26h (Fasted) of fasting as determined by ELISA (N = 23). D) Plasma levels of N-terminal ANGPTL4 after 2h (Fed) and 26h (Fasted) of fasting as determined by Western blot (N = 23). E) Plasma levels of ANGPTL8 after 2h (Fed) and 26h (Fasted) of fasting as determined by ELISA (N = 23). Individuals are depicted in the same colour in all figures.

Strikingly, *ANGPTL4* mRNA levels were markedly reduced by insulin in vivo in both insulin-sensitive and resistant individuals (**Figure 5B**). By contrast, *ANGPTL8* mRNA levels went up, especially in the insulin-sensitive individuals (Figure 5B). These data support a possible role of insulin in the regulation of ANGPTL4 expression in human adipose tissue during fasting, but do not indicate whether insulin has a direct role in regulating ANGPTL4 in human adipose tissue. To address this question, we cultured primary human adipocytes. In these cells, full length ANGPTL4 protein was easily detectable by immunoblot, whereas N-terminal ANGPTL4 was absent (**Supplemental figure 1B**). The lack of ANGPTL4 cleavage in cultured human adipocytes is supported by experiments in human Lisa-2 adipocytes (**Supplemental figure 1C**) and SGBS adipocytes (43, 48). Levels of ANGPTL4 protein declined rapidly after treatment with cycloheximide, indicating that ANGPTL4 has a fast turnover in human adipocytes (**Figure 5C, D**).

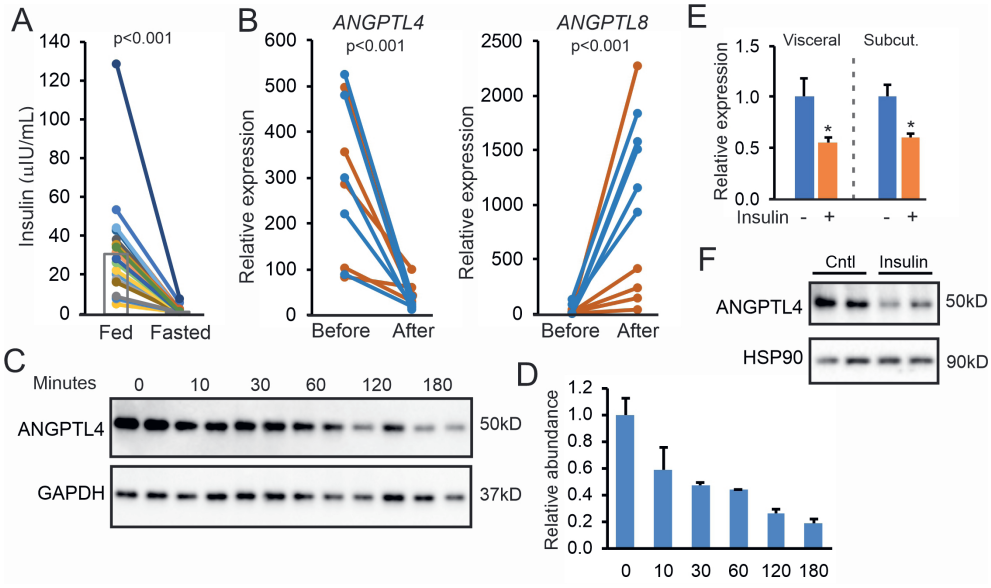


**Figure 5.** Insulin downregulates ANGPTL4 in vivo and in vitro. A) Plasma insulin concentration after 2h (Fed) and 26h (Fasted) of fasting. Each line represents one individual. Individuals are depicted in the same colour in all figures. Bars represent group means (N = 23). B) Adipose tissue mRNA levels of *ANGPTL4* and *ANGPTL8* in insulin-sensitive (blue lines) and insulin-resistant (orange lines) subjects before and after a hyperinsulinemic clamp. Data were extracted from GSE26637. C) Western blot for ANGPTL4 and GAPDH in primary human subcutaneous adipocytes treated with cycloheximide for different durations. D) Quantification of the ANGPTL4 levels relative to GAPDH. E) *ANGPTL4* mRNA in primary human visceral and subcutaneous adipocytes treated with 500 nM insulin for 24h. F) Western blot for ANGPTL4 and HSP90 in primary human visceral adipocytes treated with insulin. HSP90 was blotted as a loading control. Statistical differences for in vitro experiments were assessed using the unpaired Student's t-test. *$p$<0.05, relative to control treatment.

In primary adipocytes from visceral and subcutaneous adipose tissue, insulin significantly reduced *ANGPTL4* mRNA after 24h treatment (**Figure 5E**). In visceral adipocytes, insulin also markedly decreased ANGPTL4 protein levels (**Figure 5F**). In line with data from mouse adipocytes (35, 46), these data indicate the insulin directly suppresses ANGPTL4 gene and protein expression in human adipocytes. Besides insulin, another factor that may be involved in regulating ANGPTL4 levels during fasting is cortisol. Fasting significantly increased plasma cortisol concentrations in the human volunteers (**Figure 6A**). In the primary human adipocytes, cortisol as well as dexamethasone significantly increased ANGPTL4 mRNA and protein levels (**Figure 6B**). Interestingly, free fatty acids, plasma levels of which were elevated during fasting (Figure 2), also increased ANGPTL4 mRNA and protein levels (Figure 6B). These data suggest that the increase in ANGPTL4 production in adipose tissue upon fasting is likely mediated by increased plasma cortisol and free fatty acids, and decreased plasma insulin.

Consistent with the increase in *ANGPTL8* mRNA levels in adipose tissue after insulin infusion, insulin markedly increased *ANGPTL8* mRNA levels in primary human adipocytes (**Figure 6C**). Cortisol and dexamethasone also induced *ANGPTL8* mRNA but to a smaller extent. Intriguingly, *LPL* mRNA levels in the primary human adipocytes were significantly increased by insulin, cortisol, and dexamethasone (Figure 6C).

As ANGPTL4 and ANGPTL8 are regulated by insulin, we hypothesized that *ANGPTL4* and *ANGPTL8* mRNA levels in human adipose tissue may respond to weight loss, which is known to increase insulin sensitivity. To that end, we analysed adipose gene expression data from subjects before and after 5 weeks on a very-low-calorie diet (500 Kcal/day), followed by a 4-week weight maintenance diet based on their individual energy requirements (49). Intriguingly, after 5 weeks of very-low-calorie diet, when subjects were in a hypocaloric state and actively losing weight, *ANGPTL4* and *ANGPTL8* mRNA levels were significantly increased and decreased, respectively. By contrast, after weight loss and in a eucaloric state of weight stability, *ANGPTL4* and *ANGPTL8* mRNA had returned to pre-weight loss values. *LPL* mRNA largely followed *ANGPTL8* mRNA. These data indicate that *ANGPTL4, ANGPTL8,* and *LPL* mRNA in human adipose tissue are not affected by weight loss as such but respond to a negative energy balance.

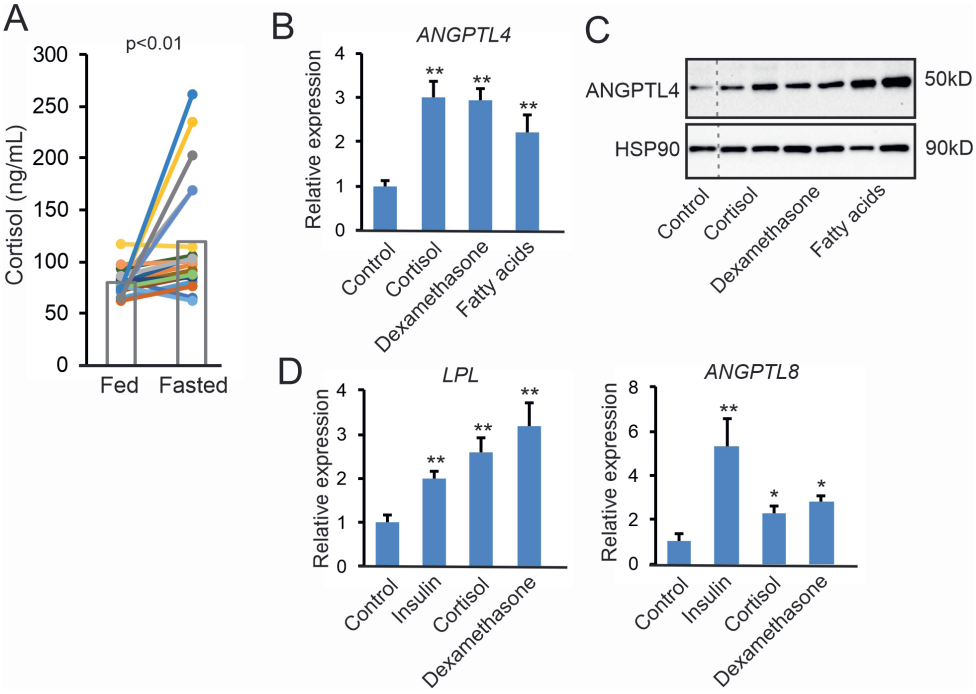


**Figure 6.** Corticosteroids and fatty acids upregulate ANGPTL4 primary human adipocytes. A) Plasma cortisol concentration after 2h (Fed) and 26h (Fasted) of fasting. Each line represents one individual. Individuals are depicted in the same colour in all figures. Bars represent group means (N = 23). *ANGPTL4* mRNA (B) and ANGPTL4 protein levels (C) in primary human visceral adipocytes treated with cortisol (1 µM), dexamethasone (1 µM), or a mixture of oleate and palmitate (2:1, 300 µM total) for 24h. HSP90 was blotted as a loading control. D) *LPL* and *ANGPTL8* mRNA in primary human subcutaneous adipocytes treated with insulin (500 nM), cortisol (1 µM), or dexamethasone (1 µM) for 24h. Statistical differences for in vitro experiments were assessed using the unpaired Student's t-test. *$p$<0.05, **$p$<0.01, ***$p$<0.001, relative to control treatment.

## DISCUSSION

In this paper we show that a 24h fast in human volunteers markedly reduces LPL activity in subcutaneous adipose tissue, concomitant with significant increases in adipose tissue *ANGPTL4* mRNA, adipose tissue ANGPTL4 protein, and plasma ANGPTL4 levels. By contrast, fasting decreases adipose tissue *ANGPTL8* mRNA and plasma ANGPTL8 levels. In cultured human adipocytes, insulin significantly decreased ANGPTL4 mRNA and protein, whereas cortisol and fatty acids had the opposite effect. Inasmuch as plasma insulin levels decrease upon fasting, and plasma cortisol and free fatty acid levels increase upon fasting, the increase in ANGPTL4 production in adipose tissue upon fasting is likely mediated by changes in these factors. Overall, our results strongly support the notion derived from studies in rodents that local upregulation of ANGPTL4 mediates the decrease in LPL activity and

associated lipid storage in adipose tissue during fasting in humans. Consistent with its role as rapidly inducible regulator of LPL activity during fasting, we found that ANGPTL4 protein in human adipocytes turns over rapidly, at a rate that is faster than the turnover rate of LPL protein and activity determined in rat adipose tissue (13, 14).

LPL activity controls plasma triglyceride clearance (6). The activity of LPL is differentially regulated in various tissues in accordance with the local physiological needs for fatty acids. In agreement with observations made in rodents (10-14), studies in human volunteers have shown that LPL activity in adipose tissue is reduced by fasting (50-54), thereby diverting circulating triglycerides to other tissues. An important and previously unaddressed question was whether the fasting-induced decrease in adipose LPL activity in humans is driven by corresponding changes in LPL expression or whether it is mainly due to a post-translational mechanism via ANGPTL4. Biochemical studies combined with studies in mice have shown that ANGPTL4 is upregulated by fasting in mouse adipose tissue (14, 26, 32, 35) and promotes the unfolding of LPL (33), thereby activating the intracellular cleavage and subsequent degradation of LPL (34, 35). The present data indicate that ANGPTL4 is upregulated by fasting in human adipose tissue, concurrent with a marked decrease in LPL activity and a lack of change in *LPL* mRNA. Although the human data are inevitably correlative, they are highly consistent with conservation of the post-translational control of adipose tissue LPL activity during fasting between rodents and humans via ANGPTL4.

Previously, we showed that ANGPTL4 promotes the degradation of LPL in adipose tissue of mice, thereby reducing the amount of LPL available on the capillary surface (34). We also found that ANGPTL4 and LPL protein levels negatively correlate in a cross-sectional analysis of human adipose tissue samples from obese individuals (40). In this study, we observed that the increase in ANGPTL4 protein levels in human adipose tissue upon fasting was not paralleled by a significant decrease in LPL protein. A number of possibilities may explain these findings. First, the method of detecting LPL via immunoblot may not be sufficiently precise to pick up small changes in LPL levels. Here, it should be noted that the mean LPL protein level was lower after fasting but this change did not reach statistical significance. Second, the immunoblot may measure the wrong LPL pool. Here, it would have been useful to be able to distinguish between EndoH-sensitive and EndoH-resistant LPL, which in mouse adipose tissue can be used to differentiate between inactive ER-resident LPL and active LPL in the

Golgi and on the cell surface, respectively. However, we were unable to visualize EndoH-sensitive and EndoH-resistant LPL in human adipose tissue. Hence, it is possible that in human adipose tissue, most of the immunoreactive LPL is inactive and in the ER. Third, the timing of sampling of the adipose tissue biopsies in relation to the meal may not have been optimal. Indeed, in mice, the level of LPL protein in adipose tissue is higher in the refed state than in the ad libitum fed state (34). Fourth, it cannot be excluded that in human adipocytes, ANGPTL4 only unfolds LPL and inhibits LPL activity, but does not regulate LPL degradation and LPL protein levels.

A number of factors may contribute to the upregulation of ANGPTL4 mRNA and protein levels in human adipose tissue during fasting. Expression of *ANGPTL4* in mouse or human adipocytes is known to be regulated via several different signals, including hypoxia (stimulatory), insulin (inhibitory) (35, 46), glucocorticoids (stimulatory) (55), tumour necrosis factor α (stimulatory) (56), and PPARγ agonists (stimulatory) (43). We show that ANGPTL4 levels in human adipocytes are also increased by fatty acids, confirming regulation in other cell types (39, 57). In addition, we find that ANGPTL4 levels in human adipocytes are decreased and increased by insulin and glucocorticoids, respectively. Induction by glucocorticoids is mediated by binding of the glucocorticoid receptor to the 3'-untranslated region of exon 7 (55). Inhibition of *Angptl4* expression by insulin in mouse adipocytes is likely mediated by the PI3K/Foxo1 pathway (46). Overall, the data suggest that the increased ANGPTL4 production in adipose tissue upon fasting is likely mediated by changes in plasma levels of insulin, cortisol, and fatty acids.

Intriguingly, ANGPTL4 was less sensitive to the suppressive effect of insulin in subcutaneous adipocytes than visceral adipocytes. This is in line with our previous finding that *ANGPTL4* mRNA levels are higher in subcutaneous adipose tissue than in visceral adipose tissue (40), which in turn is in agreement with the finding that the LPL activity/mass ratio is lower in subcutaneous than visceral adipose tissue (58). Why insulin less effectively lowers ANGPTL4 in subcutaneous adipocytes is not clear. Clearance of plasma triglycerides is promoted by insulin. Accordingly, the impaired action of insulin in type 2 diabetes leads to reduced plasma triglyceride clearance, which in turn contributes to elevated post-prandial lipid excursions and fasting dyslipidaemia (59). Taking into consideration the repression of adipocyte *ANGPTL4* mRNA by insulin (35, 46), upregulation of ANGPTL4 in insulin resistance may contribute to the postprandial dyslipidaemia in insulin-resistant individuals via inhibition of LPL. In

support, type 2 diabetics present with elevated circulating ANGPTL4 levels (60). Contradicting this scenario, however, the reduction in adipose *ANGPTL4* mRNA during a hyperinsulinemic clamp was similar in insulin-sensitive and resistant individuals. In addition, weight loss, despite an improvement in insulin sensitivity, did not influence *ANGPTL4* mRNA levels in human adipose tissue. Existing data on the relation between insulin resistance and adipose tissue LPL activity are mixed as well. In a group of 26 subjects varying in insulin sensitivity, insulin resistance was negatively correlated with adipose tissue LPL activity (61). Consistent with these data, in type 2 diabetic men, adipose tissue LPL activity was significantly reduced compared to matched non-diabetic subjects, while the differences were more modest in women (62). By contrast, Olivecrona found that the induction of adipose tissue LPL activity with feeding was similar in type 2 diabetes patients and matched healthy controls, suggesting that dysregulation of adipose LPL is not involved in the postprandial hypertriglyceridaemia in type 2 diabetes (63). Overall, these data make it difficult to assign a role for aberrant ANGPTL4 regulation in post-prandial hypertriglyceridaemia in type 2 diabetes.

