The effects of dietary saturated fatty acids on blood cholesterol response and body composition

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DECLARATION

I confirm this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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List of Publications and Presentations

Papers submitted for publication

Association between dietary saturated fat with cardiovascular disease risk markers and body composition in healthy adults- Findings from the cross-sectional BODYCON study

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Papers prepared for submission

Manuscripts have been prepared and will be submitted once the primary paper of BODYCON and RISSCI-1 study has been published.

Association between *APOE* genotype with body composition and cardiovascular disease risk markers is modulated by BMI in healthy adults: Findings from the BODYCON study

Ezgi Ozen, Rada Mihaylova, Natalie J Lord, Julie A Lovegrove and Kim G Jackson

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Impact of replacing dietary saturated fatty acids with unsaturated fatty acids on cardiovascular disease risk markers and body composition

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Impact of replacing dietary saturated fatty acids with unsaturated fatty acids on genes regulating cholesterol metabolism in peripheral blood mononuclear cells-Findings from the RISSCI-1 study

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Abstracts published in conference proceedings

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Impact of replacing dietary saturated with unsaturated fats on the expression of genes related to cholesterol metabolism in peripheral blood mononuclear cells: Findings from the RISSCI-1 study. Ezgi Ozen, Athanasios Koutsos, Rona Antoni, Gloria Wong, Laury Sellem, Barbara Fielding, Denise M Robertson, Bruce A Griffin, Julie A Lovegrove, Kim G Jackson**.** *Nutrition Society Summer Conference 2021: Nutrition in a Changing World, 6th-8th July 2021 (poster presentation)*

Impact of Dietary Fibre Intake on Body Composition and Cardiometabolic Disease Risk Markers. E. Ozen, R. Mihaylova, M. Weech, E. Read, J. A. Lovegrove and K. G Jackson. *FENS Conference, Dublin, Ireland 15-19 Oct 2019, (poster presentation)*

ABSTRACT

Reducing dietary saturated fat (SFA) intake via replacing it with unsaturated fatty acids (UFA) is a public healthy strategy for cardiovascular disease (CVD) prevention. However, with some studies reporting inter-individual variability in the low-density lipoprotein cholesterol (LDL-C) response to lowering dietary SFA, there is considerable interest in potential determinants underlying the responsiveness of LDL-C to fat intake. Human studies suggest that dietary fat composition affects body fat distribution with higher SFA intakes proposed to be associated with abdominal fat accumulation and greater CVD risk. However, it is not clear whether the variability in LDL-C response to dietary SFA intake is related to changes in body composition.

The relationship between dietary SFA intake with CVD risk markers and body composition was investigated using a cross-sectional (BODYCON, Chapter 2) and an 8-week sequential dietary intervention (RISSCI, Chapter 3) study. BODYCON included 409 healthy adults aged 18-70y, with data collected for total body composition, dietary intake, physical activity levels and circulating CVD risk markers. Although dietary SFA and abdominal visceral adipose tissue explained 9% of the variability in LDL-C, there was a lack of a dose-dependent relationship between increasing quartiles of dietary SFA%TE intake and these determinants, with total and LDL-C concentrations lower in Q2 (10.1-11.9%TE) than Q4 (14.9-38.7%TE) ($p \leq 0.05$). Of the anthropometric measures, only lean mass within the trunk (android) region was greater in Q3 (12.0-14.8%TE) compared to Q1 (1.9-10.0%TE) ($p=0.02$). Therefore, findings from the BODYCON study suggested that the effect of dietary SFA on LDL-C may be independent of body fat distribution. In the RISSCI study (n=41/109), significant reductions in fasting lipids (total, high density lipoprotein and LDL-C and triacylglycerol) (on average 10-15%, $p<0.01$) and percentage of android body fat were evident after replacing 8% dietary SFA with UFA. This suggested that, in the RISSCI study, the beneficial effects of replacing dietary SFA with

UFA on lipid CVD risk markers was associated with a reduction in central obesity in healthy men.

To provide insights into the mechanisms underlying the effects of dietary fat on LDL-C, the expression of genes involved in cholesterol metabolism were measured in a subset of the RISSCI participants (n=58/109). Compared with the high SFA diet, there was an upregulation of the LDL-receptor, ABCG1 and NR1H3 mRNA gene expression in peripheral blood mononuclear cells (PBMC) after the low SFA diet. To determine whether the variability in the LDL-C response to dietary SFA intake (-39% to +19%) was associated with changes in PBMC gene expression, the group was then stratified into responder (n=12) and non-responders (n=13) according to actual change in LDL-C in response to the change in dietary fat composition. Interestingly, prior to the start of the intervention, the fasting LDL-C concentration was 19% lower in non-responders compared to responders with a significant reduction in fasting lipids after replacing dietary SFA with UFA only evident in the responders group ($p \leq 0.05$). Although there was a non-significant tendency for the LDL-R mRNA expression to be increased in both responder and non-responders after replacing dietary SFA with UFA, it was only in non-responders that the ABCG1 and NR1H3 mRNA expressions were significantly upregulated after the low SFA diet ($p \leq 0.01$) (Chapter 4).

Studies investigating the associations between *APOLIPOPROTEIN* (*APO)E* genotype with CVD risk markers have generated inconsistent results, with a small number of studies suggesting that BMI plays an important role in this relationship. Using data from BODYCON (n=360), fasting blood lipids were found to be lower in the *APOE2/E3* than the *APOE3/E3* group and *APOE4* carriers in the normal BMI subgroup only ($p \leq 0.04$) (Chapter 5). Lower dietary fibre (g) and trans-fat (%TE) intake in the *APOE2/E3* participants than *APOE4* carriers, and a lower carbohydrate (%TE) intake relative to the *APOE3/E3* group were also evident in the normal BMI subgroup. *APOE* x BMI interactions on body weight and android fat mass were

observed ($p \leq 0.01$). While lean mass was higher in *APOE4* carriers than *APOE3/E3* in the normal BMI subgroup ($p=0.02$), the android:gynoid fat ratio was lower in *APOE4* carriers than *APOE3/E3* in the overweight/obese subgroup ($p=0.04$). Future studies should confirm the *APOE*-body composition association.

In summary, by using a combination of cross-sectional and interventional study designs, this thesis has generated novel findings into the role of dietary SFA on CVD risk markers, body fat distribution and responsiveness of genes regulating cholesterol metabolism to dietary fat manipulation. More studies are needed to determine mechanisms underlying the inter-individual variability in LDL-C response to dietary fat manipulation and to confirm *APOE*-body composition association to provide more effective personalised dietary advice.

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ABBREVIATIONS

ABCG1	ATP-binding cassette subfamily G member 1
ACAT	Acetyl cholesterol ester transferase
A/G ratio	Android:gynoid ratio
ANCOVA	Analysis of covariance
AOAC	Association of Official Analytical Chemists
APO	Apolipoprotein
BMI	Body mass index
BODYCON	Impact of physiological and lifestyle factors on body composition study
CHO	Carbohydrate
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DXA	Dual energy x ray absorptiometry
ER	Endoplasmic reticulum
HC	Hip circumference
HDL-C	High density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
LDL-C	Low density lipoprotein cholesterol
LDL-R	Low density lipoprotein receptor
MUFA	Monounsaturated fatty acid
NDNS	National Diet and Nutrition Survey
NEFA	Non-esterified fatty acids
NR1H3	Nuclear receptor subfamily 1 group H member 3
PA	Physical activity
PBMC	Peripheral mononuclear cells

PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
RCT	Randomised controlled trials
RISSCI	Reading Imperial Surrey Saturated fat Cholesterol Intervention study
SACN	Scientific Advisory Committee on Nutrition
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SFA	Saturated fatty acids
SNP	Single nucleotide polymorphism
SREBF1	Sterol Regulator Element Binding Transcription Factor 1
TAG	Triacylglycerol
TC	Total cholesterol
TE	Total energy
UFA	Unsaturated fatty acid
VAI	Visceral adiposity index
VAT	Visceral adipose tissue
VLDL	Very low density lipoprotein
WC	Waist circumference
WHR	Waist to hip ratio
WHtR	Waist to height ratio

Chapter 1: Literature review: Impact of dietary fat composition on cardiometabolic disease risk markers and body composition

The aim of this chapter is to provide a review of the current evidence on association between dietary saturated fat, body composition and cardiometabolic disease risk markers with a focus on the potential role of the *APOE* genotype on this relationship.

Contribution towards chapter 1:

EO wrote this chapter under the guidance of JAL and KGJ.

1.1 Introduction

The worldwide prevalence of obesity has nearly tripled between 1975 and 2021. Obesity is defined as excessive body fat accumulation and in the UK, an estimated 36% of adults are classified as obese compared with 13% of adults globally (1, 2). There is growing evidence that obesity is linked with non-communicable diseases such as cardiovascular diseases (CVD), some types of cancers, metabolic syndrome, and diabetes. Amongst these diseases, CVD remains the number one cause of morbidity and mortality worldwide and accounts for more than a quarter of all deaths in the UK (2, 3). However, it is becoming increasingly recognised that not all obese people diagnosed with, or at a greater risk of developing a metabolic disease, progress to CVD. This suggests that obesity per se is not always a marker for metabolic dysfunction (4). In agreement with this, clinical observations have reported a healthy metabolic profile in both lean and obese subjects related to the location of body fat storage (5). Therefore, it has become clear that body fat distribution may represent a better indicator of metabolic abnormalities than total adiposity (6, 7). Diet is one of the most important factors affecting adiposity (8), with dietary fat composition being argued to be more important than level of fat intake in relation to body fat accumulation (9, 10). Since the type of fat consumed in the diet can also affect CVD risk markers, there is now considerable interest in whether dietary fat composition has an impact on cardiovascular health via effects on the location of fat storage in the body.

Furthermore, genetic heterogeneity can contribute between 40-75% of the inter-individual variation in body mass index (BMI) (11), towards phenotype diversity and predisposition to adiposity. This genetic variation can occur when one nucleotide is replaced by another in the genome (referred to as a single nucleotide polymorphism (SNPs)) and can affect the function of the gene. The number of copies of the risk allele for a certain disease will impact on an individual's susceptibility to diseases such as obesity, with possession of 0 copies considered protective, whereas 2 copies increasing susceptibility to this disease (12). Numerous

studies have been undertaken to identify genes associated with body composition, which include candidate gene analysis and genome wide linkage studies (GWAS) (13). Using GWAS more than 1000 specific independent loci were found to be associated with obesity with FTO gene being the most commonly identified (14). Interestingly, *APOLIPOPROTEIN (APO)E,* a gene associated with CVD, has also been shown in a small number of studies to interact with BMI on CVD risk markers (15-17). This has generated an interest in potential mechanisms that explain the relationship between obesity and increased CVD risk.

In this literature review, the effects of dietary fat composition on CVD risk markers and body composition will be presented, with a focus on the potential role of the *APOE* genotype on this relationship.

1.2 Dietary Fat Composition and Cardiovascular Disease Risk

1.2.1 CVD and risk markers

CVD is the term given to the group of disorders including coronary heart disease (CHD), cerebrovascular disease (stroke), peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism (18). An estimated 18.6 million people died from CVDs in 2019 and if this trend continues it is expected to be responsible for over 23 million deaths by 2030, partly due to increasing obesity rates (19, 20). CVD is multifactorial in nature, with both modifiable (smoking status, blood pressure, blood cholesterol, obesity, physical activity levels and dietary habits) and non-modifiable (age, sex, ethnic background, and genetic susceptibility) risk factors. Of these risk factors, diet is recognised as one of the most important modifiable risk factors in relation to CVD and a cornerstone of public health recommendations for the prevention of this chronic disease. It is well-established that increased dietary SFA is linked with greater circulating low-density lipoprotein cholesterol (LDL-C) concentrations, an independent CVD risk factor (21).

Impaired lipid metabolism contributes to the development of CVD by causing atherosclerosis, in which plaque (cholesterol, cellular waste, calcium, fibrin) builds up within the arterial wall leading to an obstruction in the blood flow inside the arteries as shown in **Figure 1.1.** In healthy people, 70% of cholesterol is carried within LDL particles to deliver to the peripheral tissues in the body for cell membrane formation and hormone production by binding to the LDL-receptors (LDL-R) on the cell surface. When LDL particles are not cleared effectively from the blood stream and remain elevated, they can become oxidised and enter the arterial wall. Damaged endothelial cells can secrete cytokines which lead to recruitment of monocytes and macrophages that have scavenger receptors which can take up oxidised LDL and are not subject to the feedback regulation. Therefore, LDL can accumulate in macrophages and form foam cells which are the basis of the sub-endothelial fatty streaks. Furthermore, there is

evidence that remnants of triacylglycerol (TAG)-rich lipoprotein (such as chylomicrons and very low-density lipoproteins (VLDL)) which transport dietary and endogenous lipids around the blood stream can also be taken up by the macrophages leading to the formation of foam cells. In contrast, high-density lipoprotein (HDL) can reduce foam cell formation by a mechanism known as reverse cholesterol transport in which free cholesterol is removed from the growing atherosclerotic plaque and delivered to the liver for excretion from the body (22). Due to the roles of these lipoproteins in the development of atherosclerosis and CVD, risk predictions are based on circulating levels of blood cholesterol and triacylglycerol (TAG). The UK guidelines state that fasting TC concentration should be <5 mmol/L, HDL-C> 1 mmol/L for men and >1.2 mmol/L for women, LDL-C < 3mmol/L and TAG <1.2 mmol/L for CVD risk prevention (23).

Regulation of intracellular cholesterol and circulating LDL-C

LDL particles contain predominately esterified cholesterol and a small proportion of TAG surrounded by monolayer of phospholipids and free cholesterol, with apolipoprotein(apo) B-100 on the surface. Circulating LDL-C concentrations are regulated by LDL production and clearance (24). As mentioned previously, clearance of LDL is regulated by LDL-R which recognises apoB-100 on the surface of LDL and internalises the particles via endocytosis. After the particles are separated from LDL-R in the lysosomes, a proportion of the LDL-R recycle back to the cell surface whilst the LDL particles are degraded by lysosomes before cholesterol is released inside the cell. However, when proprotein convertase substilisin/lexin type 9 (PCSK9) binds to the LDL-R on the cell surface, LDL-R becomes internalised for degradation, therefore normal recycling of LDL-R would be disrupted (25). The LDL-R is expressed by the LDL-R gene in the liver and its activity and expression are mainly regulated by the intracellular cholesterol concentration (26). To maintain cholesterol homeostasis, when intracellular cholesterol levels are high, insulin induced gene 1 protein (Insig-1) binds to sterol regulatory

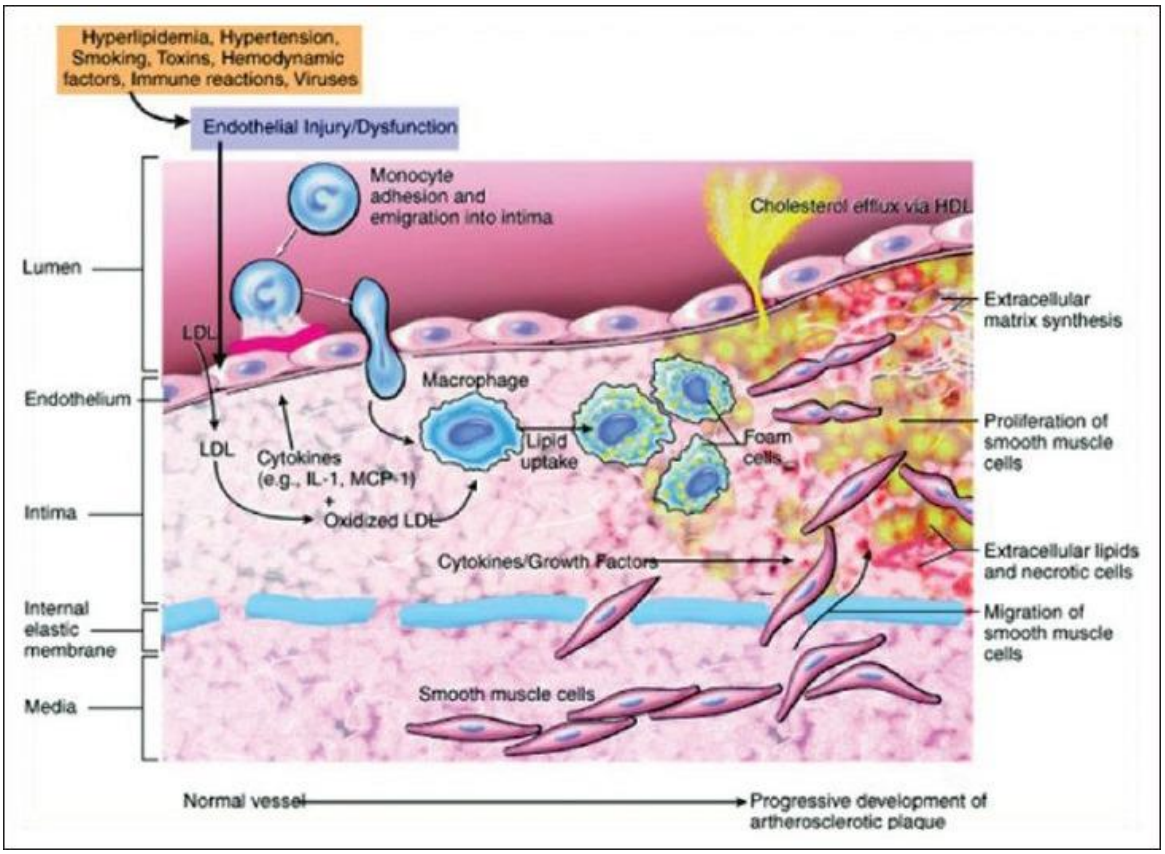


Figure 1.1 Atherosclerotic plaque formation. Elevated LDL particles become oxidised and cytokine secretion from damaged endothelial cells leads to recruitment of monocytes and macrophages within the sub-endothelium of the growing plaque which can take up oxidised cells and form foam cells (Adapted from (22)).

element binding protein-2 (SREBP-2)/SREBP cleavage-activating protein (SCAP) complex in the endoplasmic reticulum (ER), preventing it from reaching the Golgi apparatus where the release of mature SREBP-2 to the nucleus occurs. However, if the cholesterol levels in the cell decrease, Insig-1 does not bind to SREBP-2/SCAP complex allowing it to leave the ER where it matures in the Golgi apparatus, before binding to the sterol regulatory elements (SRE) in the nucleus to upregulate LDL-R gene expression (27) (**Figure 1.2**).

The nuclear receptor subfamily 1 group h member 3 (NR1H3) (also known as liver x receptor alpha (LXRα)) also plays a role in the regulation of the LDL-R expression indirectly by regulating intracellular cholesterol and lipid metabolism. They serve as cholesterol sensors and when intracellular cholesterol concentrations are increased, they stimulate the expression of ATP-binding cassette subfamily G member 1 (ABCG1), which play a significant role in reverse cholesterol transport. They also regulate the sterol regulatory element binding transcription factor 1 (SREBF1), which encodes SREBP-1c, an important transcription factor in hepatic fatty acid biosynthesis. Moreover, in human liver cells, NR1H3 agonists have been shown to induce the E3 ubiquitin ligase-inducible degrader of the LDL-R (IDOL/MYLIP) which targets LDL-R for degradation in the lysosomes (28, 29). Thus, NR1H3 and its target genes such as SREBF1 and ABCG1 play an important role in LDL-R expression and cholesterol metabolism. A summary of the pathways regulating LDL-R expression and cholesterol metabolism described above are shown in **Figures 1.2 and 1.3**.

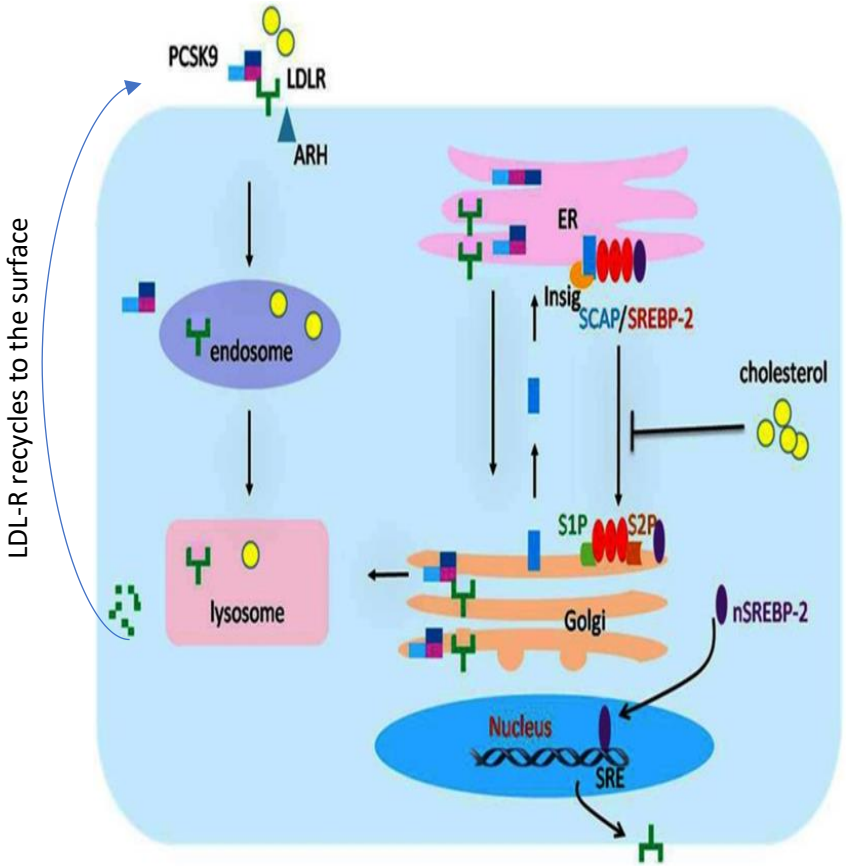


Figure 1.2. Cholesterol uptake by the low-density lipoprotein receptor (LDL-R) and transcriptional regulation of LDL-R expression. On the left-hand side of the figure, the LDL-R binds to LDL particles, LDL-R is internalised with autosomal recessive hypercholesterolemia (AHR) AHR protein, broken down in the lysosome where LDL-R is recycled to the surface. However, when proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to LDL-R it prevents recycling of LDL-R. When the intracellular cholesterol levels are high, sterol regulatory element binding protein (SREBP) cleavage activating protein (SCAP) protein binds to insulin induced gene 1 protein (Insig) which traps SREBP-2/SCAP complex in the endoplasmic reticulum (ER) membrane. In contrast, when the cells are low in cholesterol, SCAP does not interact with Insig proteins, SREPB-2/SCAP complex therefore reach the Golgi apparatus, mature SREBP-2 is released while SCAP returns to the ER. SREBP-2 enters nucleus and binds with sterol regulatory element (SRE) to activate LDL-R gene transcription (Adapted from(30)).

1.2.2 Dietary fat recommendations for CVD risk prevention

Dietary fat is a key element in the human diet in terms of its high energy content. The first hypothesis relating dietary fat with cardiovascular health and blood cholesterol levels was proposed by Keys in the 1950s (31). Subsequently, with numerous studies showing detrimental effects of dietary SFA on CVD risk, dietary fat composition gained importance in terms of its role in the development of CVD. Therefore, current dietary guidelines follow a similar pattern and suggest limiting dietary SFA intake to manage CVD. The Scientific Advisory Committee on Nutrition (SACN) report on SFA and health recommends the population average intake of SFA should remain less than 10% of total energy for CVD prevention (32). This target is reported in the UK Eat Well guide in grams as less than 30 grams of daily SFA intake for the average man and less than 20 grams for the average woman (33). Despite these recommendations to limit SFA intake to less than 10% of total energy, it still remains to be a significant source of energy in the UK diet of around 12% of total energy (25.3g/day) (34). While reducing dietary SFA intake has been a public health strategy in the UK, the replacement macronutrient will also play an important role in determining CVD risk. Studies have reported more beneficial effects on CVD risk markers after replacement of SFA with unsaturated fatty acids (UFA) than carbohydrates (CHO) or protein (35, 36). In the next section, studies investigating the effect of replacing dietary SFA with MUFA and PUFA on CVD risk markers will be presented. Detailed information about the studies included are shown in **Tables 1.1 and 1.2.**