We found that human adipose tissue and adipocytes only produce full length ANGPTL4. Based on the inability to detect full length ANGPTL4 in human plasma, it could be reasoned that ANGPTL4 produced in adipose tissue does not end up in the circulation, suggesting it has a local role. Alternatively, adipose tissue-derived full length ANGPTL4 may undergo cleavage in the circulation. As the plasma concentration of full length ANGPTL4 is probably very low, the ANGPTL4 ELISA, which is able to detect full length and C-terminal ANGPTL4 but not N-terminal ANGPTL4 (45), in essence measures plasma levels of C-terminal ANGPTL4.

In this paper we find that adipose tissue ANGPTL8 expression is markedly reduced by fasting. Most of the published data relate to the role of ANGPTL8 in the liver, where in the fed state ANGPTL8 forms a complex with ANGPTL3 and supports the inhibition of plasma triglyceride clearance by ANGPTL3 in brown adipose tissue, heart, and muscle, thereby rerouting plasma triglycerides to white adipose tissue for storage (22, 24). Recently, evidence was presented that ANGPTL8, via direct protein interaction, may interfere with the ability of ANGPTL4 to inhibit LPL (36). Presumably, in the fed state, when ANGPTL8 expression is high, ANGPTL8 suppresses ANGPTL4 function, thereby promoting adipose tissue LPL activity. The extent to which adipose tissue contributes to the changes in plasma ANGPTL8 during fasting is unclear. Interestingly, in human adipocytes, *ANGPTL8* mRNA was upregulated by

cortisol and dexamethasone, although to a lesser extent than by insulin. The impact of the induction of ANGPTL8 by glucocorticoids on LPL activity needs further investigation.

This paper has limitations. First, our study cannot demonstrate a direct causal link between the upregulation of ANGPTL4 in human adipose tissue during fasting and the decrease in LPL activity. Nevertheless, the plethora of pre-clinical data combined with our data strongly suggest that upregulation of ANGPTL4, and possibly the downregulation of ANGPTL8, causes the decrease in adipose tissue LPL activity in humans during fasting. Second, we were unable to visualize LPL protein in the human primary adipocytes. For reasons that are unclear, LPL is very hard to detect in primary adipocytes compared to adipose tissue, and its migration is dubious.

In conclusion, our data support the notion that upregulation of ANGPTL4 mediates the decrease in LPL activity and associated lipid storage in adipose tissue during fasting in humans. The increase in ANGPTL4 production in human adipose tissue by fasting is likely mediated by increased plasma cortisol and free fatty acids, and decreased plasma insulin.



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## CONFILCT OF INTEREST

There is no conflict of interest.

## AUTHOR CONTRIBUTIONS

*P.M.M.R.* Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original draft, Writing – Review & Editing, Visualization, Supervision; *C.C.J.R.M.* Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – Review & Editing, Visualization; *E.J.H.* Resources, Writing – Review & Editing; *A.P.* Resources, Writing – Review &

Editing; *G.O.* Resources, Writing – Review & Editing; *L.A.A.* Conceptualization, Writing – Review & Editing, Supervision, Project administration, Funding acquisition; *S.K.* Conceptualization, Formal analysis, Writing – Original draft, Writing – Review & Editing, Visualization, Supervision, Project administration, Funding acquisition

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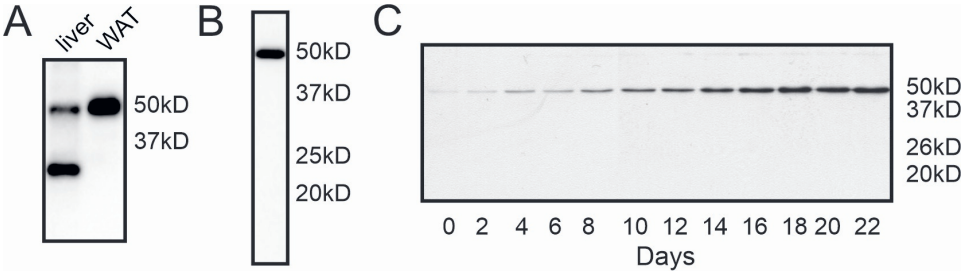
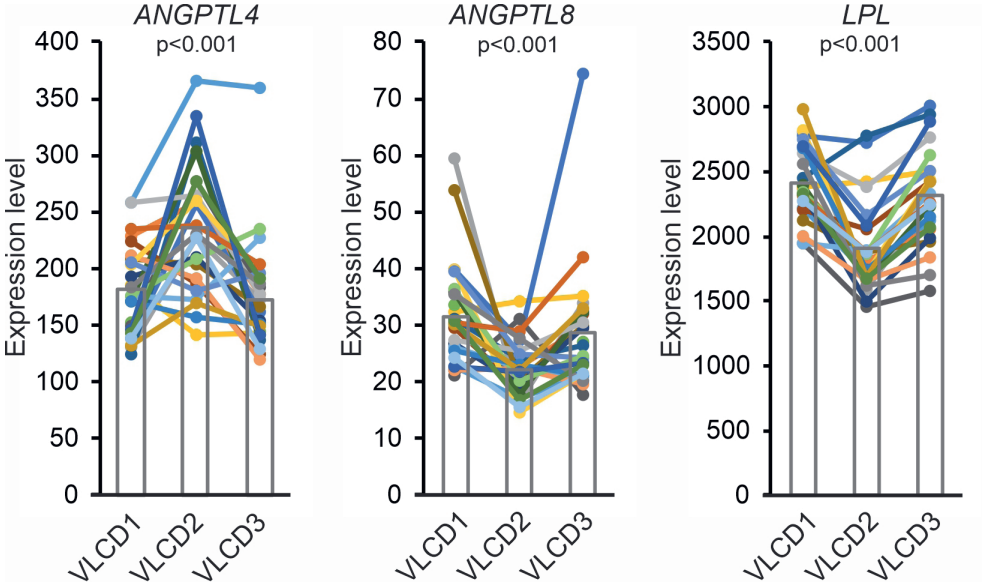
## LIST OF ABBREVIATIONS

| Abbreviation | Full description |
|---|---|
| ANGPTL4 | Angiopoietin-like 4 |
| ANGPTL8 | Angiopoietin-like 8 |
| SVF | Stromal Vascular Fraction |
| WAT | White Adipose Tissue |

## SUPPLEMENTAL MATERIAL




**Supplemental figure 1.** ANGPTL4 is produced in human adipose tissue as full length protein. A) Immunoblot for ANGPTL4 of human liver and human subcutaneous adipose tissue. B) Immunoblot for ANGPTL4 of human primary adipocytes. C) Immunoblot for ANGPTL4 during adipogenic differentiation of human Lisa-2 adipocytes [1].




**Supplemental figure 1.** Adipose tissue mRNA levels of *ANGPTL4, ANGPTL8* and *LPL* are altered during severe hypocaloric diet but are unaffected by weight loss per se. Microarray-based gene expression of *ANGPTL4, ANGPTL8* and *LPL* in adipose tissue of subjects before weight loss (VLCD1), after 5 weeks of very low calorie diet (500 Kcal/day, VLCD2), and after 4 weeks of weight maintenance (VLCD3) (GSE77962) [2].

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**Supplemental Table 1.** Primer sequences used for qPCR in this study.

| Gene | Forward primer | Reverse Primer |
|---|---|---|
| *ANGPTL4* | CACAGCCTGCAGACACAACTC | GGAGGCCAAACTGGCTTTGC |
| *ANGPTL8* | CAGAAGGTGCTACGGGACAG | AAATTCTCGGTAGGCAGGGC |
| *LPL* | CATTCCCGGAGTAGCAGAGT | GGCCACAAGTTTTGGCACC |
| *BACTIN* | AGAAAATCTGGCACCACACC | AGAGGCGTACAGGGATAGCA |

# 6

# Biomarkers of food intake for cocoa and liquorice (products): A systematic review

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## ABSTRACT

**Background:** To unravel true links between diet and health, it is important that dietary exposure is accurately measured. Currently, mainly self-reporting methods (e.g. food frequency questionnaires and 24h recalls) are used to assess food intake in epidemiological studies. However, these traditional instruments are subjective measures and contain well-known biases. Especially estimating the intake of the group of confectionary products, such as products containing cocoa and liquorice, remains a challenge. The use biomarkers of food intake (BFIs) may provide a more objective measurement. However, an overview of current candidate biomarkers and their validity is missing for both cocoa and liquorice containing foods.

**Objective:** The purpose of the current study was to (1) identify currently described candidate BFIs for cocoa (products), and liquorice, (2) to evaluate the validity of these identified candidate BFIs, and (3) to address further validation and/or identification work to be done.

**Methods:** This systematic review was based on a comprehensive literature search of three databases (PubMed, Scopus and ISI web of Science), to identify candidate BFIs. Via a second search step in the Human Metabolome Database (HMDB), the Food Database (FooDB) and phenol-explorer, the specificity of the candidate BFIs was evaluated, followed by an evaluation of the validity of the specific candidate BFIs, via pre-defined criteria.

**Results:** In total 37 papers were included for cocoa and 8 papers for liquorice. For cocoa 164 unique candidate BFIs were obtained, and for liquorice four were identified in total. Despite the high number of identified BFIs for cocoa, none of the metabolites was specific. Therefore, the validity of these compounds was not further examined. For liquorice intake, 18-glycyrrhetinic acid (18-GA) was found to have the highest assumed validity.

**Conclusions:** For cocoa specific BFIs were missing, mainly because the individual BFIs were also found in foods having a similar composition, such as tea (polyphenols), or coffee (caffeine). However, a combination of individual BFIs might lead to discriminating profiles between cocoa (products) and foods with a similar composition. Therefore, studies directly comparing the consumption of cocoa to these similar products are needed, enabling efforts to find a unique profile per product. For liquorice, we identified 18-GA as a promising BFI, however important information on its validity is missing, thus more research is necessary. Our findings indicate a need for more studies to determine acceptable BFIs for both cocoa and liquorice.

## INTRODUCTION

Several epidemiological studies have observed relationships between habitual intake of cocoa (products), liquorice and health. Beneficial effects associated to the consumption of cocoa and cocoa products intake include a positive association with flow-mediated vasodilatation, and inverse associations with blood pressure, serum insulin, HOMA-IR, calcified atherosclerotic plaques in the coronary arteries, incident cardiovascular diseases, cardiac mortality, cardiovascular mortality, and all-cause mortality (1-6). Liquorice has been widely exploited for medicinal purposes including its use as remedy in case of sore throat or cough (7, 8). However, even though beneficial effects have been reported for both the consumption of cocoa and liquorice containing products, many of these products are often energy-dense foods, high in sugar and fats. Therefore, intake of these products in high amounts is not recommended, as it is associated with obesity and related diseases (9, 10). Also, contraindications for the use of liquorice in large amounts have been reported in specific conditions, especially during pregnancy and in patients with hypertension, hypokalaemia, or with hepatic or kidney failure (11, 12).

To unravel true links between diet and health in epidemiology studies, it is essential to accurately assess dietary exposure. Currently, the use of self-reporting methods such as 24h-dietary recalls, food diaries, and food-frequency questionnaires are the most frequently used instruments to assess food exposure in epidemiological studies. However, these conventional methods are subjective measures, they contain well-known biases, such as reporting and recall biases (13, 14), and do not take into account individual characteristics and differential metabolic responses to the intake of different food components and food bioactives. Therefore, the observed relationships between nutrition and health could have been affected, thereby possibly leading to inconsistencies in the field of nutritional research. In particular, accurate estimation of the intake of cocoa and liquorice containing products is difficult. This is partly due to the fact that the moments on which these types of foods are consumed are often not planned. Furthermore, people have difficulties determining accurate portion sizes, they might underreport their intake of these types of food as they are believed to be unhealthy, and they might not be able to recall all the foods that contain these types of compounds. Hence, there is an urgent need for more accurate measurements of food intake, especially for cocoa and liquorice containing foods. As a result of the application of metabolomics in the nutrition field, candidate biomarkers of food intake (BFIs) are increasingly described in the literature (15). BFIs are objective measures of actual food

intake, and as such could be used in conjunction with the conventional methods, to improve the quality of dietary assessment in nutritional science, and to assist in examining true associations between nutrition and health (16). In the literature many different biomarker classifications schemes exist (17, 18). In this manuscript we defined BFIs according to the definition proposed by Gao *et al* (18). In short, we included all biomarkers described in the literature after the intake of cocoa products, liquorice products, or their components, that can be used to estimate recent or average intakes of these entities. Since cocoa and cocoa products are consumed all over the world, the number of potential BFIs for cocoa (products) is rapidly rising in the literature. Liquorice is also widely consumed, especially in Europe, and can be used as an ingredient in different food products. However, potential BFIs for the consumption of liquorice (products) are less well explored (19). Currently, three approaches are used for BFI identification, namely: (1) acute or chronic intervention studies where metabolic profiles are examined after a specific load of the food of interest, (2) dietary pattern studies where metabolic profiles are examined after subjects adhere to a certain dietary pattern, and (3) observational studies where metabolic profiles are compared between consumers and non-consumers of a specific food of interest (20, 21). To examine the validity of a candidate BFI, it is important that the BFI has been evaluated using all of these approaches. An overview of already identified candidate BFIs for cocoa (products) and liquorice, as well as an evaluation of their validity, is needed to identify known and accepted BFIs, as well as to identify what information is still missing and requires further investigation. Hence, the aims of this systematic review were (1) to identify currently described candidate BFIs for cocoa (products), and liquorice in the literature, (2) to evaluate the validity of these identified candidate BFIs, and (3) to address further identification and/or validation work to be done. This systematic review is performed in the frame of the FoodBAll (Food Biomarkers Alliance) project under the Joint Programming Initiative - A Healthy Diet for a Healthy Life (JPI-HDHL) (http://www.foodmetabolome.org/).

## MATERIALS AND METHODS

### *Identification of biomarkers*

In order to identify papers on BFIs for both cocoa and liquorice, we carried out an extensive literature search following the Biomarker of Food Intake Reviews (BFIRev) methodology proposed previously (22). In short, for this systematic review all elements of the PRISMA statement (23) relevant for a

literature search on biomarkers were used. Original papers and reviews were searched in Scopus, Pubmed and ISI Web of Knowledge, using the grouped search terms: (biomarker* OR marker* OR metabolite* OR biokinetics OR kinetic* OR biotransformation) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (trial OR experiment OR study OR intervention) AND (urine OR plasma OR serum OR blood OR excretion) AND (intake OR meal OR diet OR ingestion OR consumption OR eating OR drink* OR administration). For the BFI for cocoa; AND (cocoa* OR chocolate* OR cacao* OR Theobroma) was added, and for the BFI for liquorice; AND (liquorice OR liquorice) was added. Three independent reviewers (CCJRM, EAA, and EMB-B) selected the papers in a process outlined in figure 1. Only English papers were included, and no restriction was applied for the publication dates (searches for cocoa and liquorice biomarkers were done up to October 2016 and March 2017, respectively). Papers describing the effect on physiology, bio stability, and/or drug metabolism were excluded. Furthermore, we excluded papers that examined chocolate products without cocoa (for example white chocolate), and liquorice products without liquorice root extract (for example red liquorice). Animals studies were also excluded, since it remains to be determined whether animal models are valid models for examining the absorption, distribution, metabolism and excretion of compounds in humans (24). Initially, only titles and abstracts were screened to determine if they satisfied the selection criteria. Any disagreements between the reviewers were resolved through consultation. Next, full text papers were retrieved for the selected titles. Additional papers were identified from reference lists of the retrieved papers, and from the selected book chapters or reviews, called hand searches. Again, three independent reviewers (CCJRM, EAA, and EMB-B) assessed the obtained papers to ensure that they were in agreement with the inclusion criteria. A data collection form was designed to streamline the process of extracting relevant information from the selected studies. This form contained the following items: dietary factor, dose of intervention, study design, number of subjects, analytical method, sample type, discriminating metabolites (BFIs), notes, and primary reference(s).



***Specificity evaluation of the identified biomarkers***

To evaluate the apparent specificity of each of the identified candidate BFI, a second search step consisting of two parts was performed. Firstly, the compound databases HMDB (http://www.hmdb.ca/), FooDB (http://foodb.ca/), and Phenol-explorer (http://phenol-explorer.eu/) were used to screen

the identified biomarkers of food intake. If a compound was found to be present in non-cocoa or non-liquorice related foods, it was removed from the selection. Secondly, an additional search was performed for the remaining selection, using combinations of the grouped search terms ("the name and synonyms of the compound") AND (biomarker* OR marker* OR metabolite* OR biokinetics OR biotransformation) AND (trial OR experiment OR study OR intervention) AND (human* OR men OR women OR patient* OR volunteer* OR participant*) AND (urine OR plasma OR serum OR blood OR excretion) AND (intake OR meal OR diet OR ingestion OR consumption OR eating OR drink* OR administration) in any of the listed databases above, or Google Scholar. If the compound was found to be present in non-cocoa or non-liquorice related foods in this second search, it was removed from the selection.