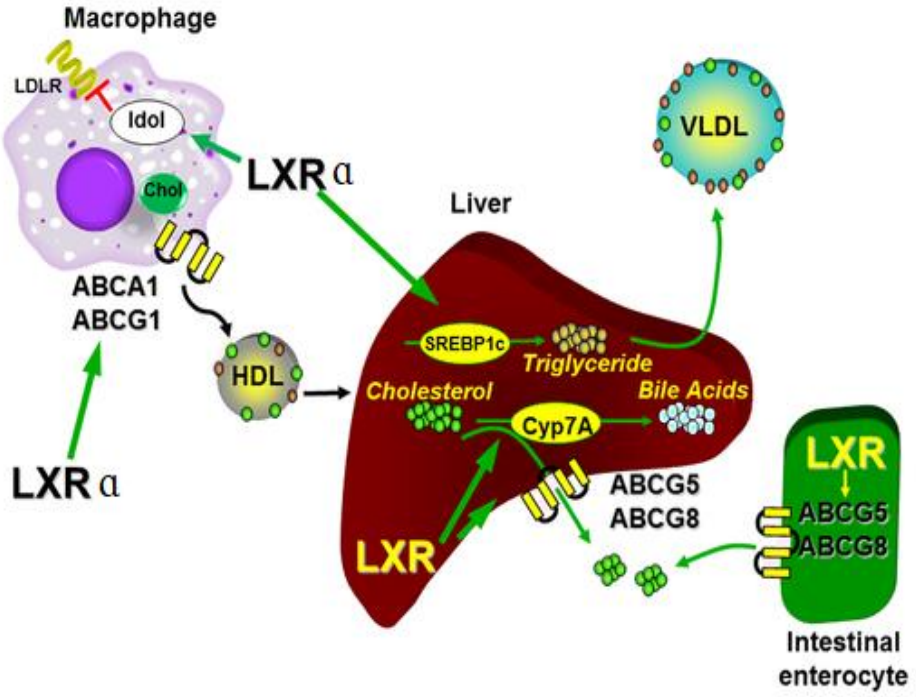


Figure 1.3. LXRα (NR1H3) regulates reverse cholesterol transport. Increased intracellular cholesterol in peripheral cells causes NR1H3 dependent upregulation of ABCG1 and ABCA1 which play a role in transporting intracellular cholesterol out of cells to HDL particles. Simultaneous regulation of IDOL downregulates the LDL-R while SREBP1c increases fatty acid synthesis and promotes secretion of VLDL. ABCA1: ATP binding cassette subfamily A member 1, ABCG1: ATP binding cassette subfamily G member 1, chol: cholesterol, Cyp7A cholesterol: 7 alpha-hydroxylase, HDL: high density lipoprotein, IDOL: inducible degrader of the LDLR, LDLR: low density lipoprotein receptor, NR1H3: nuclear receptor subfamily 1 group h member 3, SREBP1c: sterol regulatory element binding protein 1 c (adapted from (37)).

1.2.3 Replacement of SFA with n-6 polyunsaturated fat (PUFA) and monounsaturated fat (MUFA)

Substitution of SFA for PUFA on CVD risk

Perspectives from observational studies

Jakobsen et al. (38) reported an inverse association between dietary PUFA and risk of coronary events when 5% of energy (TE) from SFA was replaced with combination of n-3 and n-6 PUFA in their pooled analysis of prospective cohort studies including 344,696 subjects. They reported that substitution of SFA with PUFA reduced cardiovascular death rates by 26% while reducing the risk of cardiovascular events by 13% (38). Similarly, in a prospective cohort by Zong et al. (39) isoenergetic replacement of 1%TE from a combined group of SFA (C12:0-18:0) by PUFA was associated with a 6-8% reduction in CHD risk using a modelling approach. Comparable findings were also shown in the prospective cohort study in which Li and colleagues (40) illustrated that replacement of 5% TE from SFA with total PUFA was related to a 25% lower risk of CHD after 24 years of follow-up in the US cohort. Moreover, in this study, although CHO from refined starches/added sugars were associated with CHD, replacing 5%TE SFA with CHO from whole grains was associated with 9% lower risk of CHD. Moreover, Virtanen et al. (41) showed in their study that while SFA intake was not associated with CHD risk, when replaced with total PUFA, fatal CHD risk was reduced by 20% in 1981 men aged 42 to 60 y. However, analysis of data from the European Prospective Investigation into Cancer and Nutrition-Netherlands (EPIC-NL) cohort study in 35,597 subjects revealed that replacing SFA with total CHO, cis-MUFA or PUFA was related to increased ischemic heart disease (IHD) risk (42). Moreover, 5% TE higher SFA intake was related to a 17% lower IHD. The authors indicated that the dietary SFA source was mostly derived from dairy products containing predominantly short and medium chain SFA. Thus, these results might be influenced by the food source, matrix and the chain length of the dietary SFA instead of the actual amount of

replacement of SFA (42). In contrast, Chowdhury et al. (43) found a negative correlation between circulating margaric acid (C17:0), which is a SFA derived from dairy products, and CVD relative risk while there was no relationship between total SFA and coronary disease risk in their meta-analysis. In contrast, findings from Prospective Urban rural Epidemiology (PURE) study which included 135,335 adults aged 35-70 years from eighteen countries, suggested that high SFA intakes were inversely associated with total mortality and stroke risk. The authors claimed that a low SFA diet might even be harmful, however, the results should be interpreted carefully as the dietary intake of the population was significantly different to that of the UK, with a mean SFA intake of 8% TE and CHO intake of 60% TE (44). According to SACN, UK adult population consume 46%TE from CHO (302). Although studies have reported a reduction in blood cholesterol levels when dietary SFA was replaced with CHO, CHO is not preferred as a replacement macronutrient due to their TAG raising properties (32). It should be noted that the CHO composition of the diet rather than total CHO intake is likely to have an impact on CVD risk, therefore further studies are needed.

Although observational studies provide associations between dietary fat intake and CVD risk, they have inevitable limitations such as under-reporting of food intake which is generally assessed by diet diaries, food frequency questionnaires or dietary recall. Therefore, randomised controlled trials (RCT) which can establish causation and prevent bias are needed to determine the cause-effect relationship between dietary fat and CVD. In their meta-analysis and systematic review of RCT, Hooper et al. (45, 46) found that lower dietary SFA to result in lower CVD events and that replacing dietary SFA with PUFA resulted in significant reduction in CVD events of about 27%. In the next section, RCTs investigating the effect of dietary SFA on lipid CVD risk markers are examined.

Perspectives from intervention trials

Vafeiadou and colleagues (35) performed a RCT with 195 men and women at moderate CVD

risk (1.5 times the population average), in which participants followed one of 3 isoenergetic diets: SFA (17%TE SFA, 11%TE MUFA, 4%TE n-6 PUFA), MUFA (9%TE SFA, 19%TE MUFA, 4%TE n-6 PUFA) and PUFA (9%TE SFA, 13%TE MUFA, 10%TE n-6 PUFA). Substituting 8%TE SFA with MUFA or PUFA had a beneficial impact on fasting serum TC, LDL-C and TC: HDL-C ratio (35). Similarly, in their RCT with 14 male subjects, Kralova et al (47) showed that substituting SFA (20.8%TE SFA,13.6%TE MUFA, 5.6%TE PUFA)) with PUFA diet (10.4%TE SFA, 13.2%TE MUFA, 16.4%TE PUFA)) decreased TC, LDL-C and HDL-C concentrations (47). In good agreement, a cross over study performed by Summers et al. (9) in 17 subjects randomised to two different diets rich in SFA (20.9%TESFA, 12.0%TE MUFA, 3.5%TE PUFA) or PUFA (8.5%TE SFA, 10.3%TEMUFA, 9.2%TE PUFA) for 10 weeks showed TC, and LDL-C to decline whereas HDL-C levels were unchanged on the PUFA compared to the SFA diet (9). However, one criticism of this study was the heterogeneity of the study population which included 5 obese, 6 non-obese and 6 diabetic subjects. Moreover, another crossover study with 17 healthy subjects showed that replacing SFA (13.9%TE SFA, 15.5%TE MUFA, 4.8%TEPUFA) with PUFA (1.3%TE SFA, 16.4%TE MUFA, 14.0%TE PUFA) for only 3 days reduced TC and TAG concentrations by 8% and 11.1% respectively (48).

Hodson and colleagues (49) investigated the effect of replacing 9%TE dietary SFA with n-6 PUFA or MUFA on plasma lipids in their cross-over study. Replacement with n-6 PUFA reduced LDL-C, TC and HDL-C by 22%, 19% and 14%, respectively while substitution with MUFA reduced these lipid risk markers by 15%, 12% and 4%. However, there was a reduction in the total fat intake when SFA was replaced with n-6 PUFA and MUFA by 2.9% and 5.1 % respectively. Caution must be applied when interpreting the findings as the energy intakes were decreased when replacing dietary SFA and the subjects were nutrition students so these findings could not be generalised to an average UK population (49). Similarly replacing 1% TE from

SFA with PUFA was reported to reduce LDL-C by 0.055 mmol/L in a systematic review and regression analysis by Mensink (50). Furthermore, after re-evaluation of the Minnesota Coronary Experiment the authors argued that replacement of SFA with n-6 PUFA lowers LDL-C but did not decrease the risk of CHD deaths (51). However, in this study TFA in margarine which was used as a PUFA source might have negated the positive effect of PUFA. Similarly, a cross-over study by Tindall et al. (52) reported lower TC, LDL-C and non-HDL-C concentrations after following 3 intervention diets (walnut diet, walnut fatty acid matched diet and oleic acid-replaces-α linolenic acid diet) which replaced dietary SFA with n-3 and n-6 PUFAs, n-3 and n-6 PUFA without walnut bioactives and n-9 MUFA and PUFA, respectively.

Therefore, the evidence for the benefits of replacing dietary SFA with PUFA in the reducing the risk of CVD events risk is convincing, however it should also be noted that further evidence is needed on the effect of replacing individual/different source of SFAs.

Substitution of SFA for MUFA on CVD risk

Perspectives from observational studies

Prospective cohort studies in a healthy population (83,349 women from the Nurses' Health study (NHS) and 42,887 men from Health Professionals follow-up study (HPFS)) showed associations between lower CHD events risk and total mortality after replacing 5% energy from SFA with MUFA (40) (53). Similarly, Joris and Mensink (54) in their review suggested that MUFA can be used as a fat source to reduce CVD risk. They reported that replacing 5% of energy from dietary SFA with cis-MUFA was associated with beneficial effects on the fasting lipoprotein profile. However, they also indicated that more well designed RCT were needed to understand clearly the potential role of MUFAs on CVD hard-end points and lipid risk biomarkers (54). In contrast with these findings, in 2009 Jakobsen and colleagues (38) suggested in their pooled analysis of 11 prospective cohort studies among 344,696 subjects that

replacing SFA with n-9 MUFA does not decrease coronary events. Therefore, evidence on the effect of replacing dietary SFA with MUFA on CVD risk markers from the observational studies are limited.

Perspectives from intervention trials

In 1999, Williams et al. (55) performed a randomised crossover study with 30 healthy middle-aged men and 23 healthy young men with a family history of CHD. Subjects consumed control (38%TE fat, 16%TE SFA, 12%TE MUFA and 6% TE PUFA) and MUFA (38%TE fat, 10%TE SFA, 18%TE MUFA, and 6%TE PUFA) diets for 2 months with a washout period between the diets. The results showed that the middle-aged men were more responsive to the LDL-C lowering effects of SFA substitution with MUFA than young men (55). Similarly, in 60 abdominally obese subjects allocated to a high SFA (19%TE SFA, 11%TE MUFA, 5%TE PUFA), a high MUFA (11%TE SFA, 20%TE MUFA, 7%TE PUFA) or MedDiet (11%TE SFA, 21%TE from MUFA, 7%TE PUFA) for 8 weeks after a 2-week run-in diet high in SFA (19%TE), the MedDiet had the most benefit on LDL-C and HDL-C but other components of this diet such as fibre may have also played a role (56). In agreement with these findings, the KANWU study carried out by Vessby et al. (57) including 162 healthy subjects who followed either high SFA (17% SFA, 14% MUFA, 6%TE PUFA) or high MUFA (8%TE SFA, 23%TE MUFA, 6%TE PUFA) diet supplemented with either placebo (olive oil) or fish oil capsules for 3 months suggested that replacing SFA with MUFA reduced LDL-C by 7.7% but this was not evident when supplemented with fish oil capsules (57). Lovejoy et al. (58) conducted a crossover study in which 25 healthy participants followed controlled diets (57% CHO, 28% fat, 15% protein) enriched with SFA (9%TE), MUFA (9%TE) or TFA (9%TE) for 4 weeks. TC and HDL-C levels were significantly elevated during the SFA diet compared with the MUFA and TFA diets. However, by contrast with the previous studies, LDL-C did not differ between the 3 diets at the end of the 4 week interventions. It should be noted that palmitic acid was used as the SFA

source which has a greater LDL-C raising effect compared with the shorter chain SFA lauric acid (58, 59). Moreover, in the PREDIMED randomised controlled study conducted in Spain, Estruch et al. (60) reported that an energy unrestricted MedDiet supplemented with olive oil or nuts reduced incidence of CVD events. However, the authors discussed whether these results could be generalised or not as the n=7447 subjects already followed a similar habitual diet and had a high CVD risk (60). In 2018, the findings from the original study were retracted due to the randomisation issues of participants to the dietary interventions, but re-evaluation of the PREDIMED data excluding the 1588 participants still showed a significant benefit of the MedDiet interventions on CVD events (61). This study showed the MedDiet to have beneficial effects on CVD, however the effects observed from the MedDiet with nuts might be due to other dietary components rather than replacement of dietary SFA with n-6 PUFA since dietary SFA%TE was not different between the diets. Furthermore, van Dijk et al. (62) conducted a parallel study in which 20 abdominally overweight subjects followed a SFA (19%TE SFA, 11%TE MUFA) or a MUFA (11%TE SFA, 20%TE MUFA) diet for 8 weeks. They concluded that LDL-C and TC were higher after the SFA diet whilst HDL-C were similar at the end of both interventions which supports the beneficial effect of replacing dietary SFA with MUFA on blood lipid risk markers (62). Similarly, Smith et al. (63) conducted a 16-week parallel study in which 51 young University students were randomised to an either high MUFA or a moderate MUFA diet and concluded that both diets achieved similar decreases in fasting blood lipids from baseline to week 16, indicating the positive effect of replacing dietary SFA with MUFA was not dose-dependent (63). Moreover, in a randomised controlled crossover intervention RESET, study participants with a moderate CVD risk were asked to follow isoenergetic modified (high fat, SFA-reduced MUFA-enriched dairy products (16%TE SFA, 14%TE MUFA)) and control diets (high fat, dairy products with a typical fatty acid composition (19%TE SFA, 11%TE MUFA)) for 12-week with an 8-week washout period in between

intervention arms. Using a novel food chain approach to partially replace SFA with MUFA in dairy products, the authors concluded that the reduced SFA diet had a beneficial effect on LDL-C levels and endothelial function compared to the control diet (36). In agreement with this, in the RISCK study (64) there were significant reductions in TC and LDL-C concentrations when dietary SFA was reduced to 10%TE and MUFA intake was increased to 20%TE for 4 weeks in subjects at risk of developing metabolic syndrome.

In summary, although evidence to conclude beneficial effects of substituting SFA with MUFA on hard clinical outcomes is limited, replacement of SFA with PUFA has been shown to be associated with lower risk of CVD events and SFA replacement with MUFA and PUFA has been reported to have beneficial impacts on CVD risk markers. However, further RCT studies are needed for firm conclusions to be drawn on the optimal macronutrient to replace SFA in the diet for CVD risk reduction.

Table 1.1 Cohort studies investigating the effect of MUFA and PUFA on CVD risk markers with comparison to SFA

Reference	Study Population, age, n (M/F)	Study Design/Duration	Assessment of dietary status	Association	Significant Outcomes
Jakobsen et al., 2009 (38)	344,696 people (European and American) aged<35y	Pooled analysis of 11 cohort studies, 4-10 y follow up.	FFQ or a dietary history interview	SFA v MUFAs, PUFAs, CHO and CHD risk	Negative association: PUFA v SFA- coronary events Positive association: CHO v SFA - coronary events
Virtanen et al., 2014 (41)	1,981 Finnish M aged 42 to 60y	PCS, 21 y follow-up	4-d food diary	SFA, MUFA, PUFA, TFA, CHO and CHD risk	SFA or TFA no association. Fatal CHD positively associated with MUFA v SFA, negatively with PUFA v SFA
Li et al., 2015 (40)	84,628 F from NHS aged 30-55y and 42,908 M aged 40-75y from HPFS	PCS 24 to 30 years of follow up.	FFQ	Replacing 5%E from SFA v UFAs or CHO and CHD risk	PUFA, MUFA or CHO (wholegrains) v SFA associated with 25%, 15%, 9% ↓ CHD risk.
Praagman et al., 2016 (42)	35,597 participants (Dutch population)-17,357 F aged 49-70y from Prospect-EPIC and 22,654 subjects aged 20-65 y from MORGEN cohort	PCS, 12y follow up	FFQ	Replacing 5 %E from SFA v protein, MUFA, PUFA, CHO, individual SFA or SFA from different food source and IHD	Inverse association: total SFA, short chain SFA or SFA from dairy v IHD risk Positive association: protein, MUFA, PUFA or CHO and IHD risk**.**
Zong et al.,2016 (39)	73147 F aged 30-55 from NHS and 42635 M aged 40-75y from HPFS	Prospective longitudinal cohort study, follow up for 18yrs	FFQ	Replacing 1 %E individual SFA v MUFA, PUFA, CHO and CHD	C12:0-18:0 v MUFA, PUFA, CHO (wholegrain) or plant proteins 6-8%↓ CHD risk
Dehghan et al., 2017 (44)	135,335 individuals from 18 countries aged 35 to70y	PCS followed for 7.4 yrs	FFQ	SFA, MUFA, PUFA, CHO and CVD or CVD mortality	Inverse association**:** SFA and stroke No association: total fat, SFA, UFA.

Abbreviations: CHD: coronary heart disease, CHO: carbohydrate, CVD: cardiovascular disease, %E: percentage of energy, F: female, FFQ: food frequency questionnaire, HPFS: Health Professionals Follow-up Study, HR: hazard ratio, IHD: Ischemic heart disease, M: male, MUFA: monounsaturated fatty acids, NHS: Nurses' Health Study, PCS: prospective cohort study, SFA: saturated fatty acid, TFA: trans fatty acids, ↑**:** increased, ↓: decrease

Table 1.2 Randomised controlled trial studies investigating the effect of MUFA and PUFA on CVD risk markers with comparison to SFA

Reference	Study Population, age, n (M/F)	Study Design/Duration	Description of trial/intervention (total fat, %E)	Dietary fat composition (%E, unless specified)				Significant Outcomes
Healthy volunteers				**SFA**	**MUFA**	**PUFA**	**other**	
Williams et al., 1999 (55)	middle aged M n=30 (mean age 49.1±5.4), young M n=13 with family history of CHD (mean age 28.7±6.6)	CO, R 8 weeks per diet .	HF, Control diet (38) HF, MUFA-rich diet (38)	16 10	13 18	6 6		LDL-C and TC↓ MUFA-rich v control diet in both group (p<0.0001)
Vessby et al., 2001 (57)	30-65y n=162	RCT, PAL, 12 weeks	SFA-rich diet (37) + placebo or FO suppl MUFA-rich diet (37) + placebo or FO suppl	17 8	14 23	6 6		LDL-C ↓ MUFA diet+ placebo (p<0.001) v LDL-C↑ SFA diet+ FO suppl (p<0.01)
Hodson et al., 2001 (49)	nutrition students aged 20-41y Trial 1: n=29, Trial 2: n=42	R, CO, 18 days per diet	SFA-rich diet v (33.3) n-6 PUFA-rich (trial1) diet (30.4) SFA-rich diet v (34) high MUFA-rich (trial 2) diet (28.9)	17.5 8.5 17.7 8.4	9.6 9.5 9.7 11.6	2.7 9.1 3.0 6.1	CHO:46.7 CHO:51.2 CHO:48.7 CHO:53.8	TC, LDL-C and HDL-C ↓ n-6 PUFA (p<0.001) or MUFA (p<0.001 for TC, LDL-C, p<0.05 for HDL-C) v SFA diet
Lovejoy et al., 2002 (58)	Mean age=28±2, n=25	R, DB, CO, 4 weeks per diet	MUFA diet (9%E C18:1 cis) (28) SFA diet (9%E as palmitic acid) (27) TFA diet (9%E as C18:1 trans) (27)	5.8 11.3 7.3	15.2 9.3 8.4	6.3 6.4 4.0	 TFA:7.3	TC, HDL-C ↑ SFA v MUFA diet (p<0.05). TAG and LDL-C did not differ among the three diets.

Smith et al., 2007 (63)	18-28y n=51 Students living in a university hall of residence	PAL, SB, 16 weeks	Moderate MUFA diet (39.7) MUFA-rich diet (37.1) Run-in (8weeks) diet	13 10 16	15 18 12	6 6 6		LDL-C, TC, HDL-C similar↓ MUFA diets v run-in diet (p<0.01)
Kralova Lesna et al., 2008 (47)	18-55 y, n=14 M	CO, 4 weeks per diet	 SFA-rich diet (40) PUFA-rich diet (40)	%total fat 52 26	%total fat 34 33	%total fat 14 41		LDL-C (p<0.01) and HDL-C (p<0.05) ↓ PUFA v SFA diet
Werner et al.,2013 (65)	50-70 y, n= 38	DB, PAL, RCT, 12 weeks	Diet containing milk delivered from mountain-pasture grazing cows from Norway (G) (34) Diet containing milk fat of typical Danish composition (C) (35)	16.0 15.8	10.2 10.6	4 3.6	20%less lauric, myristic and palmitic acid and 26% higher stearic acid in G diet.	NS
Gaundal et al.,2020 (48)	18-55y, n=17	DB, CO, 3 days per diet	Two muffins+20g/d spread SFA-rich (<37) n-6 LA PUFA-rich (<37)	g/d 29.9 2.4	g/d 33.4 30.9	g/d 10.2 26.4		TC, TAG ↓ PUFA v SFA diet (p=0.002)
Subjects with obesity, metabolic syndrome, or greater CVD risk								
Summers et al., 2002 (9)	N=17 - 6 DM, 6 non-OB (BMI<27kg/m^2) and 5-OB without DM(BMI>30kg/m^2) mean age 56±10,55±13,50±9 y respectively	CO, 5 weeks per diet	 SFA diet (ND) PUFA diet (ND)	g/d 58.7 20.1	g/d 33.9 24.3	g/d 9.8 21.7		TC (p=0.001) and LDL-C (p=0.002) ↓ PUFA v SFA.

Van Dijk et al., 2009 (62)	45-60y, n=20 abdominally OW	PAL, controlled-feeding trial, 8 weeks	SFA diet (36.8) MUFA diet (39.9)	19 11	11 20	5 7		TC, LDL-C ↓ MUFA v SFA diet (p≤0.01).
Bos et al., 2009 (56)	40-65y, n= 57, mild abdominal OB no DM	R, PAL controlled-feeding trial, 8 weeks	MUFA-rich (39.9), MedDiet (40.2) SFA-rich diet (36.8)	11 11 19	20 21 11	7 7 5		TC, LDL-C ↓ MUFA v SFA diet. HDL-C↑ and TC: HDL-C↓ MedDiet v MUFA diet (p value ND)
Estruch et al., 2013 (60)	55 to 80 y, n=7447, increased CVD risk	Multicenter, PAL RCT, median follow up for 4.8y	MedDiet +EVOO (41.2) MedDiet+ mixed nuts (41.5) Control diet (37.0)	9.4 9.3 9.1	22.1 20.9 18.8	6.1 7.7 5.5		MedDiet +EVOO or nuts↓ the incidence of cardiovascular events
Vafeiadou et al., 2015 (35)	21 to 60y, n=195, moderate CVD risk	SB, PAL dietary intervention, 16 weeks	SFA-rich diet (36) MUFA-rich diet (36) n-6 PUFA-rich diet (36)	17 9 9	11 19 13	4 4 10		TC, LDL-C and TC: HDL-C↓ MUFA or n-6 PUFA v SFA diet (p≤0.001)
Miller et al., 2016 (66)	38-76y, n=39, MetS	prospective, PAL RCT, 6 months	3x PUFA or MUFA enriched muffin/day. High oleic sunflower oil muffin (38) Safflower oil muffin (35)	g/d 4.2 3	g/d 30.9 6	g/d 2.1 27.6		W and WC↓ both diets (p≤0.02). TAG↓ and FMD↑ PUFA v MUFA diet (p=0.04).
Estruch et al., 2018 (61)	55-80y, n=7447 Increased CVD risk	multicenter, PAL RCT	MedDiet +EVOO (41.2) MedDiet+ mixed nuts (41.5) Control diet (37)	9.4 9.3 9.1	22.1 20.9 18.8	6.1 7.7 5.5		MedDiet +EVOO or nuts↓ the incidence of cardiovascular events