***Validity of the identified biomarkers***

For the final selection of identified BFIs the validity was assessed. For this, we have used the method proposed by Dragsted *et al* (25). An elaborate explanation on how the validity was assessed, including the pre-defined criteria, how the validity questions were constructed, and a discussion on the strengths and weaknesses of this method, can be found in the paper the paper of Dragsted and colleagues (25). In short, the validity was assessed via answering nine questions, where possible answers were: Yes (Y), No (N), or unknown/uncertain (U) where appropriate. If Y was answered to a question, this would increase the assumed validity of that specific biomarker. The questions taken into consideration were related to current knowledge about biological, analytical and nutritional aspects of the methodology, and are based on a thorough search of previous literature. The questions were as follows: (1) Is the marker compound plausible as a specific BFI for the food or food group (chemical/biological plausibility)? (2) Is there a dose-response relationship at relevant intake levels of the targeted food (quantitative aspect)? (3) Is the single-meal time-response relationship described adequately to make a wise choice of sample type and time window (single-dose kinetics)? (4) Is the biomarker kinetics for repeated intakes of the food/food group described adequately providing the frequency of sampling needed to assess habitual intake (e.g. the cumulative aspects: does the biomarker accumulate in the body over time after repeated intakes? (5) Has the marker been shown to be robust after intake of complex meals reflecting dietary habits of the targeted population (robustness)? (6) Has the marker been shown to compare well with other markers or questionnaire data for the same food/food group (reliability)? (7) Is the marker chemically and biologically stable during bio specimen collection and

storage, making measurements reliable and feasible? (8) Are analytical variability (CV%), accuracy, sensitivity and specificity known as adequate for at least one reported analytical method? (9) Has the analysis been successfully reproduced in another laboratory (reproducibility)? In the end the number of times a Y was given per biomarker was added, in order to get insight in the validity of a selected biomarker. The higher this number, the more is known about the compound, the higher its assumed validity. This score will therefore reflect the current level of validity of that particular compound, and pinpoints what additional research is needed to increase the validity of that particular compound.

## RESULTS

### *Candidate BFIs, identification*

*Cocoa (products) BFIs*

A total of 414 potentially relevant papers were identified from searches in PubMed, Web of Science and Scopus. After a first screening of the title and abstract, 55 papers were collected as full text and assessed for further inclusion. Then, 20 papers were excluded due to either inappropriate study designs (e.g. animal studies), unreported cocoa or chocolate intakes, or unreported metabolites/markers. Finally, two additional papers were identified via hand searches and added to the list, which lead to a total inclusion of 37 papers (**Figure 1**). Intervention studies (n=34) were the most frequently employed methods to determine candidate BFIs for cocoa (products) (**Supplemental Table 1**). In these intervention studies the participants consumed: cocoa-based beverages (n=20), chocolate (n=8), a mixture of chocolate and cocoa beverages (n=2), cocoa-based nut cream and/or polyphenol capsules (n=2), and cocoa extract as part of a ready-to-eat meal (n=1). Of the 34 intervention studies, there were 16 acute crossover studies, 12 acute single dose studies, 3 crossover intervention studies (ranging from 4 to 6 weeks), 3 parallel intervention studies (ranging from 5 days to 6 months), and 2 single arm intervention studies (4 and 12 weeks). Only 2 observational studies (26, 27) using estimated self-reported dietary intake data, reported candidate BFIs for cocoa and cocoa-products consumption. The most commonly used bio samples were urine (n=26) and plasma (n=18). Urine was collected mainly as 24 hours urine (n=16), but also as spot urine (n=7), as 8 hours urine (n=1), and as 72 hours urine (n=2). Number of subjects ranged from 1 up to 59 in the intervention studies, and up to 481 for the cross-sectional study. Furthermore, 28 studies used a targeted approach to determine candidate BFIs, and 10 studies used an untargeted approach, by which more compounds could be identified.

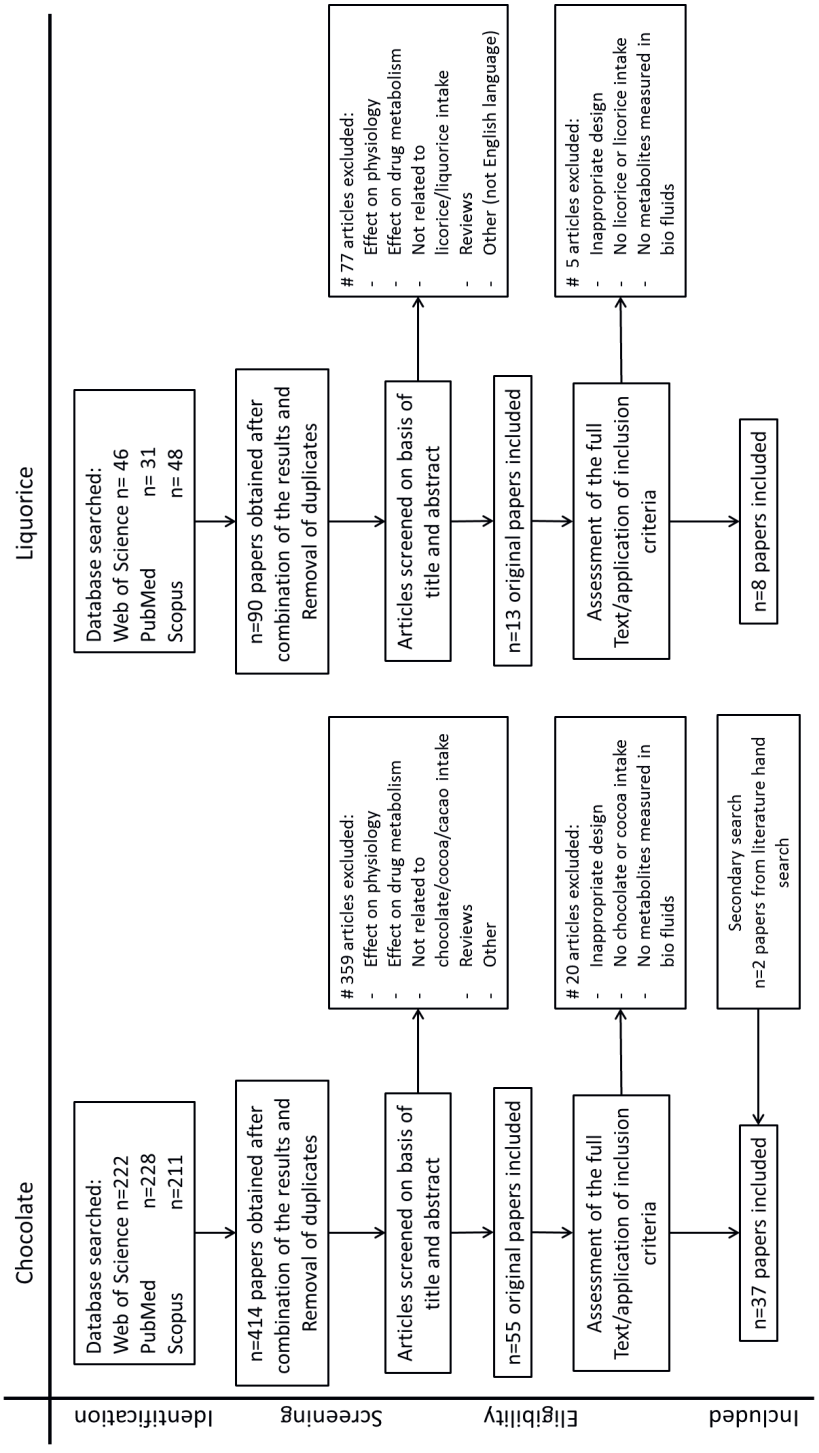


**Figure 1.** Flowchart selection of papers for biomarkers of chocolate and liquorice intake. Searches for cocoa and liquorice biomarkers were done up to October 2016 and March 2017, respectively.

Among the selected papers a total of 164 different compounds were found as candidate BFIs for cocoa (products) intake (Supplementary Table 1). (±)-Catechin and (-)-epicatechin derivatives were by far the group of metabolites most reported after cocoa intake, followed by hydroxyphenylvalerolactones, hydroxyphenylvaleric acids, and methylxanthines.

*Liquorice (products) BFIs*

A total of 90 potentially relevant papers were identified from searches in PubMed, Web of Science and Scopus. After a first screening of the title and the abstract, 13 papers were collected as full text and assessed for further inclusion. Then, 5 papers were excluded due to either inappropriate study designs (animal studies e.g.), unreported liquorice intakes, or unreported metabolites/markers. No additional papers were obtained via hand searches, leading to a total inclusion of 8 papers (Figure 1). All the included studies were intervention studies to determine candidate BFIs for liquorice (products) (**Table 1**). In these intervention studies the participants consumed: isolated compounds of liquorice (n=4), solid liquorice (n=2), liquid liquorice (n=1), or a mixture of consuming a compound or solid liquorice (n=1). In the eight papers, there were four acute single dose studies, two acute crossover studies, two parallel single dose studies, two parallel placebo controlled intervention studies (1 and 4 weeks), and one parallel intervention study (5 days). No observational study using estimated self-reported dietary intake data reported candidate BFIs liquorice (product) consumption, nor were there any longer term studies performed, as the longest study was four weeks. Again, plasma and urine were the most commonly used bio samples, n=7 and n=4 respectively. Multiple spot urine collections were used in two studies, prolonged collection of urine (for 4 and 5 days) were used in two other studies, and one study collected 24 hour urine. The number of subjects ranged from 1 up to 60. Furthermore, all studies used a targeted approach to determine candidate BFIs. Among the selected papers a total of 4 different compounds were found as candidate BFIs for liquorice (products) intake (Table 1), namely: 18-glycyrrhetinic acid (18-GA), 18-GA glucuronides, 3β-monoglucuronyl-18β-glycyrrhetinic acid (3-MGA), and glabridin.

***Specificity and validity of the identified BFIs***

*Cocoa (products) BFIs*

The specificity of each of the identified candidate BFI was evaluated via a second search step. First, the 164 candidate BFIs were screened for specificity for cocoa or cocoa products in the compound

**Table 1.** List of reported candidate liquorice biomarkers of intake, including information about dosage, study design, number of subjects, method used, sample type and the primary references. *Continues on the next page.*

| Dietary factor | | Dose of intervention | Comparable to # grams of liquorice containing 0.17% GL[1] | Study design | # subjects (#men) | Age (y) |
|---|---|---|---|---|---|---|
| **Liquorice (products)** | | | | | | |
| Solid | Katzen Kinder by Katjes (0.05% GL) | 200 grams | 135 | Acute single dose study | 4 (1) | 26-29 |
| | Solid liquorice (0.23% GL) | 50, 100, 200 g daily for 5 days | 68, 135, and 271 | Parallel intervention study (5 days) | 3 (0) | 19-23 |
| | | 500 g within 4 hours | 676 | Acute single dose study | 1 (1) | 22 |
| Isolated compounds | Glycyrrhizin (GL) | 600 mg in 2dl water (excretion study) | 353 | Acute single dose study | 6 (5) | 24-40 |
| | Glycyrrhetinic acid (GA) | 500 mg | 509[3] | Acute single dose study | 10 (10) | 24-38 |
| | Liquorice extract (LE) vs. glycyrrhizin | 21 gram LE vs. 1600 mg GL | 941 | Acute crossover study | 16(8) | U |
| | Glycyrrhetinic acid | 500, 1000, 1500 mg with water | 294, 588, and 882 | Parallel single dose study | 6 (6) | 27-31 |
| Liquid | Liquorice flavonoid oil (LFO: 90% MCT, 8% polyphenols, 1% glabridin) | 300 mg, 600 mg, and 1200 mg of LFO versus placebo | - | Parallel single dose study<br>Parallel placebo controlled intervention study (1wk)<br>Parallel placebo controlled intervention study (4wk) | 15 (15)<br>42 (21)<br>60 (30) | 20-60 |
| Mixture | Glycyrrhetic acid, Glycyrrhizin, and Solid liquorice (0.15% GL) | 130 mg 18-GA[2] in water-propyleneglycol<br>225 mg GL in water<br>150 g sweet liquorice<br>150 g salted liquorice | 132 | 4-way random acute crossover study | 16 (8) | U |

*[1] Calculated based on the report of the European Scientific Committee on Food, reporting a mean glycyrrhizin content in liquorice confectionery of 0.17% (96). This is not calculated for LE and LFO, as it is unclear what the exact percentages of these compounds are in liquorice itself [2]If BMI was reported in the article, otherwise only height and/or weight were reported. If nothing was reported at all we have written down: U [3]Amount GA was first converted to amount GL, as 130 mg GA is equivalent to 225 mg GL, according to (50) *HMDB-ID is only reported once for the same candidate biomarker of food intake. Abbreviations: 18-GA, 18(β)-glycyrrhetic acid or 18(β)-glycyrrhetinic acid; 3-MGA, 3β-monoglucuronyl-18β-*

| Dietary factor | | BMI range[2] (kg/m$^2$) | Analytical method | Approach | Sample type and Time | Candidate Biomarkers of food intake | HMDB ID* | Ref. |
|---|---|---|---|---|---|---|---|---|
| **Liquorice (products)** | | | | | | | | |
| Solid | Katzen Kinder by Katjes (0.05% GL) | 18.6-28.6 | LC-MS/MS (LC-ESI-MS) | Targeted | Blood (0, 0:05, 1,2,3,5 and 7h) Urine (regularly) | Blood: 18-GA (highest after 6h; 150 – 434 ng/ml) Urine: - | HMDB 0011628 | (53) |
| | Solid liquorice (0.23% GL) | U<br>U | GC-MS | Targeted | Urine (all up to 5 days) | Urine: 18-GA | | (52) |
| Isolated compounds | Glycyrrhizin (GL) | U | HPLC-UV | Targeted | Urine (all up to 4 days) | 18-GA<br>3-MGA (Cmax 0.49-2.69 ug/ml) | HMDB 0037827 | (56) |
| | Glycyrrhetinic acid (GA) | U | LC analyser | Targeted | Serum (0, 2, 4, 7, 10, 24h)<br>Urine (0, 2, 4, 7, 10, 24h) | Serum: 18-GA (max after 2-4h 13.4 umol/l)<br>Urine: - | | (54) |
| | Liquorice extract (LE) vs. glycyrrhizin | U | HPLC-UV | Targeted | Plasma (up to 36h) | 18-GA (both after LE and GL consumption) | | (55) |
| | Glycyrrhetinic acid | H: 176-180 cm W: 65-73 kg | HPLC | Targeted | Plasma (-0.5h, every 30 min up to 8h, 9, 10, and 12h; 1000mg additionally at 14, 24h; 1500mg additionally at 48h) Urine (24h) | Plasma: 18-GA (Cmax; 500, 4.5 mg/l; 1000, 7 mg/l; 1500, 9 mg/l, tmax 3-4h)<br>Urine: 18-GA, and 18-GA glucuronides (only at 1500 mg dose) | 18-GA glucuronides: not in HMDB | (51) |
| Liquid | Liquorice flavonoid oil (LFO: 90% MCT, 8% polyphenols, 1% glabridin) | BW >50 kg men >40 kg women | SPE-LC-MS/MS | Targeted | Plasma (0, 2,4,6,8, 24h) Plasma (0, 4, 24h on day1 and day7) Plasma (0h on day1, day14, day28) | glabridin (Cmax at 4h, around 0.8-2.1 ng/ml depending on the dose) | HMDB 0034188 | (57) |
| Mixture | Glycyrrhetic acid, Glycyrrhizin, and Solid liquorice (0.15% GL) | U | HPLC | Targeted | Plasma (-1, 2, 2.5, 4, 5.5, 7, 8.5, 10, 11.5, 13, 14.5, 18, 22, 32, 48 and 56h) | 18-GA (Cmax about 1000 ug/L, same after salty or sweet tasting liquorice) | | (50) |