Tindall et al., 2019(52)	30-65y, n= 36 OW and OB (BMI 25-40 kg/m^2)	R, CO ,6 weeks per diet	WD (35) WFMD (35) Control diet (34) ORAD (35)	7 7 12 7	9 9 12 12	16(2.7 ALA) 16(2.6 ALA) 7 14(0.4 ALA)	CHO:48, protein:17 CHO:50 Protein:16 CHO:48, protein:17 CHO:48, protein:17	TC, non-HDL-C and LDL-C ↓ after each diet v baseline (p<0.0001)
Vasilopoulou et al., 2020 (36)	20-70y, n=54, moderate CVD risk	DB, R, CO, 12 weeks per diet	Control diet (38) Modified diet (38)	19 16	11 14	6 5		LDL-C ↓ modified v control diet (p=0.03)

Abbreviations: ALA: α-linolenic acid **,** BP: Blood pressure**,** CO: crossover**,** DB: double blind**,** DM: diabetes mellitus**,** F: female**,** FMD: flow mediated dilution**,** FO: fish oil**,** HDL-C: high density lipoprotein cholesterol**,** LA: linoleic acid**,** LDL-C: low density lipoprotein cholesterol**,** M: male**,** MedDiet: Mediterranean Diet**,** MUFA: monounsaturated fatty acids**,** NS: not significant**,** ND: not determined, ORAD: oleic acid-replaces α-linolenic acid diet, PAL: parallel, OB: obese, OW: overweight, PUFA: polyunsaturated fatty acids, R:randomised, RCT: randomised controlled trial, SB: single blind, SFA: saturated fatty acid, TAG: triacylglycerol, TFA: trans fatty acid, TC: total cholesterol, W:weight, WC: waist circumference, WD: walnut diet, WFMD: walnut fatty acid matched diet, ORAD: oleic acid replaces ALA diet,↑: increased, ↓ decreased

1.2.4 Potential mechanisms behind the effect of dietary SFA on LDL-C

As highlighted previously, hepatic LDL-R expression plays an important role in the clearance of LDL particles and therefore impacts on the circulating LDL-C concentration. Replacing dietary SFA with PUFA or to a lesser extend with MUFA is a public health strategy to lower LDL-C and prevent the development of CVD in the UK. Thus, it is important to understand the potential mechanisms behind this effect of dietary fat composition on LDL-C. There are several suggested potential mechanisms such as cholesterol absorption and bile acid synthesis, but in this section, I will focus on the impact of dietary fat on the expression of genes involved in hepatic cholesterol regulation.

It is well documented that gene expression is regulated in response to the environmental factors such as diet (67-69). This has led researchers to investigate the molecular mechanisms behind the effect of dietary SFA on LDL-C concentrations. Several animal and in vitro studies have reported a decrease in mRNA expression of the LDL-R after a high SFA diet (70). Although it is not totally understood, it was suggested that increased dietary SFA decrease acyl:CoA cholesterol acyltransferase (ACAT) activity and therefore increase intracellular cholesterol concentrations, preventing SREBP-2 from leaving the ER and suppressing the expression of the LDL-R (71). Moreover, dietary SFA have been shown to be associated with smaller, more dense LDL particles (occur when TAG-enriched LDL becomes a good substrate for hepatic triglyceride lipase resulting in depletion of the lipid core) which are reported to have a decreased affinity for the LDL-R (72-74). In addition, the greater competition between LDL particles and larger, apoE-rich TAG-rich particles isolated after a high SFA meal was shown to reduce hepatic LDL uptake in HepG2 cells (75). Since these findings are predominately from animal and in vitro studies, it makes it difficult to transfer these findings to humans due to the differences in lipid metabolism in rodent models. To overcome this limitation, studies have started to isolate circulating peripheral mononuclear cells (PBMCs, includes lymphocytes and

monocytes) as a surrogate marker of hepatic cholesterol metabolism due to the presence of the LDL-R on the surface of these cells and regulated control of intracellular cholesterol levels. As these cells are exposed to nutrients circulating in the blood and easily accessible, PBMCs may serve as a good model to investigate molecular mechanisms of the effect of dietary components on cardiovascular health (76, 77). As seen in **Figure 1.4**, PBMCs can serve as a marker of hepatic lipid metabolism, however it should be noted that there could be some limitations such as differences in lipid regulation compared with liver cells. In the next section studies examining the effect of dietary fat composition on genes involved in cholesterol metabolism in PBMCs will be presented.

Effect of dietary SFA on PBMC LDL-R gene expression and others related to hepatic lipid regulation

In an early cross-over study by Mustad et al. (78) 25 healthy subjects followed one of the 3 diets: an average American Diet (34%TE fat, 15%TE SFA 13%TE MUFA 6%TE PUFA), a step-one diet (29%TE fat, 9%TE SFA 13%TE MUFA 6%TE PUFA) or a very low SFA diet (25%TE fat, 6%TE SFA 13%TE MUFA 6%TE PUFA) for 8 weeks. LDL-R protein abundance was found to be 6% and 9% higher after the step-one and very low saturated fat diets, respectively. Moreover, the change in LDL-R protein abundance and change in LDL-C concentration were found to be negatively associated. In line with this, a RCT by Ulven et al. (79) reported an upregulation of LDL-R expression in 99 healthy subjects with moderate hypercholesterolemia after they replaced 6.5% TE SFA with n-6 PUFA for 8 weeks. They also reported an upregulation of genes involved in hepatic cholesterol metabolism (SREBF1, ABCG1 and NR1H3). Therefore, findings from a limited number of human studies in PBMCs are in line with the previous animal and in vitro studies suggesting a role for dietary SFA on LDL-R expression and intracellular cholesterol regulation expression.

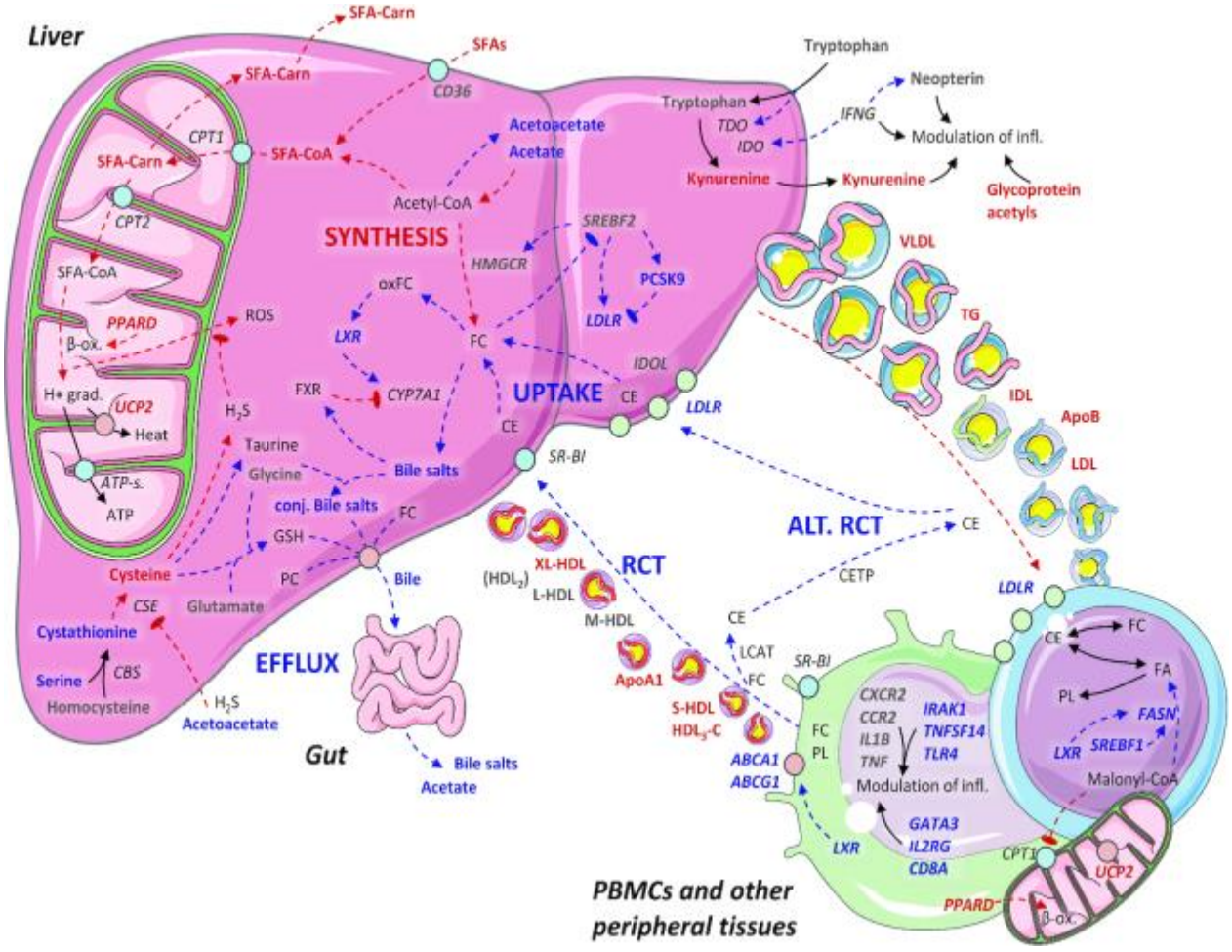


Figure 1.4. Cholesterol regulation pathways in liver and PBMC. Replacing dietary SFA with UFA increase LDL-R expression via SREBF2, which causes a higher hepatic uptake of apoB100-containing lipoproteins in the liver. This inhibits further cholesterol synthesis and activates cholesterol efflux to the gut. Cholesterol-derived oxysterol activate LXR, which activates bile salt production. In PBMCs, LXR activity increase reverse cholesterol transport (ABCG1, ABCA1) and fatty acid synthesis or cholesterol esterification which inhibits β-oxidation. (Red: reduced/lower, blue: increased/higher) ABCA1 ATP binding cassette subfamily A member 1, ABCG1 ATP binding cassette subfamily G member 1, LDL-R low density lipoprotein receptor, LXR liver x receptors, SFA saturated fatty acid, SREBF2 sterol regulatory element binding transcription factor 2, PBMC peripheral blood mononuclear cells, PUFA polyunsaturated fatty acid (Adapted from (79)).

1.3 Body Composition and CVD risk

1.3.1 Obesity and CVD risk

As previously mentioned, obesity is one of the major contributors to the global burden of CVD. It is a heterogenous disorder, and the pattern of body fat deposition have been associated with different cardiometabolic disease risk profiles. In android obesity, excess fat accumulation occurs around the abdominal region of the body whereas in gynoid obesity excess fat accumulates in the lower part of the body, around hips and thighs (80). Studies have shown that while android type is associated with CVD, gynoid fat accumulation could be protective against this disease (81).

Adipose tissue consists of white, brown and beige adipose tissue (82). Main total fat stores in humans are shown to be white adipose tissue which has two different metabolic characteristic compartments, subcutaneous adipose tissue (SAT) (deep and superficial) and visceral adipose tissue (VAT). SAT represents a major component of body fat and is located under the skin whereas VAT can be defined as the accumulation of adipose tissue around or within organs such as omental (intestines and organs in the lower abdomen), mesenteric (intestines and bowel), epicardial (heart) and mediastinal (thoracic cavity) regions (83). Importantly, there are anatomical, cellular, and molecular differences between SAT and VAT. In terms of cellular differences, VAT contain larger adipocytes which are dysfunctional, insulin resistant, hyperlipolytic and not subject to the anti-lipolytic effect of insulin compared to SAT which contains small adipocytes (84). Studies have reported that gynoid fat accumulation is mostly subcutaneous whereas abdominal fat accumulation includes increased amount of VAT mass which is associated with increased risk of developing chronic metabolic diseases (85).

The link between VAT accumulation and the development of chronic diseases is explained by insulin resistance which can be defined as impairment of insulin action on glucose, lipid and protein metabolism (86). A few potential mechanisms have been proposed to explain

the link between insulin resistance and VAT. First of all, the portal theory suggests that due to its anatomical position increased levels of non-esterified fatty acids (NEFA) and pro-inflammatory factors released from hypertrophic dysfunctional adipocytes of VAT are delivered directly to the liver via the portal vein where they can mediate their effects by increasing glucose production and exacerbating insulin resistance (87-89). Secondly, according to the spill over hypothesis, the limited ability of SAT to expand after it reaches saturation causes spill over of NEFA into the visceral fat and to non-adipose tissue increasing the size of these fat depots leading to insulin resistance, cell lipotoxicity, ER stress and secretion of inflammatory cytokines (**Figure 1.5**) (87).

Although the underlying mechanisms to explain the relationship between VAT and insulin resistance are not totally understood, inflammation plays an important role in the hypotheses described above. This was first demonstrated by Hotamisligil (90) in obese mice as adipocyte-derived TNF-α, a pro-inflammatory cytokine, has a direct role in obesity-induced insulin resistance. This has also been proven in a study which showed higher levels of pro-inflammatory cytokines in obese states to be negatively correlated with insulin sensitivity (91). Moreover, although the underlying reason is not clear, lower adiponectin levels, which are commonly found in subjects with VAT accumulation, are associated with insulin resistance. Additionally, as hypertrophic adipocytes have impaired ability to take up NEFAs, they are directed to peripheral tissues. According to the Randle hypothesis, high levels of NEFA oxidation results in increasing acetyl-CoA that causes inhibition of acetyl CoA supply from pyruvate. That causes glucose-6 phosphate accumulation and that inhibits glucose uptake by the cell causing insulin resistance (92). Increased NEFA inhibits insulin clearance, as a vicious cycle, hyperinsulinemia downregulate insulin receptors. It also promotes insulin resistance inhibiting insulin receptor substrate. In addition, during high NEFA supply, excessive β oxidation of lipids leads to mitochondrial dysfunction as well as accumulation of NEFA-

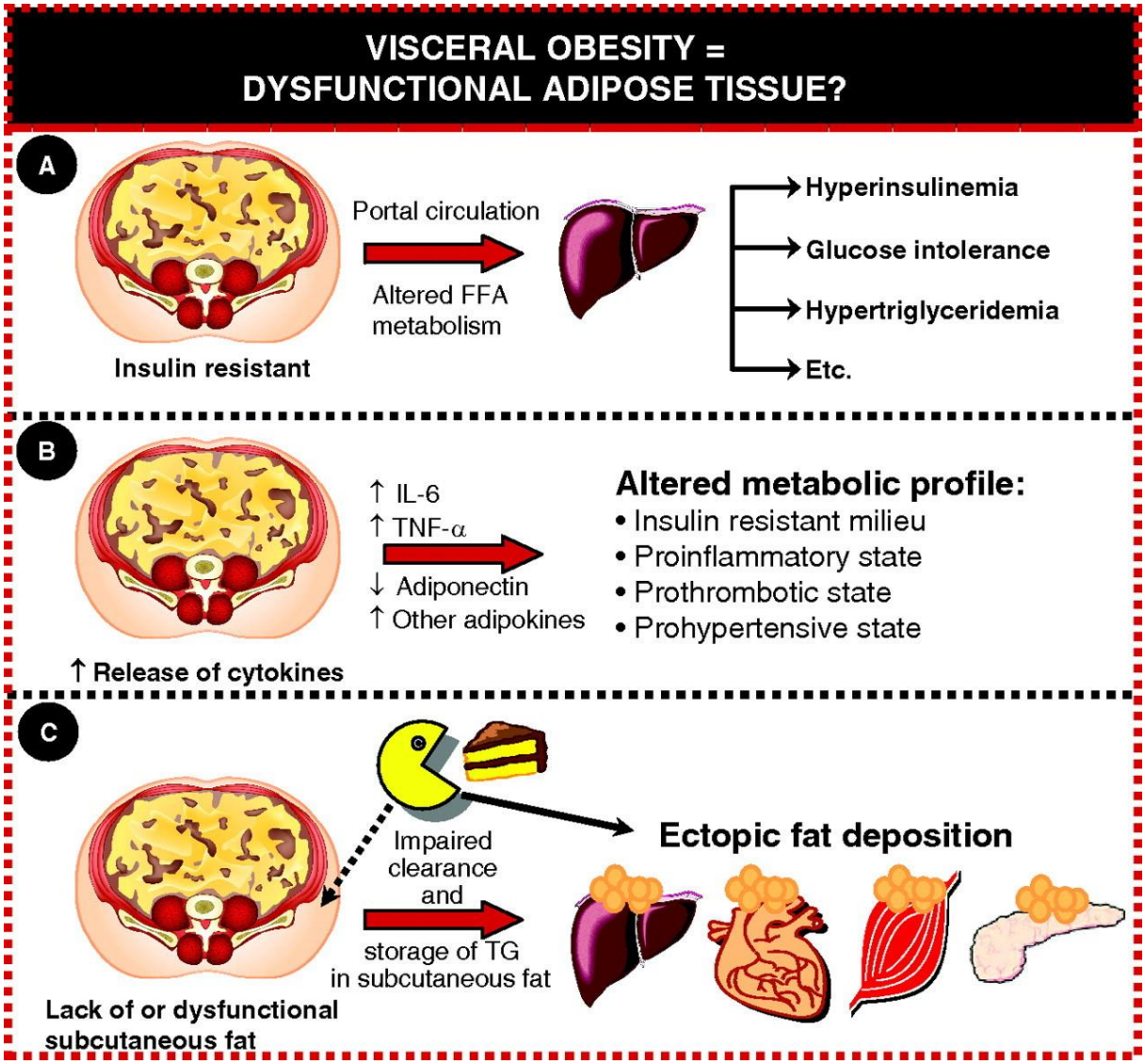


Figure 1.5. An overview of three potential mechanisms explaining the relationship between visceral adipose tissue (VAT) and cardiovascular disease. A) hypertrophic dysfunctional adipocytes of VAT deliver non-esterified fatty acids (NEFA) directly to the liver via the portal vein where they cause hyperinsulinemia, glucose intolerance, hypertriglyceridemia etc. B) release of pro-inflammatory factors from VAT such as interleukin (IL)-6, tumour necrosis factor (TNF)-α which contribute to insulin resistant, pro-inflammatory state of obesity C) lack of or limited ability of subcutaneous adipose tissue (SAT) to expand causes spill over of NEFA leads to ectopic fat deposition (adapted from (93)).

derivatives such as diacylglycerol (DAG) and ceramides which cause impairment in insulin signalling (94). In addition to these, hypertrophic adipocytes can lead to hypoxia. Inadequate vascularization for the expanded adipose tissue can lead to ER stress which triggers the inflammatory pathways, cytokine and chemokine production which impair the insulin signalling pathway (95). Moreover, stressed hypertrophic adipocytes and adipocyte apoptosis attract macrophages into the stromal vascular fraction, causing a crown like structure. Obesity also causes macrophages to undergo polarized differentiation from non-inflammatory M2 type to pro-inflammatory M1 type. The pro-inflammatory features of M1 type contribute to release of pro-inflammatory cytokines from adipose tissue (96).

Thus, adipocyte dysfunction and interaction with other cells within adipose tissue such as immune cells (e.g. macrophages) appear to play an important role in the development of metabolic abnormalities such as insulin resistance (96). Moreover, Gregor and Hotamisligil (97) pointed out in their review paper that inflammation is a key link between obesity and metabolic diseases such as type 2 diabetes. In support of these findings, Kelly and colleagues reported that following bariatric surgery, there is an improvement in the profile of inflammatory markers and adipokines (such as adiponectin) associated with observed weight loss which suggested potential reductions in CVD and type 2 diabetes (98). This finding supports the association between obesity and inflammation and involvement of inflammatory markers in metabolic disease development and progression.

In summary, there are several possible mechanisms linking obesity and CVD such as elevated NEFA, pro-inflammatory cytokine release and ER stress which are thought to be related to insulin resistance and inflammation (**Figures 1.6 and 1.7**). Although available literature suggests that inflammation and insulin resistance are important determinants of the relationship between body fat distribution and metabolic diseases, there is currently insufficient scientific evidence to draw a firm conclusion.

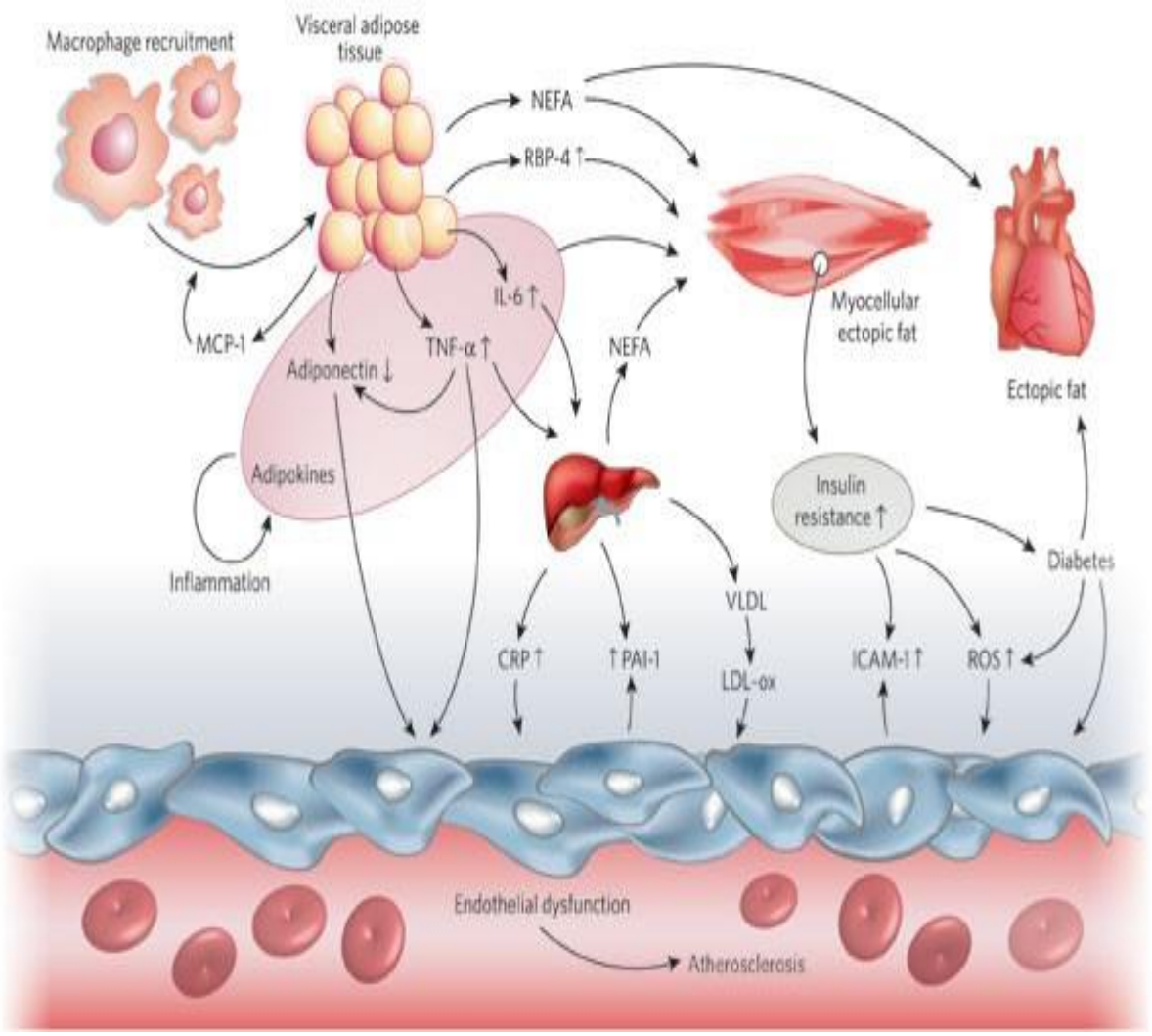


Figure 1.6. The relationship between visceral adipose tissue (VAT), inflammation and insulin resistance. Expansion of the fat mass leads to hypertrophic dysfunctional adipocytes of VAT which start inflammation process through releasing non-esterified fatty acids (NEFA) and proinflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α). NEFA, cytokines and retinol binding protein 4 (RBP-4) induce insulin resistance which can induce endothelial dysfunction (plasminogen activator inhibitor-1 (PAI-1), 1intracellularadhesion molecule-1 (ICAM-1)) and oxidative stress (reactive oxygen species (ROS)). TNF-α and adiponectin, which are secreted after macrophage recruitment via monocyte chemoattractant protein-1 (MCP-1), affect endothelial dysfunction. TNF-α and IL-6 also affect endothelial dysfunction and c-reactive protein (CRP) indirectly. Together these create inflammatory state which contributes to insulin resistance and atherogenic dyslipidaemia (Adapted from (99)).