*glycyrrhetinic acid; BW, Body Weight; GC-MS, Gas Chromatography - Mass spectrometry; GL, glycyrrhizin; H, height; HPLC, High-performance liquid chromatography; LC-ESI-MS liquid chromatography-electrospray ionization-tandem mass spectrometry; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; LE = Liquorice extract; LFO, Liquorice flavonoid oil; Ref., Reference; SPE-LC-MS/MS, solid phase extraction liquid chromatography tandem mass spectrometry; U, Unkown: values not reported in the article; UV, Ultraviolet; W, weight.*

databases HMDB, FooDB, and Phenol-explorer. Based on presence in other foods, 63 markers were removed from the selection. Three turned out to have an unclear formulation (e.g. missing information on the place of side chains) and were therefore excluded. Based on the search results in the compound databases the following metabolites were removed because of their presence in other food; epicatechins, catechins, and vanillic acid. Important to note is that most of their biotransformation products (e.g. glucuronides and sulfates), a total of 27 metabolites, were not found in the compound databases. As it is known that these biotransformation products are all related to epicatechins, catechins or vannilic acid, they were also removed from the selection. Furthermore, 11 metabolites of caffeine (theobromine, paraxantine, theophylline, and their biotransformation products) were removed from the selection, since these metabolites are produced in the gut microbiota upon intake of food products containing caffeine, such as coffee, tea, or cola (source: HMDB), and they were therefore suspected not to be specific BFIs for chocolate. The specificity of the remaining 61 candidate BFIs was further examined by performing additional searches in Scopus, ISI Web of Science, PubMed or Google Scholar, following the syntax explained in methods section. Hydroxyphenylvaleric acids and hydroxyphenylvalerolactones were found to be microbial derived metabolites from polyphenol intake (flavan-3-ols, flavonols and flavanones) (28-30), and were therefore suspected not to be specific BFIs for chocolate intake, but markers for all polyphenol-containing foods, such as almonds or tea (28) (removal of 30 metabolites). N-phenylpropenoyl-L-amino acids, are known to be particularly common in cocoa, but also in coffee and other plant-based foods such as red clover (31, 32), and were therefore removed from the selection (removal of 13 metabolites). Furthermore, a ketone body (3-hydroxybutyrate) and other endogenous metabolites (tyrosine sulfate, N-methylguanine, methylglutarylcarnitine, guanidinoacetate) were removed from the selection (27, 33-35). And lastly, metabolites related to protein intake (xanthurenic acid, indoxyl-sulfate, 4-cresol sulfate (36, 37)), vegetable intake (phenylacetylglutamine (38, 39)), coffee intake (furolglycine (40)), tea intake (cyclo(Ser-Tyr), cyclo(Pro-Pro)(41)), beer intake (cyclo(Propylalanyl), cyclo(Pro-Pro)(42)), aspartame intake (cyclo(Aspartyl-Phenylalanyl)(43)), nicotinic acid metabolism (hydroxynicotinic acid (44)), and food packaging (di-iso-nonyl phthalate, di-(2-ethylhexyl)phthalate (45, 46)) were also removed from the selection. This meant that none of the currently identified BFIs for cocoa (products) made the final selection, and therefore the validity was not checked for any of the candidate BFIs.

*Liquorice (products) BFIs*

The specificity of each of the identified candidate BFI for liquorice (products) was also evaluated via a second search step. First, the 4 candidate BFIs were screened for specificity for liquorice (products) in the compound databases HMDB, FooDB, and Phenol-explorer. The compound 18-GA glucuronide could not be found in any of the compound databases. However, it is known to be the product of the hepatic metabolism of 18-GA (47), and therefore it was excluded from further evaluation. The other three compounds were found in the databases HMDB and FooDB, and were linked to the presence in herbs/spices (18-GA, glabridin), or were known as a sweetener (3-MGA). 3-MGA is also a metabolite of GA, and is known to be excreted via urine in small quantities, and to be a possible biomarker for liquorice-induced adverse-effects (19, 48). Therefore, we decided to include 3-MGA for further evaluation. In Table 1 an overview is presented of the studies on liquorice BFIs, including details on dose used in the study, number of subjects included, which analytical method was used to analyse the samples, which BFIs were found and the primary reference. The specificity of all 3 candidate BFIs was further examined by performing additional searches in Scopus, ISI Web of Science, PubMed or Google Scholar; which gave us no indication that these metabolites were related to the intake of other food products. On the basis thereof, and on the 8 originally included papers, the validity of the 3 selected candidate BFIs for liquorice (products) was evaluated (**Table 2**). As can be seen in Table 2 the compound 18-GA in urine after liquorice (product) consumption had the highest assumed validity, however uncertainty still remains about its specificity, since liquorice root extracts itself is used in many other products, such as chewing gum, other confectionary, or beverages (question 1) (19, 47, 49, 50). Krahenbuhl *et al.* (51) observed a clear dose-response relationship for 18-GA in plasma, however the concentration GA used in this study was comparable to an intake of liquorice ranging from 1.7-5.2 kg, therefore no relevant intake levels of the targeted food were examined in this study. In the study of Kerstens *et al.* (52) 50-200 grams of liquorice were consumed, showing a dose-response relationship of 18-GA in urine (question 2). 18-GA was found to be traceable in urine after the intake of 50 grams of solid liquorice, and around 0.04% of the total intake could be traced back after 51h in total (52). A peak in 18-GA concentration was measured after 6 hours in blood after the consumption of 200 grams of solid liquorice (53), or between 1.5 – 40 hours in urine after the consumption of 600 mg of the compound glycyrrhizin (content comparable to 353 gram of liquorice, table 1) (19). 18-GA was also traceable in 24h urine after the consumption of 1500 mg of the compound GA (content comparable to 882 gram of liquorice, table 1) (51). In serum a peak was observed after 2-4h of the consumption of 500 mg of the compound GA (54), and in plasma the peak time was after 3-4h after the consumption

**Table 2.** Evaluation of the validity of the identified candidate biomarkers of food intake for liquorice.

| Metabolite | Bio fluid | Q1 | Q2 | Q3 | Q4 | Q5 | Q6 | Q7 | Q8 | Q9 | Sum | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18-glycyrrhetic acid | Plasma | U | U | Y | U | Y | N | U | Y | N | 3 | (50, 51, 55) |
| | Serum | U | U | Y | U | Y | N | U | Y | N | 3 | (54) |
| | Blood | U | U | Y | U | N | N | U | Y | N | 2 | (53) |
| | Urine | U | Y | Y | U | Y | N | U | Y | N | 4 | (51-53) |
| 3β-monoglucuronyl-18β-glycyrrhetinic acid | Urine | U | U | N | U | Y | N | U | Y | N | 2 | (19) |
| Glabridin | Plasma | U | U | U | U | N | N | U | N | N | 0 | (57) |

Y = Yes, N = No, U = Unknown

Q1: Is the marker compound plausible as a specific BFI for the food or food group (chemical/biological plausibility)?

Q2: Is there a dose-response relationship at relevant intake levels of the targeted food (quantitative aspect)?

Q3: Is the single-meal time-response relationship described adequately to make a wise choice of sample type and time window (single-dose kinetics)?

Q4: Is the biomarker kinetics for repeated intakes of the food/food group described adequately providing the frequency of sampling needed to assess habitual intake (e.g. cumulative aspects)?

Q5: Has the marker been shown to be robust after intake of complex meals reflecting dietary habits of the targeted population (robustness)?

Q6: Has the marker been shown to compare well with other markers or questionnaire data for the same food/food group (reliability)?

Q7: Is the marker chemically and biologically stable during bio specimen collection and storage, making measurements reliable and feasible?

Q8: Are analytical variability (CV%), accuracy, sensitivity and specificity known as adequate for at least one reported analytical method?

Q9: Has the analysis been successfully reproduced in another laboratory (reproducibility)?

of 21 grams of liquorice extract (55), or 500 mg and higher concentrations of the compound GA (51). Best sampling time for 3-MGA in urine was unknown, since time to maximum peak height ranged from 1.5h to 39.5h. This high interindividual variability in time to maximum peak height was likely caused by differences in metabolism rate and enterohepatic cycling of the compound (19) (question 3). None of the studies examined repeated intakes of liquorice, or isolated compounds of liquorice roots (question 4). In plasma, serum, and urine, 18-GA was measured after subjects continued with their habitual diet during the measurements (question 5) (50-55). Since none of the studies was an observational study, or included questionnaire data, the answers to question 6 were “no” for all candidate BFIs. Kerstens *et al.* (52) did examine whether 18-GA could be used to detect whether two patients had consumed liquorice, even if the patients denied having eaten liquorice containing products. They observed that it was indeed possible to detect liquorice intake via measuring 18-GA in urine, after which the patient indeed admitted to have eaten liquorice containing products. Unfortunately, data on actual liquorice intake (via for example food frequency questionnaires, or 24h recalls) were not recorded in this study. Question 7 concerned the chemical and biological stability during specimen collection and

storage. In the eight included papers it was described that urine samples were stored at 4 degrees (19, 54) or -20°C (52, 53), and blood, plasma, and serum samples were stored -20°C (50-53, 55) before continuing with the analyses. In some cases no information was available about the storage temperature of the samples (57), and for all 8 studies it was unclear how long the samples had been stored. Analytical variability for measuring 18-GA in plasma was 3% (51), in urine CV% was between 6-9.3% (51, 53), in serum there was a 2.1% within assay variability, and 8.5% between assay variability (54), and in blood CV% was between 4.6-6.3% (53). For 3-MGA the repeatability of the measurements within and between-days was tested, with peak height 4.88 and 7.21 RSD% respectively, and peak area 2.61 and 6.21 RSD% respectively (19). In the papers of Raggi *et al.* (55), Aoki *et al.* (57), and Ploeger *et al.*(50) no details about analytical variability were presented (question 8). Lastly, none of the analyses described in the eight selected papers were reproduced by another laboratory. The analyses used were either described in the paper for the first time (19, 50, 52, 53, 55), or had previously been performed at their own lab (51, 54, 57) (question 9).

## DISCUSSION

The present systematic review examined the current status of candidate BFIs for the consumption of cocoa (products) and liquorice. In total, 37 relevant papers were included for cocoa (products) (26, 27, 32-34, 44, 58-88), and 8 relevant papers were included for liquorice (products) (19, 50-55, 57). For cocoa (products) 164 different compounds were identified as candidate BFIs in the 37 obtained papers. After evaluating the specificity of these compounds, none of these candidate BFIs turned out to be specific for cocoa (products). Therefore, the validity of these compounds was not further examined. For liquorice (products) 4 different compounds were identified as candidate BFIs in the 8 obtained papers. After evaluating the specificity and the validity of these 4 compounds, 18-GA in urine was found to have the highest assumed validity.



Regarding the 164 cocoa (products) BFIs (Supplementary table 1), none of the identified compounds was specific. Our results indicated that most of the identified BFIs for cocoa (products) were also found or are expected to be found after the consumption of foods such as tea, coffee, or red wine. This is due to similarities in the composition of these foods, such as a high polyphenol content or the presence of caffeine compounds (89-92). Only a few randomized trials (both short and long term) have compared the metabolite profile after consumption of cocoa (products) with other products showing

a similar metabolite profile, such as the metabolite profiles observed after tea (64, 72), or coffee (64). Up till now, no metabolites were obtained that could discriminate between these food products. However, only a small amount of metabolites was measured in these studies via targeted approaches. With untargeted approaches a higher number of compounds can be measured in bio fluids, and therefore might elucidate compounds that are specific and valid BFIs for cocoa (products). Before any conclusion can be drawn about the specificity of the identified BFIS for cocoa (products) more randomized intervention studies comparing food products with a similar metabolite excretion pattern, using untargeted approaches, are needed. Furthermore, it remains to be explored whether the dose-response relationship is equal after consuming for example same amounts of tea and a cocoa drink (64). Another important point to consider is that the way of processing of the cocoa beans, the cocoa variety, and the origin of the cocoa bean can affect the final concentration of compounds in the cocoa product (93). In addition, cocoa beans are subject to seasonal variation, again affecting concentrations of several compounds in the beans (94, 95). In this review we focused on single BFIs for cocoa intake, which turned out to be nonspecific. A combination of several biomarkers, a so called biomarker profile or panel of biomarkers might however increase the specificity for cocoa intake. Garcia-Aloy and colleagues (96) have examined this possibility in a untargeted study, and found a combined model for cocoa consumption that included 7-methylxanthine and dihydroxyphenylvalerolactone glucuronide. This combined model was a better discriminant for cocoa consumption compared to all individual metabolites. It is essential to explore whether this combination of compounds will also discriminate cocoa (products) intake from tea intake, or coffee intake.

The four identified liquorice BFIs (Table 1), 18-GA, 18-GA glucuronides, 3-MGA, and glabridin, were only described in relation to liquorice intake or liquorice roots extracts. However, liquorice root extracts are known to be used in a variety of products, such as different sweets, chewing gums, chewing tobacco, tea, or even in (alcoholic) drinks, and medicinal products, often as sweetening or flavouring agent (19, 47, 49, 50). It will therefore be necessary to examine the relative content and contribution of liquorice extract in these products and in the habitual consumed diets overall. An important point to consider is that the content of liquorice root extracts may vary from product to product, depending on the characteristics the producer desires for that product, which causes variation in the final concentrations of liquorice root extracts in these products. For example, in the solid liquorice that was consumed in the included studies the GL content varied from 0.05% up to 0.23%. 18-GA in urine was the most

promising and most studied candidate BFI out of the three candidates for liquorice (products). To increase its validity, studies on repeated intake, habitual food consumption, dietary patterns, stability during storage, and reproducibility of the methods between labs, are still needed. Important to note is that in all studies examining liquorice biomarkers, subjects consumed 68 grams of liquorice or more (when standardized to the average glycyrrhizin content in liquorice confectionary of 0.17% (97), this is equivalent to an intake of GL of 116 mg), while the European Scientific Committee on Food advises that ingestion of liquorice should not exceed 58 grams per day (equivalent to an intake of 100 mg GL per day) (97). Therefore it is crucial to investigate whether 18-GA is still a reliable biomarker when measured after the intake of a low dose of liquorice, or after repeated intake of low doses of liquorice. This is especially important since some studies were unable to measure 18-GA in urine, because the amount was below the detection limit at lower concentrations of intake (51, 54). Clearly lacking in the literature for all candidate BFIs for liquorice were long term intervention studies, dietary pattern studies, and observational studies. Currently, the longest study had a duration of 4 weeks. However, this study was done using liquorice flavonoid oil in which glycyrrhizin was almost removed from the product (<0.005%) (57). Consequently, this study did not give us information about the consumption of solid liquorice or other products using liquorice root extracts, which have a higher glycyrrhizin content. The lack of observational and dietary pattern studies makes it impossible to properly validate any of the 3 found candidate BFIs, therefore these studies are urgently needed. Moreover, we only found the polyphenol glabridin as candidate BFI for liquorice intake; however there are more polyphenols known to be present in liquorice roots. For example Vaya *et al.* (98) have isolated hispaglabridin A, hispaglabridin B, 4'-O-methylglabridin, formononetin, and glabridin itself from liquorice roots. It should be further examined what the exact contribution of these polyphenols is in liquorice (products), and whether these compounds could be possible BFIs for liquorice (products). For 18-GA and glabridin, commercial standards are available, for 18-GA also a non-commercial standard is available through FoodComEx (Food Compound Exchange, foodcomex.org), an online catalogue of pure compounds made available by academic laboratories (99).



## CONCLUSIONS

In this paper, we have identified potential BFIs for cocoa (products) and liquorice (products). For cocoa (products) none of the individual BFIs were found to be specific. However, a combination of individual BFIs might lead to discriminating profiles between cocoa (products) and foods with a similar

composition. This needs to be further explored. We did identify 18-GA as a promising candidate BFI for liquorice, however important information on its validity is still missing, and therefore more research is needed. This systematic review shows that there is still an urgent need for research to identify specific and valid biomarkers of the consumption of cocoa (products) and liquorice (products).

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## CONFILCT OF INTEREST

There is no conflict of interest.