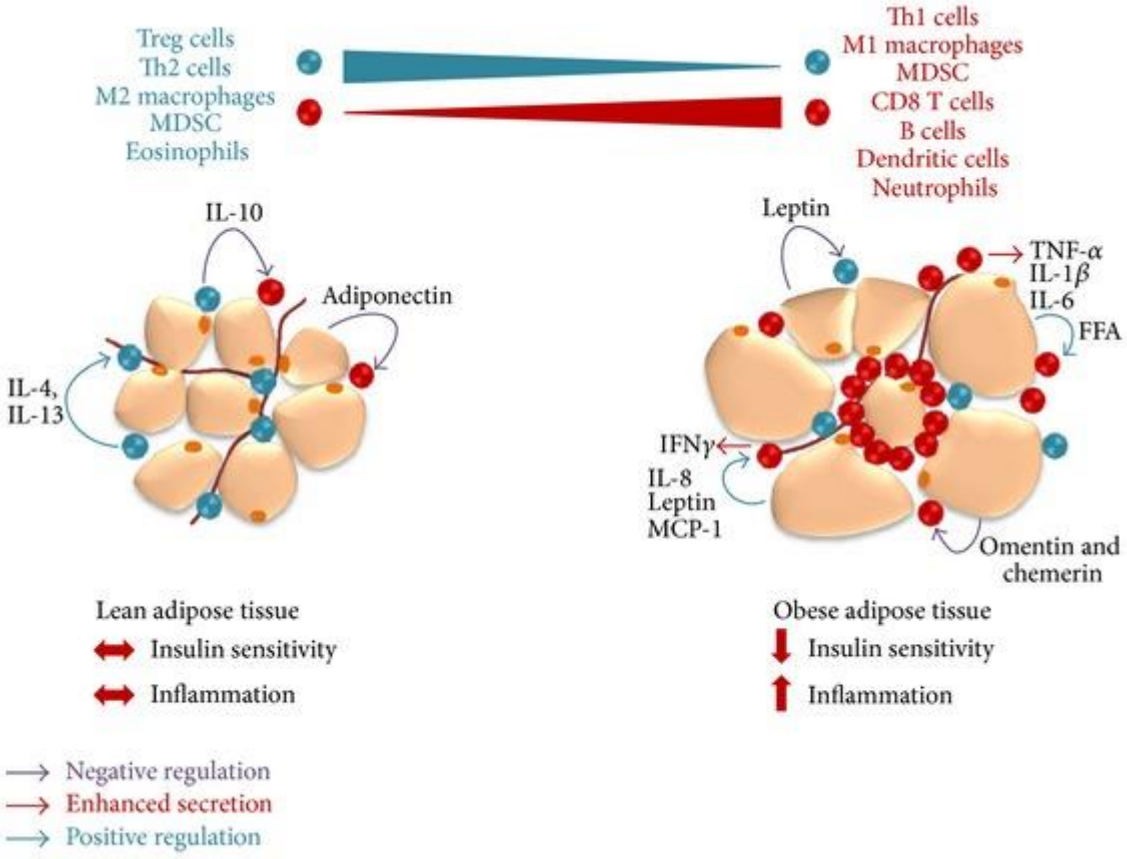


Figure 1.7. Lean and obese adipose tissue as endocrine organs. In obese adipose tissue secretion of pro-inflammatory cytokines increase (tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6)). M2 macrophages switches to M1 macrophage type. These changes induce insulin insensitivity and inflammation (Adapted from (100)).

1.3.2 Dietary Fat Intake and Body Composition

Although higher dietary fat intake was initially reported to be associated with adiposity, obesity rates continued to increase despite decreasing fat intakes (101). Therefore, the quality of dietary fat has gained importance but studies investigating the role on body fat content and distribution are limited. This was echoed in the review of Melanson et al. (101) published in 2009 which proposed that SFA had negative effects on body weight but that more research was needed. Replacing dietary SFA with UFA has been shown in some studies to have a positive effect on body composition and abdominal VAT mass without a weight loss (9, 102). In this section of the literature review, studies examining the effect of dietary fat composition on abdominal obesity will be presented.Findings from a cross-sectional study conducted in 128 men (mean BMI of 28 kg/m^2) from the Quebec Family study concluded that dietary MUFA was associated with trunk skinfolds and waist circumference (WC), SFA intake with body fat mass whereas PUFA showed no relationship with the adiposity markers (WC and body fat mass) (103). Later, in a crossover study performed by Summers et al. (9) with 17 subjects (6 with type 2 diabetes, 6 non-obese and 5 obese individuals), substituting SFA (20.9%TE SFA,12.0%TE MUFA, 3.5%TE PUFA) with n-3 and n-6 PUFA (8.5%TE SFA, 10.3%TE MUFA, 9.2%TE PUFA) decreased abdominal fat accumulation specifically subcutaneous fat using magnetic resonance imaging (MRI) in female subjects without diabetes whilst there was no difference in the waist to hip ratio (WHR) and body fat percentage. However, findings from this study need to be interpreted with caution due to the small sample sizes within each of the subgroups and lower total fat intake on the PUFA diet. During a 16-week RCT, 75 participants with a BMI of 28-40 kg/m^2 followed either a control diet (their habitual diet) or a low-fat vegan diet which was high in PUFA and low in total fat, SFA and TFA. The results suggested that a decrease in SFA intake in the vegan diet was associated with reduced fat mass. However, this finding was not significant after adjustment for BMI showing that the level of adiposity of the

participants at study entry was an important determinant of the response to dietary fat intake (105). In a cross-sectional study conducted by Neville et al. (10), the authors concluded that women aged 20-50 y with a higher dietary SFA intake were found to have higher total body fat percentage and trunk fat mass. In this study body composition was measured using BIA which is more prone to bias compared to dual energy x ray absorptiometry (DXA), computed tomography (CT) or MRI scans (10).

In a randomised crossover study, substituting dietary SFA (24%TE SFA, 13%TE MUFA, 3%TE PUFA) with MUFA (11%TE SFA, 22%TE MUFA, 7%TE PUFA) using two isoenergetic diets for 4 weeks resulted in 1.7 kg reduction in fat mass in 8 overweight or obese male subjects. The authors stressed that on the SFA diet participants gained fat around the abdominal area, whereas during the MUFA diet, the fat loss was similar from the trunk and limbs, however, the subjects were free living so differences in physical activity levels and food intake between diets may have influenced these findings. Moreover, due to the small sample size, these results must be interpreted with caution (102). In the parallel PREDIMED study participants with high CVD risk and a mean BMI of 29.5 kg/m^2, a reduction in WC by 5% was observed in the MedDiet with nuts group after 1 year compared to baseline without a significant change in body weight. However, there was no difference after the MedDiet with EVOO and the low-fat control diet groups which indicates that increased n-6 PUFA compared to MUFA might be more beneficial for obesity (106).

In the LIPOGAIN parallel RCT performed in 39 free living subjects, eating isocaloric muffins high in SFA for 7 weeks was found to increase visceral adiposity whereas muffins rich in PUFA increased lean tissue mass suggesting that dietary fat composition may influence fat distribution and body composition (107). It should be noted that both groups gained 1.6 kg in weight. In another randomised controlled crossover feeding study, 101 men with central obesity followed 5 isocaloric diets (50%TE CHO, 35%TE fat (18%TE from treatment fats) 15%TE protein)

containing Canola, CanolaOleic, CanolaDHA, Corn/Safflower and Flax/Safflower for 4 weeks with a 2–4-week washout period. Reduction in abdominal obesity (3.09±0.1 kg) was evident after CanolaOleic diet (6.5%TE from SFA, 19.3%TE from MUFA, 6.9%TE from PUFA) compared with Flax/Safflower diet (6.8%TE from SFA, 9.6%TE from MUFA, 16.3%TE from PUFA) (108). Raatz et al. (8) argued in their study that SFA intake was positively associated with BMI, however this observation needs to be interpreted with caution as food intake was self-reported and BMI has limitations as a marker of adiposity. Similarly, the isocaloric replacement of a high SFA diet (47% CHO, 38% fat- 20% SFA, 12% MUFA, 6% PUFA) by a MedDiet (47% CHO, 38%fat- <10% SFA, 22% MUFA, 6% PUFA) or CHO diet (57% CHO, 28% fat- <10% SFA, 12% MUFA, 6% PUFA) had a positive effect on total body fat mass in 34 hypercholesterolemic male subjects with a mean BMI of 28.2 kg/m^2 in a crossover study (109). However, the type of carbohydrate they used in the diets was not clear, therefore findings should be interpreted with caution. It should be noted that observed inconsistencies in the literature may arise from different methodologies used to determine body fat distribution in these studies (e.g., anthropometric measures, BIA, DXA, CT, MRI), sample size and different dietary approaches (e.g., supplementation, replacement, and self-reported intakes). Moreover, as it was discussed earlier, other behavioural and lifestyle factors in addition to diet such as sedentary lifestyle as well as non-modifiable risk factors such as the effects of genetic make-up may influence the relationship between body composition and CVD.Mechanisms behind the effect of dietary fat composition on body composition

DiNicolantonio and O'Keefe (110) speculated on the potential mechanisms on how dietary fatty acids influence body composition in their review paper. As long chain SFA have a lower oxidation rate than MUFA and long chain PUFA, they tend to stay in the liver longer whereas UFAs are more likely to be packaged into VLDL and exported from liver. This may explain the reason behind the line of thinking that SFA increase abdominal obesity more than UFA. In other

words, dietary fat composition could impact on energy expenditure. Studies in humans have indicated higher increased β oxidation and diet-inducted thermogenesis is associated with higher levels of long chain PUFA and MUFA (245-249). Moreover, it has also been claimed that n-6 PUFA may cause more fat accumulation than n-3 PUFA by inhibiting the positive effects of n-3 PUFA on obesity such as increasing fatty acid oxidation and prevention of adipocyte proliferation, differentiation, and lipogenesis (110, 111). Finally, it has been argued that dietary fatty acid composition may affect appetite and satiety differently. For example, in a randomised controlled study, adults aged 18-35y experienced a positive change in physiological markers of hunger and satiety after following a PUFA rich diet for a 7 day (304). Moreover, although the studies looking at the effect of dietary fat composition on body lean mass in humans are limited, dietary PUFA has been shown to reduce protein oxidation, promote cell differentiation and growth in animal studies (243). Therefore, the reasons behind the differential effects of dietary fatty acids on body composition could be explained by a combination of different factors. However, future studies in humans are needed to draw a firm conclusion on how these factors work together.

Table 1.3. Summary of the studies investigating the effect of SFA intake on body composition

Reference	Study population, age,n, M/F	Study Design/ Duration	Method to assess body composition	Dietary evaluation method	Description of trial/intervention (Total fat (%E))	Dietary fat composition (%E, unless specified)				Significant outcomes
						SFA	MUFA	PUFA	other	
Doucet et al.,1998 (103)	M, mean age 54.6±0.6 n=128, mean BMI 28 kg/m^2	Cross-sectional	W, WC, HC, SFT, and body density (under water weighing)	3-day food diary	-				CHO:47 Fat:35.5 Protein:16.2	Positive association: SFA intake with body fat mass, BMI, WC
Summers et al., 2002(9)	N=17 - 6 DM, 6 non-OB (BMI<27kg/m^2) and 5-OB without DM(BMI>30kg/m^2) mean age 56±10,55±13,50±9 y	R, CO, 5 weeks per diet	MRI	Dietary intervention/ 3-day food diary to check adherence	SFA-rich (ND) PUFA-rich (ND)	58.7 20.1	33.9 24.3	9.8 21.7		Subcutaneous fat ↓ PUFA v SFA diet.
Fernandez de la Puebla et al.,2003(109)	18-63y, hypercholesteraemic OW (mean BMI 28.2±2.6 kg/m^2) M n=34	A randomised CO, 28days per diet	BIA	Dietary intervention	MUFA-rich (30) CHO-rich (28) Run-in diet (38)	<10 <10 20	22 12 12	6 6 6	CHO:47 CHO:57 CHO:47	Body fat mass ↓CHO or MUFA diet v SFA. Lean body mass ↑ CHO v SFA diet

Piers et al.,2003(102)	24-49y, OW or OB (BMI 25.5-31.3 kg/m^2), n=8	R, CO, 4 weeks per diet	DXA	Dietary intervention	SFA-rich (40) MUFA-rich (40)	24 11	13 22	3 7		Body fat mass↑ SFA v MUFA diet, body fat mass ↓ MUFA v SFA diet
Bjermo et al.,2012(104)	30-65y, abdominally OB (WC: F:>88 cm, M:>102cm) n=61	PAL, RCT,10 weeks	W, MRI for liver fat content, BOD POD for body fat mass	Dietary intervention	SFA (42) n-6 PUFA (40)	20 9	ND ND	ND ND		liver fat↓ PUFA v SFA diet. No difference between diets for abdominal VAT or SAT.
Neville et al.,2012 (10)	20-50y, M n= 49, mean BMI 24±3 kg/m^2, F n=51, mean BMI 22±3 kg/m^2	Cross-sectional	W, BIA	7-day food diary						Positive association: SFA intake with body fat and trunk fat in women
Damascen o et al., 2013(106)	High CVD risk PREDIMED subgroup (n=169) M; aged 55-80, F: aged 60-80 mean BMI 29 kg/m^2	PAL, RCT, 1 year	W, WC	Dietary intervention	medDiet+EVOO medDiet+nuts control diet	9.4 9.3 9.1	22.1 20.9 18.8	6.1 7.7 5.5		WC↓ MedDiet+mixed nuts v baseline
Rosqvist et al.,2014(107)	20-38y, n=39, BMI 18-27 kg/m^2	PAL RCT, 7 weeks	MRI	Dietary intervention	SFA-rich (36.8) n-6 PUFA rich (40)	16.4 11.5	12.9 12.4	4.5 12.9		lean body mass↑ PUFA diet**,** liver fat, body fat and visceral fat content ↑ SFA diet