## AUTHOR CONTRIBUTIONS

*C.C.J.R.M.* Performed systematic database search, selected papers and critically reviewed whether they adhered to the selection criteria, on both the cocoa and liquorice part, and wrote the manuscript; *E.A.A.* selected papers and critically reviewed whether they adhered to the selection criteria, of the cocoa part, and critically revised the manuscript; *E.M.B.-B.* selected papers and critically reviewed whether they adhered to the selection criteria of the liquorice part; *M.U.S.* and *L.A.A.* critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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## LIST OF ABBREVIATIONS

| Abbreviation | Full description |
|---|---|
| 18-GA | 18- glycyrrhetinic acid |
| 3-MGA | 3β-monoglucuronyl-18β-glycyrrhetinic acid |
| BFIRev | Biomarker of Food Intake Reviews |
| BFIs | Biomarkers of Food Intake |
| FooDB | Food DataBase |
| FoodBAll | Food Biomarkers Alliance |
| FoodComEx | Food Compound Exchange |
| HMDB | Human Metabolome DataBase |
| HOMA-IR | Homeostatic Model Assessment – Insulin Resistance |

## SUPPLEMENTAL MATERIAL

**Supplemental Table 1.** Overview of the studies on biomarkers for cocoa (products), including information about dosage, study design, number of subjects, method used, sample type and the original references. *Continues on the next pages.*

| Dietary factor | Dose of intervention | Study design | # subjects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| **Cocoa (products)** | | | | | | | | |
| **Solids** Dark chocolate | 6 mg/kg/day theobromine | Acute single dose study | 12 | HPLC | Targeted | Blood (0 - 24 h after intake) | Theobromine | (83) |
| [8-$^{14}$C] theobromine | 10 mg/kg theobromine sodium acetate (equivalent to 6 mg/kg) with 60 µLCi [8-$^{14}$C]theobromine | | | HPLC-radioactivity monitor | Targeted | Urine (0 - 72 h after intake) | Theobromine<br>6-Amino-5(N-methylformylamino)-1 methyluracil<br>Methylxanthine<br>7-methyluric acid<br>7-methylxanthine<br>3-methylxanthine | |
| Dark chocolate | 40 g and 80 g with bread and water | Acute single dose study | 8 | HPLC-UV<br>HPLC-FLD | Targeted | Plasma (0 - 8 h after intake) | (-)-Epicatechin<br>Theobromine | (72) |
| Procyanidin-rich chocolate | 80 g of procyanidin-rich chocolate in the form of 105 g of M&M's | Acute single dose study | 13 | HPLC-ECD | Targeted | Plasma (0 - 6 h after intake) | (-)-Epicatechin (sum free and conjugated metabolites) | (100) |
| Flavanol-rich chocolate | 80 g with bread and water | Acute single dose study | 11 | GC-MS<br>HPLC-MS/MS | Targeted | Urine (24 h before and 3 - 48h after intake) | 3,4-dihydroxyphenylpropionic acid<br>m-hydroxyphenylpropionic acid<br>Ferulic acid<br>3,4-dihydroxyphenylacetic acid<br>m-hydroxyphenylacetic acid<br>Phenylacetic acid<br>vanillic acid<br>m-hydroxybenzoic acid<br>p-hydroxybenzoic acid<br>p-hydroxyhippuric acid<br>Hippuric acid | (74) |
| Dark chocolate | 100 g | Acute single dose study | 5 | UPLC-MS/MS and LC-MS | Targeted | Urine (5 and 10h after intake) | 3'-O-methyl-(-)-epicatechin-5-O-sulphate<br>3'-O-methyl-(-)-epicatechin-7-O-sulphate<br>4'-O-methyl-(-)-epicatechin-5-O-sulphate<br>4'-O-methyl-(-)-epicatechin-7-O-sulphate | (58) |
| Dark chocolate | 100 g | Acute single dose study | 5 | UPLC-MS/MS | Targeted | Urine (0 - 24 h after intake) | (-)-epicatechin-3'-β-D-glucuronide<br>(-)-epicatechin-4'-β-D-glucuronide<br>(-)-epicatechin-7-β-D-glucuronide<br>(-)-epicatechin 3'sulfate<br>(-)-epicatechin 4'-sulfate<br>(-)-epicatechin 5-sulfate<br>3'-O-methyl-(-)-epicatechin 4'-sulfate<br>3'-O-methyl-(-)-epicatechin 5-sulfate<br>3'-O-methyl-(-)-epicatechin 7-sulfate | (59) |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 4'-O-methyl-(-)-epicatechin 5-sulfate | |
| | | | | | | | 4'-O-methyl-(-)-epicatechin 7-sulfate | |
| | | | | | | | 3'-O-methyl-(-)-epicatechin-β-D-glucuronide (a) | |
| | | | | | | | 3'-O-methyl-(-)-epicatechin-β-D-glucuronide (b) | |
| | | | | | | | 3'-O-methyl-(-)-epicatechin-β-D-glucuronide (c) | |
| | | | | | | | 4'-O-methyl-(-)-epicatechin-β-D-glucuronide (a) | |
| | | | | | | Plasma (0 - 24h after intake) | (-)-epicatechin-3'-β-D-glucuronide | |
| | | | | | | | (-)-epicatechin-4'-β-D-glucuronide | |
| | | | | | | | (-)-epicatechin-7'-β-D-glucuronide | |
| | | | | | | | (-)-epicatechin 3'-sulfate | |
| | | | | | | | (-)-epicatechin 4'-sulfate | |
| | | | | | | | 3'-O-methyl-(-)-epicatechin 4'-sulfate | |
| | | | | | | | 3'-O-methyl-(-)-epicatechin 5-sulfate | |
| | | | | | | | 3'-O-methyl-(-)-epicatechin 7-sulfate | |
| | | | | | | | 4'-O-methyl-(-)-epicatechin 5-sulfate | |
| | | | | | | | 4'-O-methyl-(-)-epicatechin 7-sulfate | |
| Dark chocolate | 50 g/d | Parallel intervention study (1 wk: regular consumers of chocolate vs indifferent consumers) | 20 | $^{1}$H NMR | Untargeted | Urine (morning spot) | Butyrate | (65) |
| | | | | | | | 3-Hydroxybutyrate | |
| | | | | | | | 3-Hydroxyisovalerate | |
| | | | | | | | Alanine | |
| | | | | | | | 4-Cresol sulfate | |
| | | | | | | | Trimethylamine | |
| | | | | | | | Dimethylglycine | |
| | | | | | | | Acylcarnitines | |
| | | | | | | | Carnitine | |
| | | | | | | | trimethyl-amine-N-oxide | |
| | | | | | | | Taurine | |
| | | | | | | | Guanidinoacetate | |
| | | | | | | | Creatinine | |
| | | | | | | | Urea | |
| | | | | | | | 3-(3-hydroxyphenyl)-propionate | |
| | | | | | | | 4-hydroxyphenylacetate | |
| | | | | | | | Phenylacetate | |
| | | | | | | | Phenylacetylglutamine | |
| | | | | | | | Indoxyl-sulfate | |
| | | | | | | | Hippurate | |
| | | | | | | | 7-Methylxanthine | |
| | | | | | | | Theobromine | |

| Dietary factor | | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | HPLC-MS | Untargeted | | Trigonelline<br>2S-1-(3-4-dihydroxyphenyl)-3-(2-4-6-trihydroxyphenyl)-propan-2-ol methylated sulfate<br>5-(3,4-dihydroxyphenyl)-valeric-acid glucuronide<br>5-(3,4-dihydroxyphenyl)-valeric-acid methylated sulfate<br>5-(3,4-dihydroxyphenyl)-valeric-acid sulfate<br>5-(3-4-dihydroxyphenyl)-valerolactone<br>5-(3-4-dihydroxyphenyl)-valerolactone glucuronide<br>5-(3-4-dihydroxyphenyl)-valerolactone methylated glucuronide<br>5-(3-4-dihydroxyphenyl)-valerolactone methylated sulfate<br>5-(3-4-dihydroxyphenyl)-valerolactone sulfate<br>5-(3-hydroxyphenyl)-valerolactone glucuronide<br>5-(3-hydroxyphenyl)-valerolactone sulfate<br>5-(4-hydroxy(3-4-dihydroxyphenyl)-valeric-acid<br>5-(4-hydroxy(3-4-dihydroxyphenyl)-valeric-acid glucuronide<br>5-(4-hydroxy(3-4-dihydroxyphenyl)-valeric-acid methylated glucuronide<br>5-(4-hydroxy(3-4-dihydroxyphenyl)-valeric-acid methylated sulfate<br>5-(4-hydroxy(3-4-dihydroxyphenyl)-valeric-acid sulfate<br>epicatechin glucuronide<br>Methylated epicatechin sulfate | |
| | Chocolate bar with different flavanol content and sweeteners | 25 g serving containing 48 mg, 251mg, or 266mg of total cocoa flavanols | Acute crossover study | 15 | HPLC-FLD/UV | Targeted | Plasma (0 - 4 h, after intake) | (-)-Epicatechin<br>3'-*O*-Methyl-epicatechin<br>4'-*O*-Methyl-epicatechin | (77) |
| **Drinks** | Cocoa beverage | 75 g of cocoa powder in 500 mL of water | Acute single dose study | 6 | HPLC-FLD | Targeted | 10 mL of gastric sample (0 and intervals of 10 min until stomach was emptied) | Catechin<br>(–)-Epicatechin<br>Proacyanidin B2<br>Proacyanidin B5<br>Proacyanidin C1 | (73) |
| | Flavanol-rich cocoa beverage | 0.375 g cocoa/kg body weight | Acute single dose study | 5 | HPLC-MS/MS | Targeted | Plasma (0 - 6h after intake) | Procyanidin dimer B2<br>Catechin<br>Epicatechin | (63) |
| | Cocoa beverage | 10 g of cocoa powder in 200 ml of water | Acute crossover study | 9 | HPLC-ESI-MS/MS | Targeted | Urine (0 - 24h after intake) | Epicatechin | (64) |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| Cocoa beverage | 40 g of cocoa powder in 250 mL of whole milk vs. whole milk only | Acute crossover study | 5 | HPLC-MS/MS | Targeted | Plasma (0 and 2 h after intake) | (–)-Epicatechin sulfates<br>(–)-Epicatechin glucuronide<br>(–)-Epicatechin methylglucuronide<br>(–)-Epicatechin sulfoglucuronide<br>(–)-Epicatechin methyl sulfoglucuronide | (80) |
| | | | | | | Urine (0 and 6 h after intake) | (–)-Epicatechin glucuronide | |
| Flavanol cocoa drinks | Cocoa beverage powder containing 917 mg (high-flavanol cocoa drink) and 37 mg (low-flavanol cocoa drink) of total flavanol in 300 mL of water | Acute crossover study | 10 | HPLC-MS<br>$^{1}$H-NMR<br>$^{13}$C-NMR | Targeted | Plasma (0 - 6 h after intake) | 4'-*O*-Methyl-epicatechin-7-O-β-D-glucuronide<br>4'-*O*-Methyl-epicatechin<br>3'-*O*-Methyl-epicatechin-5/7-O-β-D-glucuronide<br>3'-*O*-Me-epicatechin<br>Epicatechin-7-O-β–D-glucuronide<br>Epicatechin<br>Catechin<br>4'-O-Methyl-catechin<br>3'-O-Methyl-catechin | (82) |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of whole milk or water | Acute crossover study | 21 | HPLC-MS/MS | Targeted | Plasma (0 - 6 h after intake) | (–)-Epicatechin-glucuronide | (81) |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of whole milk | Acute crossover study | 21 | HPLC-MS/MS | Targeted | Urine (0 - 24 h after intake) | (–)-Epicatechin-glucuronide<br>(–)-Epicatechin-sulfates | (78) |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of whole milk or water | Acute crossover study | 21 | HPLC-MS/MS | Targeted | Urine (0 - 24 h, after intake) | (–)-Epicatechin-glucuronide<br>(–)-Epicatechin-sulfates | (79) |
| Cocoa beverage | 20.3 of alkalized cocoa powder in 400 mL of water | Acute single dose study | 8 | HPLC-MS/MS<br>NMR | Targeted | Urine (0 - 8 h after intake) | N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-aspartic acid<br>N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-dopa<br>N-[3',4'-dihydroxy-(E)-cinnamoyl]-L-tyrosine<br>N-[4'-hydroxy-(E)-cinnamoyl]-L-aspartic acid<br>N-[4'-hydroxy-(E)-cinnamoyl]-L-glutamic acid<br>N-[4'-hydroxy-(E)-cinnamoyl]-L-dopa<br>N-[4'-hydroxy-(E)-cinnamoyl]-L-tyrosine<br>N-[4'-hydroxy-3'-methoxy-(E)-cinnamoyl]-L-aspartic acid<br>N-cinnamoyl-L-aspartic acid<br>N-[4'-hydroxy-(E)-cinnamoyl]-L-tryptophane<br>N-[4'-hydroxy-3'-methoxy-(E)-cinnamoyl]-L-tyrosine | (32) |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of milk or water | Acute crossover study | 10 | HPLC-qTOF-MS | Untargeted | Urine (baseline, 0 - 24 h after intake) | Tyrosine<br>Hydroxynicotinic acid<br>Trigonelline<br>Hydroxyacetophenone<br>Vanillic acid | (44) |

| Dietary factor | Dose of intervention | Study design | # subjects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Vanilloylglycine<br>Cyclo(Pro-Pro)<br>Epicatechin-O-sulfate<br>*O*-Methylepicatechin<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone-sulfate<br>3,5-Diethyl-2-methylpyrazine<br>Caffeine<br>6-amino-5-[*N*-methylformylamino]-1-methyluracil<br>7-methyluric acid<br>7-methylxanthine<br>3-methyluric acid<br>3-methylxanthine<br>3,7-dimethyluric acid<br>Theobromine<br>Cyclo(Ser-Tyr)<br>4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone glucuronide<br>5-(3',4'-dihydroxyphenyl)-g-valerolactone glucuronide<br>3'-methoxy-4'-hydroxyphenylvalerolactone glucuronide<br>3'-methoxy-4'-hydroxyphenylvalerolactone | |
| Cocoa beverage | 10 g of green and black's organic cocoa in 250 ml of milk or water | Acute crossover study | 9 | HPLC-PDA-MS/MS | Targeted | Urine (0 - 24 h after intake)<br><br>Plasma (0 - 24h after intake) | (epi)catechin-O-sulfate,<br>(-)-Epicatechin-*O*-glucuronide,<br>*O*-Methyl-(epi)catechin-O-sulfate<br>(epi)catechin-O-sulfate,<br>*O*-Methyl-(epi)catechin-O-sulfate | (67) |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of water for humans, and 4.8 g natural cocoa powder/kg/day for rats | Acute crossover study | 21 | HPLC-MS/MS | Targeted | Urine (0 and 24 h after intake) | 3,4-butyrate acid<br>m-Coumaric acid<br>p-Coumaric acid<br>Caffeic acid<br>Ferulic acid<br>3,4-Dihydroxyphenylacetic acid<br>3-Methoxy-4-hydroxyphenylacetic acid<br>3-Hydroxyphenylacetic<br>Phenylacetic acid<br>Protocatechuic acid<br>Vanillic acid<br>4-Hydroxybenzoic acid<br>3-Hydroxybenzoic acid | (85) |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 4-Hydroxyhippuric acid<br>Hippuric acid;<br>Enterodiol;<br>Enterolactone;<br>(−)-Epicatechin;<br>Procyanidin B2;<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone<br>5-(3'-methoxy,4'-hydroxyphenyl)-γ-valerolactone | |
| Cocoa beverage | 40 g of cocoa powder in 500 mL of skimmed milk per day | Crossover Intervention study (4 wk of control beverage and 4 wk of cocoa beverage) | 42 | HPLC-MS/MS | Targeted | Urine (baseline and 24 h, after intervention) | Epicatechin-O-glucuronides;<br>Epicatechin-O-sulfates;<br>O-Methyl-epicatechin-O-glucuronides;<br>O- Methyl-epicatechin-O-sulfates;<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone-O-glucuronides<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone-O-sulfates<br>5-(3'-methoxy,4'-hydroxyphenyl)-γ-valerolactone-O-glucuronides<br>5-(3'-methoxy,4'-hydroxyphenyl)-γ-valerolactone-O-sulfates<br>(−)-Epicatechin<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone<br>3,4-Dihydroxyphenylacetic acid<br>3-Hydroxyphenylacetic acid<br>Vanillic acid | (86) |
| | | | | | | Plasma (baseline and 24 h, after intervention) | 5-(3',4'-dihydroxyphenyl)-γ-valerolactone-O-glucuronides<br>5-(3'-methoxy,4'-hydroxyphenyl)-γ-valerolactone-O-glucuronides<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone<br>3,4-Dihydroxyphenylacetic acid | |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of milk | Acute single dose study | 10 | HPLC-qTOF-MS | Untargeted | Urine (before (-2 and 0 h) and 2 - 24 h after intake) | N-methylguanine<br>Vanilloylglycine<br>Dihydroxyphenyl valerolactone glucuronide<br>Furoylglycine<br>7-methylxanthine<br>3-methylxanthine<br>Theobromine<br>Xanthurenic acid | (34) |
| Cocoa beverage | 40 g of cocoa powder in 250 mL of milk or water | Acute crossover study | 21 | HPLC-MS/MS | Targeted | Urine (0 - 24 h after intake) | 3,4-Dihydroxyphenylpropionic acid<br>m-Coumaric acid | (84) |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | p-Coumaric acid<br>Caffeic acid<br>Ferulic acid<br>3,4-Dihydroxyphenylacetic acid<br>3-Methoxy-4-hydroxyphenylacetic acid<br>3-Hydroxyphenylacetic<br>Phenylacetic acid<br>Protocatechuic acid<br>Vanillic acid<br>4-Hydroxybenzoic acid<br>3-Hydroxybenzoic acid<br>4-Hydroxyhippuric acid<br>Hippuric acid | |
| Cocoa beverage | 46 g of cocoa powder in 250 mL of whole milk | Acute single dose study | 1 | HPLC-CEAD | Targeted | Plasma (0 and 2 h after intake) | (–)-Catechin*<br>(+)-Catechin* | (75) |
| Cocoa beverage | 0.5 g/kg bw low flavanol cocoa powder, 5 g/kg bw milk (1% fat), and 22 mg/kg bw cocoa extract containing 47.7% (w/w) flavanols | Acute single dose study | 10 | HPLC-MS/MS | Targeted | Plasma (0 - 4 h, after intake) | (–)-Epicatechin<br>(–)-Epicatechin-3'-β-D-glucuronide<br>(–)-Epicatechin-3'-sulfate<br>(–)-Epicatechin-5-sulfate<br>(–)-Epicatechin -7-sulfate<br>3'-*O*-Methyl-epicatechin-5/7-sulfates<br>4'-*O*-Methyl-epicatechin-5/7-sulfates<br>4'-*O*-Methyl-epicatechin-7-β-D-glucuronide<br>3'-*O*- Methyl-epicatechin*<br>4'-*O*- Methyl-epicatechin* | (70) |
| Cocoa beverage | 40 g/d of cocoa powder in 500 mL of skimmed milk or only skimmed milk | Crossover intervention study (4 wk of control beverage and 4 wk of cocoa beverage) | 20 | HPLC-qTOF-MS | Untargeted | Urine (baseline and 24h after intervention) | Hydroxynicotinic acid<br>7-methyluric acid<br>Tyrosine sulfate<br>3-methyluric acid<br>Butyrylcarnitine<br>7-methylxanthine<br>Methylglutarylcarnitine<br>3-methylxanthine<br>3,7-dimethyluric acid<br>Cyclo(propylalanyl)<br>3,5-diethyl-2-methylpyrazine<br>Theobromine<br>Vanillic acid glucuronide | (33) |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Vanilloylglycine<br>4-hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide<br>3'-methoxy-4'-hydroxyphenylvalerolactone<br>4-hydroxy-5-(hydroxy-methoxyphenyl)-valeric acid glucuronide<br>N-[4'-hydroxycinnamoyl]-l-aspartic acid<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone glucuronide<br>(Epi)catechin glucuronide<br>Methoxyhydroxyphenylvalerolactone glucuronide<br>N-[4'-hydroxy-3'-methoxy-E-cinnamoyl]-l-aspartic acid<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone sulfoglucuronide<br>5-(3',4'-dihydroxyphenyl)-γ-valerolactone-sulfate<br>Hydroxyphenyl-γ-valerolactone glucuronide<br>Vanillic acid sulfoglucuronide,<br>4-hydroxy-5-(dihydroxyphenyl)-valeric acid sulfate<br>Epicatechin sulfoglucuronide<br>Methyl-(epi)catechin sulfate<br>Hydroxyphenylvalerolactone sulfate<br>5-(hydroxy-methoxy-phenyl)-γ-valerolactone sulfate<br>4-hydroxy-5-(phenyl)-valeric acid sulfate | |
| Cocoa beverage | 15 g of cocoa powder (control) and 25 g of cocoa powder enriched in methylxanthines, in 200 mL of semi-skimmed milk | Acute crossover study | 13 | LC-QTOF and LC-DAD | Targeted | Urine (baseline (-2-0h), 0 - 24 h after intake) | 1-methylxanthine<br>3-methylxanthine<br>7-methylxanthine<br>1,7-dimethylxanthine<br>3,7-dimethylxanthine<br>1,3-dimethylxanthine<br>1,3,7-trimethylxanthine<br>1-monomethyluric acid<br>1,3-dimethyluric acid<br>1,7-dimethyluric acid<br>3,7-dimethyluric acid<br>1,3,7-trimethyluric acid | (66) |
| | | | | HPLC-DAD | Targeted | Plasma (0 - 8h after intake) | Caffeine<br>Paraxanthine<br>Theobromine<br>Theophylline<br>3-methylxanthine<br>7-methylxanthine | |

| Dietary factor | | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|---|
| | Fruit-flavored cocoa powder beverage | Cocoa powder containing 5.3 mg and 10.7 of cocoa-flavanol/kg BW in 500 mL of water | Acute crossover study | 40 | HPLC-FLD-UV-ECD | Targeted | Plasma (0-24 h, after intake) | Epicatechin-3'-β-D-glucuronide<br>Epicatechin-3'-sulfate<br>3'-*O*-Methyl-epicatechin-5-sulfate<br>3'-*O*-Methyl-epicatechin-7-sulfate<br>(–)-Epicatechin<br>Epicatechin-5-sulfate<br>Epicatechin-7-sulfate | (76) |
| | | | | | | | Urine (0 - 24 h after intake) | Epicatechin-3'-β-D-glucuronide<br>Epicatechin-3'-sulfate<br>3'-*O*-Methyl-epicatechin-5-sulfate<br>3'-*O*-Methyl-epicatechin-7-sulfate<br>5-(3,4-dihydroxyphenyl)-L-valerolactone* | |
| **Cocoa products** | Average Danish Diet (including chocolate) and. New Nordic Diet | ADD contained cocoa products, NDD free of cocoa products | Parallel intervention study (6 months) | 181 | UPLC-qTOF-MS | Untargeted | Urine (24h in week 0, 4, 12, 20 and 26) | Theobromine<br>7-methyluric acid<br>6-amino-5-[N-methylformylamino]-1-methyluracil<br>3,7-dimethyluric acid<br>7-methylxanthine | (61) |
| | Average Danish Diet (including chocolate) vs. New Nordic Diet | ADD contained cocoa products, NDD free of cocoa products | Parallel intervention study (6 months) | 107 | UPLC-qTOF-MS | Untargeted | Urine (24h in week 0, 12 and 26) | Theobromine,<br>7-methyluric acid<br>6-amino-5-[N-methylformylamino]-1-methyluracil | (60) |
| | Cocoa-containing food items | Based on (24h-dietary recall) | Observational study Cross-sectional | 481 | UPLC-qTOF-MS | Untargeted | Urine (24h-collection) | Methyl(epi)catechin sulfate<br>Vanillic acid sulfate | (26) |
| | Cocoa-containing food items | Based on FFQ (non-consumers vs. consumers) | Observational study Cross-sectional | 64 | HPLC-qTOF-MS | Untargeted | Spot urine | Xanthine<br>6-amino-5[N-methylformylamino]-1-methyluracil<br>6-amino-5[N-methylformylamino]-1-methyluracil isomer<br>3-methyluric acid<br>7-methylxanthine<br>3- methylxanthine<br>3,7-dimethyluric acid<br>Theobromine<br>Furoglycine<br>cyclo(aspartyl-phenylalanyl<br>Aspartyl-phenylalanine<br>Vanillin sulphate<br>(Epi)catechin glycuronide<br>Vanillic acid<br>(Epi)catechin sulphate<br>4-hydroxy-5-(dihydroxyphenyl)valeric acid glucuronide | (27) |

| Dietary factor | | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 4-hydroxy-5-(hydroxy-methoxyphenyl)-valeric acid glucuronide<br>Methoxyhydroxyphenylvalerolactone,<br>5-(3',4'-Dihydroxyphenyl)-valerolacetone sulfoglucuronide<br>5-(3',4'-Dihydroxyphenyl)-valerolacetone glucuronide<br>4-hydroxy-5-(dihydroxyphenyl)valeric acid<br>4-hydroxy-5-(dihydroxyphenyl)valeric acid sulphate<br>5-(3',4'-Dihydroxyphenyl)-valerolacetone glucuronide<br>4-hydroxy-5-(hydroxymethodyphenyl)-valeric acid sulphate<br>Methoxyhydroxyphenylvalerolactone glucuronide<br>Hydroxyphenyl-valeroacetone glucuronide<br>5-(3',4'-Dihydroxyphenyl)-valerolacetone sulphate<br>Hydroxyphenyl-valeroacetone sulphate<br>4-hydroxy-5-(hydroxyphenyl)-valeric acid sulphate<br>4-hydroxy-5-(phenyl)-valeric acid sulphate<br>Methylglutarylcarnitine | |
| **Miscellaneous** | Chocolate and cocoa | 96 g of chocolate and 66 g cocoa | Acute crossover study | 5 | HPLC-MS | Targeted | Urine (0-24 h after intake)<br>Plasma (0- 24 h after intake) | Epicatechin (non)methylated - free/glucuronide/sulfate/sulfoglucuronide<br>Methylepicatechin- sulfoglucuronide | (62) |
| | Chocolate bars and cocoa beverages | 40 g bars and 250 mL beverages | Acute crossover study | 6 | RP-HPLC-ECD | Targeted | Serum (0 - 6 h after intake) | (±)-Catechin<br>(–)-Epicatechin | (68) |
| | Cocoa in nut cream and capsules | Capsules: cocoa polyphenol extract in high-amylose maize starch 1:9 (w/w)<br>Cocoa-nut creams: 20% (w/w) cocoa for control cream, and 1·5% of free or encapsulated cocoa polyphenol extract | Acute crossover study | 12 | HPLC-MS/MS | Targeted | Serum (0 - 24 h after intake) | Chlorogenic acid<br>Caffeic acid<br>Hippuric acid<br>Hydroxybenzoic acid<br>4-hydroxyphenylacetic acid<br>3-(4-hydroxyphenyl)propionic acid<br>5-(3',4',-dihydroxyphenyl)- γ -valerolactone | (87) |
| | | | | | | | Urine (0 - 24 h after intake) | (epi)catechin<br>Epigallocatechin<br>Procyanidin<br>Protocatechuic acid<br>Vanillic acid<br>Ferulic acid<br>Caffeic acid<br>Coumaric acid<br>Chlorogenic acid<br>Hippuric acid<br>Homovanillic acid | |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Hydroxybenzoic acid | |
| | | | | | | | 3,4-dihydroxyphenylacetic acid | |
| | | | | | | | Dihydrocaffeic acid | |
| | | | | | | | Dihydroferulic acid | |
| | | | | | | | 3-(4-hydroxyphenyl)propionic acid | |
| | | | | | | | 4-hydroxyphenylacetic acid | |
| | | | | | | | 5-(3',4',-dihydroxyphenyl)- γ -valerolactone | |
| | | | | | | Faeces (on the day of experiment) | (Epi)catechin | |
| | | | | | | | Epigallocatechin | |
| | | | | | | | Procyanidin | |
| | | | | | | | Protocatechuic acid | |
| | | | | | | | Vanillic acid | |
| | | | | | | | Ferulic acid | |
| | | | | | | | Caffeic acid | |
| | | | | | | | Chlorogenic acid | |
| | | | | | | | Homovanillic acid | |
| | | | | | | | Dihydrocaffeic acid | |
| | | | | | | | Dihydroferulic acid | |
| | | | | | | | 3-(4-hydroxyphenyl)propionic acid | |
| | | | | | | | 5-(3`,4`,-dihydroxyphenyl)- γ -valerolactone | |
| Cocoa flavanols-capsules | Part 1: Cocoa flavanol extract: 1000 mg/d (2 capsules/d in wk 1+2), 1500 mg/d (3 capsules/d in wk 3+4), and 2000 mg/d (4 capsules/d in wk 5+6) | Crossover intervention study (Open-label, intake-amount escalated, 6 wk) | 33 | HPLC-UV/Vis-FLD-ECD | Targeted | Plasma (0-2 h, after intake and on days 1,15,29,43, and 57 of the intervention) | Ec-3'-β-D-glucuronide | (69) |
| | | | | | | | Ec-3'-sulfate | |
| | | | | | | | 3'-*O*-Methyl-epicatechin-5-sulfate | |
| | | | | | | | 3'-*O*-Methyl-epicatechin-7-sulfate | |
| | | | | | | | 4'-*O*-Methyl(−)-epicatechin-5-sulfate | |
| | | | | | | | 4'-*O*-Methyl(−)-epicatechin-7-sulfate | |
| | | | | | | | (–)-Epicatechin | |
| | | | | | | | 5-(3',4'-dihydroxyphenyl)- γ -valerolactone | |
| | | | | | | | Theobromine | |
| | | | | | | | Caffeine | |
| | | | | | | | Paraxanthine | |
| | Part 2: Cocoa flavanol extract: 1000 mg/d (2 capsules/d in wk 1), 1500 mg/d (3 capsules/ d in wk 2), and 2000 mg/d (4 capsules/d in wk 3-10) | Single arm Intervention study (12 wk) | 59 | HPLC-MS/MS | Targeted | Plasma (0-2 h, after intake and on days 1,43,85, and 99 of the intervention) | 5-(3',4'-dihydroxyphenyl)- γ –valerolactone | |
| | | | | | | | Theobromine | |

| Dietary factor | Dose of intervention | Study design | # sub-jects | Analytical method | Approach | Sample type | Candidate Biomarkers of Food Intake | Primary Reference |
|---|---|---|---|---|---|---|---|---|
| Cocoa extract as part of a ready-to-eat meals | 1.4 gram cocoa extract | Single arm Intervention study (4 wk, part of a weight loss diet) | 47 | HPLC-qTOF-MS | Untargeted | Urine (24h, before and after the study) | 3-methylxanthine<br>3-methyluric acid<br>l-Beta-Aspartyl-L-phenylalanine<br>2,5,7,3',4'-Penthahydroxyflavanone 5-O-glucoside<br>7,4'-Dimethoxy-6-C-methylflavanone<br>3-methoxy-4-hydroxyphenylglycol sulphate<br>Uridine monophosphate | (101) |

Abbreviations: $^{1}$H NMR, Proton nuclear magnetic resonance; $^{13}$C NMR, Carbon-13 (C13)nuclear magnetic resonance; CEAD, coulometric electrode array detector; DAD, diode array detector; ECD, Electrochemical detection; ESI, Electrospray ionization; FLD, fluorescence detector; GC-MS, Gas Chromatography - Mass spectrometry; GL, glycyrrhizin; HPLC, High-performance liquid chromatography; LC, liquid chromatography; MS/MS, Liquid chromatography-tandem mass spectrometry; PDA, photodiode array detector; RP-HPLC, Reversed-phase high-performance liquid chromatography; SPE, solid phase extraction liquid chromatography tandem mass spectrometry; UPLC: Ultra performance liquid chromatography; UV, Ultraviolet ; VIS, visible light

# 7

# General Discussion

## AIM OF THE THESIS

The aim of this thesis was to identify potential dietary exposure and effect biomarkers, using various omics approaches.

## DIETARY EXPOSURE AND EFFECT BIOMARKERS

In all chapters, we measured various dietary exposure and effect biomarkers by using different study designs. One of the points we observed was that although biomarkers of exposure were consistently increased, this was not always paralleled by changes in effect biomarkers in all individuals. This finding points towards a good individual compliance with the dietary intervention that not always resulted in a diet-induced effect in each individual. For example, in **chapter 3** we observed that consumption of the Mediterranean diet resulted in a consistent increase in omega 3 fatty acids to total fatty acid ratio in the circulation, thereby reflecting the increased intake of fatty fish. However, the diet-induced significant decrease on effect biomarkers, such as several VLDL fractions, was not robustly changed in all individuals. This indicates that even though each individual in the Mediterranean diet group consumed fatty fish, as shown by the consistent increase in the exposure biomarker, this did not always translate into a change in effect biomarkers, such as the VLDL fractions. Interestingly, in the fenofibrate versus fish oil study (**chapter 2**), the fish oil intervention robustly increased exposure biomarkers, e.g. cholesterol esters and lyso-phosphatidylcholines containing EPA and DHA, which were quite consistently paralleled by induced changes in effect biomarkers, e.g. a decrease in the saturated TG species. However, also in this study the increase in exposure markers reflecting fish oil intake was not paralleled by a decrease in saturated TG species in one individual. Hence, if an individual does not respond to a dietary intervention, this does not necessarily indicate that this individual was not compliant with the dietary intervention. Furthermore, this indicates that large variation in individual responses can occur upon dietary interventions. Also in other chapters we observed these differences in responses. For example, in **chapter 5** most individuals showed a decrease in their glucose levels upon the 26-hour fast, but surprisingly a couple of individuals showed an increase in their glucose levels. Slight inter-individual variations were also observed in several other measured parameters in this chapter, such as LPL and ANGPTL4 gene and protein expression in the subcutaneous adipose tissue. The highest inter-individual variation was observed in **chapter 4**, where the microRNA responses differed considerably in size as well as direction among the individuals.

Several factors may be responsible for this inter-individual variation in response. First, genetics may play a role. For example, carriers of the *APOE4* genotype show a larger decrease in plasma TG after supplementation with fish oil compared to non-carriers (1). Additionally, by using a transcriptomics approach it was observed that carriers of the *APOE4* genotype induced a higher expression of cholesterol biosynthesis pathways upon fish oil supplementation, compared to non-carriers (2). As we did not examine genetic variations, we cannot be sure whether this might have played a role in the inter-individual variation in response observed in our intervention studies. Secondly, inter-individual variation in response might be caused by habitual dietary intake, as some individuals might already have had a higher intake of the diet/intervention foods at the start of the study. Therefore, these individuals are less likely to respond to the intervention. However, in the Mediterranean diet study (**chapter 3**) habitual dietary intake has likely not played a role, as this study included a run-in period of two weeks, thereby adjusting for prior dietary habits (3). Thirdly, early developmental state of metabolic disease such as diabetes type II or CVD might have affected metabolic pathways (4), and thereby either the response to the intervention, or baseline levels at start of the intervention. In our studies apparently healthy participants were included, but as disease onsets often occur long before actual symptoms of a disease are present, it is possible that some participants might already have had (unknown) light disturbances in their metabolism. For example, increased amounts of triglycerides in the liver, e.g. a fatty liver, can cause disturbances in hepatic lipid metabolism (5, 6), thereby influencing the response to a dietary intervention with subsequent consequences for secretion of circulating lipoproteins, such as VLDL. In addition, high levels of plasma TG at baseline have shown to lead to a bigger decrease in plasma TG upon fish oil supplementation (7-9). It is therefore possible that if an individual has low levels of circulating TG at baseline, the expected fish oil induced effects on TG levels will be smaller, or even absent. In this thesis, we observed that the four individuals who showed an increase (instead of the average decrease) in serum TG and VLDL fractions upon the Mediterranean diet intervention (**chapter 3**), had significantly lower TG levels at baseline (0.81 ± 0.39 mmol/L, n=4), compared to the baseline TG levels (1.56 mmol/L, n=10) of individuals who showed a decrease in serum TG, and VLDL fractions ($p$-value = 0.042). This suggests that indeed baseline differences can influence the response in the effect biomarkers.

Furthermore, this thesis showed that the higher the stressor/exposure the participants were exposed

to, the lower the inter-individual variation. The prolonged fast (**chapter 5**) was a stringent exposure and induced the biggest and most consistent effects, compared to effects seen in dietary interventions using a whole diet approach, such as the study on the Mediterranean diet and MUFA described in **chapter 3**, and the study on nutrient quality and energy restriction described in **chapter 4**. As expected, the pharmacological agent fenofibrate also induced consistent effects, and more surprisingly so did the fish oil intervention (**chapter 2**). The dosage used in the fish oil group however, was very high (18.5 times the recommended daily intake), which may have led to these consistent almost drug-like results. The less consistent effects on diet effect biomarkers using whole diet approaches (**chapters 3** and **4**), are quite commonly observed in human nutrition studies with intakes within the boundaries of the recommended daily intakes. In the literature this has often been attributed to a possible lack of compliance in the non-responding individuals (10). However, the whole diet interventions applied here were either fully controlled (**chapter 3**), or well monitored (**chapter 4**), and in the Mediterranean diet study (**chapter 3**) adherence to the diets was confirmed by the observed increase in the exposure biomarker: omega 3 to total fatty acids ratio. The observed higher inter-individual variation in response in the studies using a whole diet approach in this thesis, were therefore likely not due to a lack of compliance, and point towards real differences in response to diet between individuals.

The question remains how robust these responses to the same diet are within an individual. As in most dietary intervention studies, we measured the response to an intervention only once. To unravel the robustness of an individual response to a dietary intervention, the intervention needs to be repeated in the same individual. If we know to what extend individuals respond consistently to a dietary intervention, personalized dietary advice can be given.

***Ambiguity in Biomarker Categories***

In this thesis we examined dietary exposure and effect biomarkers. An effect biomarker reflects the functional response of the human body to a (dietary) exposure, while an exposure biomarker reflects short or long term dietary intake (11, 12). Therefore, the reported increase in the ketone body 3-hydroxybutyrate upon the consumption of chocolate found in the literature review (**chapter 6**), cannot be categorized as an exposure biomarker, but may rather reflect an effect biomarker. Besides dietary exposure and effect biomarkers, there is another category of biomarkers, namely health biomarkers. A health biomarker is defined as a biomarker associated with an increased or decreased

risk of developing a particular disease. Diet-induced changes in health biomarkers therefore represent the functional effects of nutrition on health status. An example of a health biomarker examined in this thesis can be found in **chapter 3**, namely the small LDL subclass. This small LDL subclass is associated with an increased risk for developing cardiovascular diseases, and as such represents not only an effect biomarker, but can also be seen as a health biomarker. This illustrates that distinctions between the different categories of biomarkers are not always straightforward. Several biomarkers that we identified in this thesis could be placed into more than one biomarker category. Another example: the observed increase in unsaturated lipid species in **chapter 2**, was likely the result of an increased incorporation of the consumed fish oils (13-15), and therefore represents a good exposure biomarker. However, it is also very well possible that fish oil activation of PPARα has induced several genes involved in fatty acid desaturase and/or elongase, such as *FADS1* or *ELOVL2* (16), resulting in an increase in these unsaturated lipid species (4, 17, 18). Therefore these unsaturated lipid species may also be viewed as effect biomarkers. Additionally, an increase in these unsaturated lipid species have been inversely associated with obesity and insulin resistance (19-23), and may therefore also reflect health biomarkers. Taken together, the observed increase in unsaturated lipid species can be categorised as an exposure, effect, and health biomarker, illustrating the existing ambiguity among the different biomarker categories. In the literature it is also recognized that overlap exists between the different biomarker categories (11, 24). As a result, a new concept is proposed to classify the biomarker based on its intended application (11). Thus, depending on the study purpose, a biomarker can be viewed as a dietary exposure, effect, or health biomarker. For example, while glucose measurements can also fall within all three categories, the purpose of the study in **chapter 5** was to examine the functional response of the human body to a prolonged fast, therefore the glucose measurement in this particular study can be classified as a biomarker of effect. This thesis has confirmed that indeed for several biomarkers there is ambiguity among the different categories. Furthermore, it highlighted that it is relevant to explore which markers merely present food intake, which markers are reflecting cellular or systemic changes for example via the activation of molecular pathways, which markers are proxies for health, and which markers can reflect multiple conditions.



***Lack of validation for exposure biomarkers***

In our systematic review (**chapter 6**), we identified many potential exposure biomarkers for cocoa and liquorice in the literature. However, very little research has focused on subsequent validation of

these identified biomarkers. In line with this, the exposure biomarkers used in the rest of this thesis have been identified as exposure biomarkers in the literature, but also the level of validation for these biomarkers is still subject to further research. For example, the cholesterol ester(20:5) and lyso-phosphatidylcholine(22:6) species for fish oil intake in **chapter 2**, and the CLA/FA ratio for butter intake in **chapter 3**, have been identified as exposure biomarkers for these foods in the literature. However, their current level of validity is unclear. Within the Food Biomarkers Alliance (FOODBAll[1]) consortium (25), massive efforts have been undertaken to 1) obtain overviews of identified biomarkers of intake for several food products, and 2) to examine their current level of validity (26-33). **Chapter 6** was a part of this consortium, and in accordance with our observations, it was evident that also for the other reviewed food products, such as dairy, legumes, fruit, and vegetables, most research had focussed on the identification of biomarkers, rather than its validation. As a consequence well-validated biomarkers are still lacking. To advance the field of exposure biomarkers, future research needs to focus on validating the identified biomarkers in the literature, according to the criteria outlined by Dragsted and colleagues (34). Once validated exposure biomarkers are obtained, they can provide unbiased estimates of dietary exposure, which will aid the long-term goal to unravel the true links between exposure and health. This may ultimately contribute to less reported inconsistencies on the relationships between dietary exposure and health, and might thereby increase the consumers trust in nutrition research (35).

## USE OF METABOLOMICS FOR BIOMARKER IDENTIFICATION

In this thesis, metabolite changes were assessed using various metabolomics technologies, such as LC-MS, GC-MS, and NMR (**chapter 2,3,6**). We revealed that metabolomics is a powerful tool to identify dietary exposure and effect biomarkers, such as cholesterol ester22:6 and total omega 3 fatty acids in **chapter 2**, and the different LDL and VLDL fractions in **chapter 3**. However, the metabolomics field still encounters several challenges.

First, unlike for transcriptomics, for metabolomics there is no single technology that is able to provide a complete analysis of the metabolome, instead the different technologies give complementary information. As a result, multiple technologies are often used in parallel in metabolomics studies. For example, in **chapter 2**, we combined a GC-MS platform with two LC-MS platforms, resulting in

1 http://www.foodmetabolome.org/

a better coverage of the circulating metabolites. However, even when using multiple metabolomics technologies, the degree of completeness that is reached in transcriptomics analysis cannot be reached in metabolomics analysis, as most of the metabolome is yet to be discovered (36).

Second, for untargeted metabolomics there is still a challenge to elucidate the structural identification of small molecules (36). In **chapter 2**, also several metabolites were listed as 'unidentified', and six of these metabolites were statistically significantly changed by either the fish oil or the fenofibrate intervention. This indeed indicates that part of the information collected in this study has not been unveiled, and highlights the challenging need for improvements for the identification of unknown metabolites in metabolomic studies.

Lastly, another challenge with using metabolomics technologies is the shortage of proper reference standards. Currently, often unmetabolized parent compounds are used as a reference to quantify metabolites, but studies have shown that this can lead to large under and overestimations of the metabolite concentrations (37). This exposes the importance of using structurally-identical analytical reference standards when quantifying metabolite concentrations. In terms of food derived metabolites, advancements have been made by the initiation of the online catalogue FoodComEx (38), that facilitates sharing of standards, but this collection is still far from complete. The use of proper analytical reference standards will also be important to validate identified exposure biomarkers, to obtain absolute instead of relative concentrations of metabolites (39), and to increase comparability between different studies.



## MICRORNAS AS BIOMARKERS

Apart from exploring the diet-induced effects on exposure and effect biomarkers using metabolomics, we also examined diet-induced effects on microRNA expression in human subcutaneous adipose tissue. MicroRNAs form an interesting layer of control, as they can influence gene expression by inducing cleavage of mRNAs or by inhibiting protein translation. Exploring the role of microRNAs within the effects of human nutrition is still in its infancy. In **chapter 4** we explored the effects of energy restriction, and the effects of energy restriction in combination with a change in nutrient quality, on the expression of microRNAs in human subcutaneous adipose tissue. Interestingly, we observed that the high- and low-quality 25% energy restricted diets induced shared, as well specific effects on microRNA expression in human subcutaneous adipose tissue, thereby indicating that nutrition may

indeed be able to affect microRNA expression in human subcutaneous adipose tissue. Links between diet-induced microRNA and gene expression changes were shown by subsequent integration of the microRNA data with the available gene expression data in the same participants. In this chapter we showed that both diet and weight-loss are indeed able to affect microRNA expression in the adipose tissue that are also linked to changes in gene expression, and hence may affect metabolism via changes in pathways or signalling routes. The microRNA profiling yielded a wealth of information, but also revealed several challenges that need to be recognized.

First of all, because of its novelty within nutrition research there are currently little to no biological and experimental study replicates of the effects of diet on microRNA expression in human subcutaneous adipose tissue. Only two other human intervention studies have examined the effect of diet-induced weight loss on subcutaneous adipose tissue microRNA expression (40, 41). However, none of these studies reported a change in the microRNAs that were affected by weight loss and nutrient quality in our study (**chapter 4**). Additionally, the results of the two other studies also did not show any overlap. This might partly be explained by differences in study designs and populations, but without enough studies to verify this we cannot fully explain these differences. This thesis thereby highlights the need to replicate studies, and to examine the robustness of these observed diet-induced effects on microRNA expression in subcutaneous adipose tissue.

A second point we would like to raise is that for many of the microRNAs that were changed by energy restriction or a combination of energy restriction and nutrient quality, little is known about their functional or health effects in the literature. For most of these microRNAs only few target genes were described. Additionally, possible associations with health or disease status were lacking. This stresses the need for research that focusses on the link between microRNA expression, cellular pathways, and health. Once we know more about the functionality of these microRNAs, we will be able to interpret the meaning of the observed diet-induced microRNA expression changes in the subcutaneous adipose tissue.

Third, we used microRNA array plates of Affymetrix, that had 100% coverage of miRBase v20 (42). However, currently there are no clear criteria for microRNA annotation, and the online repository miRBase does not curate the input of microRNAs, but only serves as an open access repository for

published microRNA sequences. This means that the miRBase database can potentially contain many false positives. Fromm and colleagues (43) even argued that nearly two-thirds of the miRBase entries for human microRNAs are false positives, i.e. sequences not derived from microRNA genes, which would clearly obstruct microRNA research. In order for the field to move forward, clear agreements must be made on which criteria should be met in order for a microRNA to be evaluated as a real microRNA.

Lastly, and possibly the biggest challenge of microRNA research in the field of nutrition, is that the interpretation of microRNA data is not straightforward. It is known that each microRNA has the potential to target the expression of numerous genes (44), and can therefore be involved in many different pathways. Accordingly, the biological function of a single microRNA can be diverse, and can differ depending on which organ is examined (45). This creates a challenge to pinpoint the exact biological function of each microRNA. Moreover, to investigate these functional roles of microRNAs, it is critical to identify which genes are targeted by which microRNA. Unfortunately, information on biological targets of most microRNAs is still missing, is only partly known, or is based on computational methods only. The latter is troublesome, since computational methods can give rise to many false positives, and at the same time can miss important gene targets, as some microRNAs are known to be only partially complementary to their targets (46). There are several databases available for placing microRNAs into gene-regulatory networks by using both predicted (in silico) as well as validated microRNA targets, for example: TargetScan (47), DIANA-microT (48), and miRDB (49). However, also in these databases unravelling microRNA functionality remains largely restricted by the current limited knowledge and predictions of target genes. Taken together, more detailed molecular data on the effects of the interaction between microRNA, genome, and health is needed to be able to interpret the biological meaning of observed changes in microRNA expression.



***Adipose tissue versus circulating microRNAs***

We explored the diet-induced effects on microRNA expression in the adipose tissue, as the subcutaneous adipose tissue is an important fat storage depot of the body that is affected early in the development of obesity, and can be beneficially affected by weight loss (50). Interestingly, the adipose tissue has shown to excrete microRNAs in extracellular vesicles, specifically in exosomes (51). The majority of the microRNAs in the circulation is present in these exosomes (52-54). These exosomal

microRNAs have shown to regulate the metabolism in distant tissues, such as the liver and skeletal muscle (51, 55). As a result, they have been proposed as novel adipokines. It will be interesting to examine which of the currently measured microRNAs in the subcutaneous adipose tissue has the potential to be secreted into these exosomal vesicles in the circulation. This might pave the way for the use of these exosomal microRNAs as either effect, or health biomarkers of disturbed or improved subcutaneous adipose tissue function.

## FUTURE RECOMMENDATIONS

In this discussion, we made several recommendations for future research. For dietary exposure biomarkers, we proposed to focus future research on increasing the validation of already identified exposure biomarkers in the literature. For microRNA expression there is a need for 1) replication of studies to examine te robustness of the observed diet-induced effects, 2) clear agreements on criteria to evaluate microRNAs as a real microRNA, and 3) additional research to obtain more detailed molecular data on the interaction between microRNA, genome and health, which thereby enables the interpretation of the biological meaning of the observed diet-induced changes in microRNA expression. Additionally, we suggested to investigate which of the subcutaneous adipose tissue microRNAs are also present in exosomal vesicles in the circulation, and can thereby serve as effect biomarker of disturbed or improved subcutaneous adipose tissue function.

In the beginning of this discussion, we also touched upon the possibility for tailoring dietary advice, based on the robustness of inter-individual variation. In the future, this could even be taken a step further. Instead of measuring whether an individual responds to an intervention, and subsequently tailor his/her/their dietary recommendation based on this information, it would be interesting to examine if we can predict why certain individuals do or do not respond to a specific intervention. For this, the integration of multi omics approaches, and the integration of omics data with phenotypic data, could lead to a machine-learning algorithm that will enable predicting responses to dietary intakes. Ultimately, this may lead to personalised nutrition advice based on personal data. This is an ambitious challenge for the future, both to integrate multiple omics, and omics with phenotypic data, into meaningful data as well as to translate the resulting data into accurate predictions of beneficial effects for individuals.

To eventually extrapolate personalized dietary advice to the general public, it will be crucial to be able to

obtain samples in which biomarkers can be assessed in a non-invasive way at home. For this, the use of Dried Blood Spots (DBS) is promising. DBS are collected drops of whole blood on filter paper that can easily be obtained at home from a single finger prick (56). Through advancements of techniques it is possible to measure several dietary exposure and effect biomarkers in these DBS, such as fatty acids, HDL, or vitamin D (57, 58). First studies even indicate that also transcriptomic and metabolomics analyses may be performed in DBS (59-61), highlighting their great potential to be used within the field of biomarker identification. Also given the current restrictions inflicted by the coronavirus pandemic, these DBS are of great interest, as participants do not have to travel to a clinic to have their samples taken, but they can be obtained safely at home. Besides DBS, the application of sensors is promising, as sensors can be relatively easy applied, and can be used to monitor biomarker excursions during the day. A lot of research has been done with continuous glucose monitors. Ultimately, this information could add to the knowledge upon which a machine learning algorithm for personalised nutrition can be based.

## CONCLUSION

In this thesis, we identified potential dietary exposure and effect biomarkers using various omics approaches. Based on the findings in this thesis, we conclude that metabolomics is a powerful tool to identify both diet-induced effect biomarkers and exposure biomarkers. The combination of both types of biomarkers within a study let us to conclude that the high variation in response to a dietary intervention cannot always be ascribed to a lack of dietary compliance, but rather paves the way for research on personalised dietary advice. This thesis also confirmed the ambiguity that exists among the different biomarker categories, and highlighted the relevance to explore whether a biomarker is an exposure, effect, or health biomarker, or can reflect multiple conditions. We further concluded that although many potential exposure biomarkers have been identified in the literature, well-validated exposure biomarkers are missing, hampering their current use in nutrition research. Lastly, we showed that microRNA expression in the subcutaneous adipose tissue can be affected by type of diet and weight-loss, demonstrating the potential future use of microRNA profiling in nutrition research. However, before microRNAs can provide a useful addition to the omics arsenal within nutrition research, several challenges need to be overcome, such as the current limited knowledge on the interaction between microRNA, genome, and health. If these challenges can be overcome, the future

of microRNA analysis within nutrition research holds strong promise.

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# Summary

Nutrition can play a pivotal role in preventing and sometimes even reversing obesity and associated diseases. Traditionally, the effects of nutrition on health were assessed by the use of a few single biomarkers, known as classical biomarkers of a disease state. While the low hanging fruits of diet-induced effects on health have been identified using these markers, new approaches are needed to capture the more subtle diet-induced effects of nutrition on health. Hence, there is a need for more sensitive and comprehensive biomarkers. These biomarkers are called diet effect biomarkers, and reflect the functional response of the human body to an exposure, i.e. nutrition. Developments in the omics field have paved the way to explore and identify these diet effect biomarkers. Additionally, these omics techniques can be used to improve measurements of dietary intake, by providing objective measurements for short and long term dietary intake, called exposure biomarkers. In this thesis, we aimed to identify potential diet effect and exposure biomarkers, by applying several different dietary exposures and study designs, and using various omics approaches

A comparative analysis between the effects of a drug treatment versus a dietary intervention on plasma metabolomics, using GS-MC and LC-MS was performed in **Chapter 2**. In this chapter, we examined the effects of a placebo controlled cross-over intervention trial with either 3.7 g/d n-3 LCPUFA (fish oil), or 200 mg fenofibrate for 6 weeks on plasma metabolomics. Fenofibrate as well as fish oil, altered the metabolomic plasma profile markedly by reducing the saturated state of the triglyceride fraction. Fish oil additionally increased several unsaturated lipid species, which was likely caused by an increased incorporation of the consumed unsaturated fatty acids in these circulating lipid species, and possibly by changes in several regulatory pathways induced by fish oil. This study pointed towards the power of consuming a high dose of a nutrient to change the circulating lipid profile in a potentially beneficial way.

In **Chapter 3** we disentangled the effect of the MUFA content in a Mediterranean diet, from the other components of a Mediterranean diet on serum metabolomics, as measured by NMR. We found that after 8 weeks of intervention, the MUFA content reduced several LDL subclasses and fractions, and was therefore responsible for a less atherogenic lipid profile. The addition of the other dietary components as provided by the Mediterranean diet, including fatty fish, reduced the larger subclasses of VLDL, VLDL-triglycerides, and serum triglycerides. A Mediterranean diet therefore resulted in additional favourable effects on risk factors for CVD.

In **Chapter 4**, we explored the effects of a high quality ER-diet and a low quality ER-diet for 12 weeks, on microRNA expression in human subcutaneous adipose tissue. We found that microRNA expression in human subcutaneous adipose tissue was influenced both by diet-induced weight loss and by the nutrient quality of the diet. These microRNA expression changes were integrated with gene expression changes, which resulted in differences in expression of the microRNA targeted genes between the two ER diets. Our explorative study showed that microRNAs are likely affected by diet. The exact functional significance of the nutrition-induced effects on microRNA expression in human SAT needs to be further elucidated.

In **Chapter 5**, the effects of a prolonged fast on several metabolic regulators in the subcutaneous adipose tissue were examined. We observed a significant decrease in LPL activity in human adipose tissue, concurrent with an increase in ANGPTL4 mRNA and protein expression, In line with the adipose tissue data, plasma ANGPTL4 levels increased upon fasting, as did plasma cortisol levels and fatty acids levels. By contrast, insulin levels significantly decreased. Supported by additional analyses in primary human adipocytes, we hypothesized that the increase in ANGPTL4 in adipose tissue is potentially regulated via a fasting-induced increase in plasma cortisol and free fatty acids, and a fasting-induced decrease in plasma insulin, ultimately leading to a decrease in LPL activity.

In **Chapter 6**, the literature was systematically reviewed to obtain an overview of candidate food biomarkers for cocoa (products) and liquorice, to evaluate their validity, and to address further validation or identification work to be done. No specific biomarkers of food intake were found for cocoa, but 18-glycyrrhetinic acid was identified as a promising candidate for liquorice, although further validation work is still necessary. Our findings indicated that currently no acceptable biomarkers for cocoa and liquorice exist, and more studies are needed to be able to assess dietary intake of these specific food products in human biospecimens.

In this thesis, we identified potential dietary exposure and effect biomarkers using various omics approaches. Based on the findings in this thesis, we conclude that metabolomics is a powerful tool to identify both diet-induced effect biomarkers and exposure biomarkers. The combination of both types of biomarkers within a study let us to conclude that the high variation in response to dietary intervention cannot always be ascribed to a lack of dietary compliance, but rather paves the way for research on

personalised dietary advice. This thesis also confirmed the ambiguity that exists among the different biomarker categories, and highlighted the relevance to explore whether a biomarker is an exposure, effect, or health biomarker, or can reflect multiple conditions. We further concluded that although many potential exposure biomarkers have been identified in the literature, well-validated exposure biomarkers are missing, hampering their current use in nutrition research. Lastly, we showed that microRNA expression in the subcutaneous adipose tissue can be affected by type of diet and weight-loss, demonstrating the potential future use of microRNA profiling in nutrition research. However, before microRNAs can provide a useful addition to the omics arsenal within nutrition research, several challenges need to be overcome, such as the current limited knowledge on the interaction between microRNA, genome, and health. If these challenges can be overcome, the future of microRNA analysis within nutrition research holds strong promise.

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## About the author

## CURRICULUM VITAE

Charlotte C.J.R. Michielsen was born on July 25th, 1991 in Eindhoven, the Netherlands. In 2009, she started the bachelor program Nutrition and Health at the Wageningen University. During this program she composed her own minor, which she called 'Psychology – Mind blowing' that consisted of several courses from the bachelor program of Psychology at the Maastricht University. During her bachelor she also wrote a BSc thesis that examined the effect of epigenetic modifications on cancer, influenced by diet. Apart from her academic life, Charlotte joined the student association K.S.V. Sint Franciscus Xaverius, where she made life-long friends and became a member of the official year club 'JC Konfett!'.

In 2012 she received her degree and continued her academic career by joining the master program Nutrition and Health at the Wageningen University, where she combined the specializations Molecular Nutrition & Toxicology and Epidemiology & Public Health. After successful completion of two MSc theses examining the effects of chocolate and flavonoid intake on microRNA expression, Charlotte moved to Ireland, where she did her internship at University College Dublin on the usability of the biomarker proline betaine to measure orange juice intake. This internship resulted in her first publication. During her MSc program Charlotte was a member of the Pas-, the LEx-, and the Almanac-committee of the study association Di-Et-Tri. End of 2015 she was appointed as a PhD candidate in the Food Biomarker Alliance project in the group of Prof. dr ir Edith Feskens, under the supervision of dr ir Lydia Afman. Here, Charlotte identified candidate biomarkers for cocoa and liquorice intake, and she examined the effects of a Mediterranean diet on metabolomics. For the latter, she won a poster price during the Nutrigenomics week in Denmark 2016, and a year later she presented her work during an oral presentation at the Nutrigenomics week in Bulgaria. In 2017, she, together with Lydia Afman, received a grant from the Alpro Foundation to continue her PhD project with the focus on nutrition and microRNA expression. As gradually her interests had shifted from data analyses to project leading, she was given the opportunity by Prof. dr ir Sander Kersten and Lydia Afman to lead a human intervention trial, in which the effects of fasting in human adipose tissue were examined. Thereafter, Charlotte concluded her PhD project with another human trial exploring the acute effects of differences in fat structure on several health markers. During her PhD project, Charlotte was also involved in teaching activities, including supervising several MSc and BSc students. Next to this, she was a member of the PhD committee of the Division of Human Nutrition and Health, and she helped organize the PhD tour to the U.K. in 2017. In 2019, she was selected to participate in the 25th Essentials seminar of the European Nutrition Leadership Platform. Additionally, Charlotte has been an active member of the Dutch Academy of Food Sciences (Nederlandse Academie voor Voedingswetenschappen, NAV), where she is part of the Young NAV committee that organizes several events throughout the year for professionals in the field of Nutrition. After the completion of her PhD in October 2020, Charlotte has continued her work as a postdoctoral researcher at the Division of Human Nutrition and Health at the Wageningen University, and she became a member of the works council of the Agrotechnology and Food Sciences group.

## LIST OF PUBLICATIONS

### *This thesis*

**Michielsen, C.C.J.R.**, Hangelbroek, R.W.J., Mensink, R.P., Afman, L.A., Comparative analysis of the effects of fish oil and fenofibrate on plasma metabolomic profiles in overweight and obese individuals, *In preparation.*

**Michielsen, C. C. J. R.,** Hangelbroek, R. W., Feskens, E. J. M., Afman, L. A., Disentangling the Effects of Monounsaturated Fatty Acids from Other Components of a Mediterranean Diet on Serum Metabolite Profiles: A Randomized Fully Controlled Dietary Intervention in Healthy Subjects at Risk of the Metabolic Syndrome. *Molecular Nutrition and Food Research 2019; 63; 1801095; https://doi.org/10.1002/mnfr.201801095*

**Michielsen, C.C.J.R.**, Hangelbroek, R.W.J., Hooiveld, G.J.E.J, Steinbusch-Coort, S.L.M., Afman, L.A., Effect of two energy restricted diets differing in nutrient quality on microRNAs, a parallel human intervention trial, *In preparation.*

Ruppert, P.M.M.*, **Michielsen, C.C.J.R.***, Hazebroek, E.J., Pirayesh, A., Olivecrona, G., Afman, L.A., Kersten, S., Fasting induces ANGPTL4 and reduces LPL activity in human adipose tissue. *Molecular Metabolism 2020; https://doi.org/10.1016/j.molmet.2020.101033*

**Michielsen, C.C.J.R.,** Almanza-Aguilera, E., Brouwer-Brolsma, E.M., Urpi-Sarda M., Afman L.A., Biomarkers of food intake for cocoa and liquorice (products): a systematic review. *Genes and Nutrition 2018; 13, 22; https://doi.org/10.1186/s12263-018-0610-x*

### *Other*

Gibbons, H., **Michielsen, C.J.R**., Rundle, M., Frost, G., McNulty, B.A., Nugent, A.P., Walton, J., Flynn A., Gibney, M.J., Brennan, L., Demonstration of the utility of biomarkers for dietary intake assessment; proline betaine as an example, *Molecular Nutrition and Food Research 2017; 61; 10; 1700037; https://doi.org/10.1002/mnfr.201700037*

Defour, M., **Michielsen, C.C.J.R.**, O'Donovan S., Afman, L.A., Kersten, S., Transcriptomic signature of fasting in human adipose tissue, *Physiological Genomics 2020; https://doi.org/10.1152/physiolgenomics.00083.2020*

Burton, K.J., Pimentel, G., Hughes, M., **Michielsen, C.C.J.R.**, Fatima, A., Vionnet, N., Pralong, F.P., Afman, L.A., Roche H.M., Brennan, L., Ibberson, M., Vergères, G., Discriminating dietary responses by combining transcriptomics and metabolomics data in nutrition intervention studies, *Accepted for publication in Molecular Nutrition and Food Research.*

Schutte, S., Esser, D., Daanje, M., **Michielsen, C.C.J.R.**, Matualatupauw, J., Siebelink, E., Boshuizen, H., Mensink, M., Afman, L.A., The diverging effects of nutrient quality within weight loss diets on metabolic health: a randomized controlled trial in subjects with abdominal obesity. *Submitted for publication.*

**Michielsen, C.C.J.R.***, Kroon, P.*, Hollman, P., Feskens, E.J.M., Brouwer-Brolsma, E.M.*, Measuring flavonoid intake using FFQ and triplicate 24-hour recall: a validation study within the NDARD-project. *In preparation.*

Gijbels A., **Michielsen C.C.J.R.**, Afman, L.A., Plasma FGF21 levels are not associated with weight loss or improvements in metabolic health markers upon 12 weeks of energy restriction in abdominally obese subjects, *In preparation.*

*authors contributed equally

## OVERVIEW OF COMPLETED TRAINING ACTIVITIES

### *Discipline specific activities*

*Courses*

- Personalised Nutrition: Use of large scale data in dietary intervention studies
  Københavns Universitet, Copenhagen (DK), 2016
- Introduction to Nutritional Metabolomics
  Københavns Universitet, Copenhagen (DK) 2016
- Metabolomics course
  BBMRI, Leiden (NL) 2016
- Nutrigenomics studies in humans: from epidemiology to intervention
  NuGO / Universitat de Barcelona, Barcelona (ES) 2015

*Conferences*

- Nutrition 2020 LIVE ONLINE
  American Society for Nutrition, Online 2020
- Nederlandse Academie van Voedingswetenschappen (NAV) meetings
  NAV / young NAV, Maastricht (NL) 2016, Utrecht (NL) 2017, Driebergen-Zeist (NL) 2018, 2019, Utrecht (NL) 2018, 2019, Online 2020
- (Dutch) Nutritional Science Days
  Independent / rotating organization, Heeze (NL), 2016, 2017 (*oral*), 2018 (*oral*), 2019
- NuGO week Mitochondria, Nutrition and Health
  NuGO / Newcastle University, Newcastle upon Tyne (UK), 2018 (*poster*)
- Metabolomics Consortium Meeting
  BBMRI, Leiden (NL), 2018
- NuGO week Molecular nutrition – understanding how food influences health
  NuGO / Medical University of Varna, Varna (BG), 2017 (*oral*)
- Alpro 20 years symposium The moment for plant-based eating is now
  Alpro foundation, Brussels (BE), 2017
- NuGO week Phenotypes and prevention: The interplay of genes, lifestyle factors and gut environment
  NuGO / Københavns Universitet, Copenhagen (DK), 2016 (*poster - First runner-up poster prize*)
- NuGO week Mechanisms of a long-life health
  NuGO / Universitat de Barcelona, Barcelona (ES), 2015

*General courses and activities*

- European Nutrition Leadership Platform 25th Essentials Seminar
  ENLP, Luxembourg City (L), 2019
- ICH Good Clinical Practice course
  Profess medical consultancy BV, Wageningen (NL), 2016, 2019
- Basic Course R
  Molecular Medicine, Rotterdam (NL), 2018
- Scientific Writing 3
  Wageningen in'to Languages, Wageningen (NL), 2017

- Master Class Mixed Models
  VLAG / Division of Human Nutrition and Health, Wageningen (NL), 2017
- Publish for Impact
  WGS, Wageningen (NL), 2017
- Reviewing a scientific paper
  WGS, Wageningen (NL), 2016
- Chemometrics
  VLAG / BIOMETRIS, Wageningen (NL), 2016
- VLAG PhD week
  VLAG, Soest (NL), 2016

*Optional activities*

- Weekly group meetings and orals
  Division of Human Nutrition and Health, Nutrition, Metabolomics and Genomics group and Nutrition and Pharmacology group 2015-2020
- Staff seminars
  Division of Human Nutrition and Health 2015-2020
- PhD study tour to the United Kingdom
  2017
- PhD study tour organisation
  Committee member (finances), Division of Human Nutrition and Health 2015-2017
- Monthly group meetings Paperclip
  Division of Human Nutrition and Health
  Global Nutrition group 2015-2017
- Preparation of research proposal
  2015

## COLOPHON

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