Liu et al.,2016(108)	20-65y, n= 101 central OB, BMI 22-40 kg/m^2	R, CO, 4weeks per diet	DXA	Dietary intervention	Canola (35.5) CanolaOleic (35.5) CanolaDHA (35.5) Corn/Safflower (35.5) Flax/Safflower (35.5)	6.6 6.5 6.9 6.7 6.8	17.6 19.3 17.8 9.5 9.6	9.1 6.9 8.0 16.3 16.3	Oleic acid: 15.7 18.0 16.5 8.3 8.4	central OB↓ CanolaOleic v Flax/Safflower diet
Kahleova et al.,2019 (105)	N=75 OW, BMI 20-40 kg/m^2, mean age 53.2±12.6.	PAL, RCT, 16 weeks	DXA	Dietary intervention/3-day food dairy to check adherence	Vegan diet (17.5) Control diet (35)	19 27	33 37	41 30		W, fat mass & visceral fat volume ↓ Vegan v control diet after adjustment for BMI

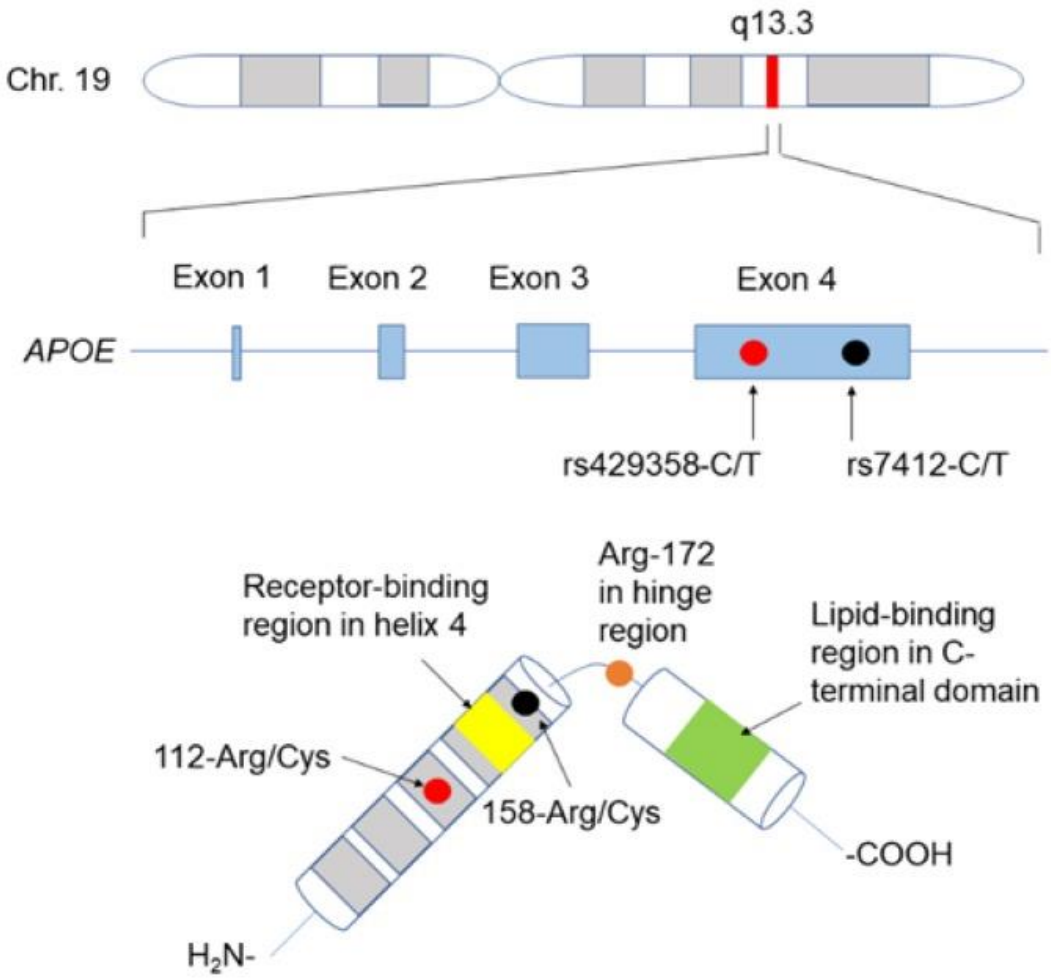
Abbreviations: BMI: body mass index**,** BIA: Bioelectrical impedance analysis**,** CO: cross over study**,** DXA: dual x ray absorptiometry**,** F: female**,** HC: Hip circumference**,** M: male**,** MUFA: monounsaturated fatty acids**,** MRI: magnetic resistance imaging**,** OB: obese**,** OW: overweight**,** PAL: parallel**,** PUFA: polyunsaturated fatty acids**,** R: randomised, SAT: subcutaneous adipose tissue, SFA: saturated fatty acids, SFT: skin fold thickness, VAT: visceral adipose tissue, W: Weight, WC: Waist circumference, ↓: reduced, ↑: increased

1.4 Association between the *APOE* gene, CVD risk factors and body composition

Although it is known that environmental and lifestyle factors (e.g., physical activity, socioeconomic status, parental dietary behaviours, food environment) play an important role in the development of obesity, individuals react differently to these obesogenic environments. Thus, the effect of genotype on obesity is inevitable. GWAS are conducted to identify human obesity genes and until 2020 there were approximately 1,200 genetic loci where sequence variance is found to be associated with obesity traits with commonly identified SNPs include those in the FTO gene (14). However due to the studies showing BMI to have an impact on the differences in blood lipids between *APOE* genotype groups and also differential responsiveness of CVD risk markers to dietary SFA intake in *APOE4* carriers, the involvement of changes in body composition needs to be investigated.

ApoE is a 34-kDa arginine rich protein consisting of 299 amino acids and encoded by the *APOE* gene. It is involved with clearance of TAG-rich lipoprotein particles such as VLDL and chylomicrons and their remnants from the circulation by serving as a receptor binding ligand (112). The *APOE* gene is found at position 13.32 on the long arm (q) of chromosome 19 at the cytogenic location 19q13.32. There are 3 major allelic variants of *APOE* gene; ε2, ε3, ε4, caused by a single base substitution of the two nucleotides (SNPs; at position 112 (rs429358) and at position 158 (rs7412)) resulting in the substitution of arginine or cysteine amino acids in the apoE protein (113) (**Figure 1.8**). These 3 alleles gives rise to 6 possible *APOE* genotypes, *APO E2/2*, *E2/4*, *E4/4*, *E2/3*, *E3/4*, *E3/3* and *APOE3/E3* is the most common genotype in population and also knowns as wild type (114). Regarding the frequency, approximately 66% of population carry two copies of *E3* allele, 20% of population carry one copy of *E4* allele, while *E2* carriers account for only 12-15% in Caucasian populations (115).

A)



B)

Allele	ε2	ε3	ε4
Haplotype	rs429358-T rs7412-T	rs429358-T rs7412-C	rs429358-C rs7412-C
Residue combination	112-Cys 158-Cys	112-Cys 158-Arg	112-Arg 158-Arg

Figure 1.8. Polymorphisms underlying the three main *APOE* variants in humans. (**A**) Chromosome location, gene structure, identity of the mutating sites in the gene, and the corresponding mutating residues in the context of the protein structure. In yellow, it is indicated as the receptor-binding region in helix 4 and, in green, it is the lipid-binding region in the C-terminal domain. Red and black dots indicate the genetic variants in *APOE* and their position in the genomic and protein sequences, respectively. (**B**) Table reporting the haplotypes and corresponding residue combination associated to each *APOE* allele (116).

The association between *APOE* gene polymorphisms and CVD risk has been broadly studied. Higher fasting TC (6.6 to 18.9%) and LDL-C (9.2 to 27.5%) concentrations in *APOE4* carriers compared to *APOE2* carriers have been reported in many studies (117-120). This was explained by the variance in the LDL-R binding activity of isoforms, with apoE4 having the greatest and apoE2 having the least affinity (121). It has been proposed that greater affinity of apoE4 for the LDL-R causes competition between TAG-rich lipoproteins (which are enriched with multiple copies of apoE) and LDL particles for LDL-R mediated clearance and therefore increase circulating LDL-C concentrations. In an invitro study the greater affinity of TAG-rich lipoproteins for the LDL-R after dietary SFA in *APOE4* carriers compared to *APOE3/E3* group was reported (122). However, lower binding affinity of the apoE2 isoform possibly reduces the clearance of TAG-rich lipoproteins, increasing the uptake of LDL particles by the liver and peripheral tissues (123, 124). In addition, in *APOE2* carriers, the lipolytic conversion of VLDL to LDL has been reported to be impaired which increase fasting TAG concentrations (125) (126, 127). Moreover, it has been suggested that *APOE2* carriers have a decreased intestinal cholesterol absorption and increased bile acid synthesis compared to *APOE4* carriers and the-wild type group, which may cause lower circulating cholesterol concentrations in *APOE2* carriers (128). However, these findings are mainly derived from animal and modelling studies, therefore studies in humans are needed to draw any firm conclusions.

Several studies investigating the effect of diet on body weight and composition in *APOE*$^{+/+}$ and *APOE* $^{-/-}$ mice concluded that *APOE* plays a role in obesity (129, 130) and that *APOE* deficiency is protective against obesity via suppression of fat accumulation in the liver and fat tissues (131). In a study by Huebbe et al. (132), *APOE4* and *APOE3* female mice were fed with low and high fat diets (5% from soy and 21.2 % from milk respectively) for 10 months, with female *APOE3* mice having a significantly higher body weight during both diets compared to female *APOE4* mice. Arbones-Mainar et al. (133) showed that male *APOE4* mice had less

body weight gain and VAT fat compared to *APOE3* mice on a high fat western type diet (21% fat from milk) while SAT accumulation was the same for both genotypes. In agreement with this, female *APOE3* knock in mice were found to be more prone to diet-induced obesity compared to the wild type mice while *APOE* $^{-/-}$ mice were resistant to this after 24 weeks of consuming a western type diet (21% fat). Moreover, *APOE3* and C57BL/6 mice liver fat content were found to be higher than *APOE* $^{-/-}$ mice showing the *APOE3* genotype to be associated with an increased fat accumulation in liver in response to a western type diet (134). These results were confirmed in another study where *APOE3* targeted replacement mice on a high fat diet (45%TE) gained more body weight and visceral fat compared to *APOE4* mice whereas when supplementing the high fat diet with long chain n-3 PUFA for 8 weeks body weight gain was reduced only in *APOE3* mice (135). Similarly, when female mice were fed with a high fat diet (60% fat from lard) for 6 months, *APOE3* mice had a higher weight gain and VAT accumulation compared to *APOE4* in two different studies by Johnson et al. (136, 137). In the latter study, SAT accumulation was reported to be the same for mice of both genotypes (137). In contrast, another study reported the same rate of increase in body weight in female *APOE3* and *APOE4* mice after a high fat (45%TE) than low fat (10%TE) diet whereas there was a non-significant trend for body weight to increase more in *APOE4* than *APOE3* male mice. In addition, a role of sex on the changes in body fat was proposed, with only an increase in VAT found in male *APOE4* mice after the high fat diet whereas VAT increased in both female *APOE3* and *APOE4* mice (138). Furthermore, another study demonstrated that *APOE2* mice had higher adiposity than *APOE3* mice when fed for 4 weeks with high fat high cholesterol western type diet (21% fat) (139). Although findings from animal studies appear to support an association between *APOE* genotype and obesity, there is currently not a consensus on which genotype group is more prone to obesity. This may be due to the difficulty in comparing studies due to the sex (as females tend to maintain greater adipose tissue and store it in different regions compared to

males) or age of mice, diet composition/length, and so these findings should be interpreted carefully.

The role of *APOE* genotype in obesity has been investigated in only a small number of human studies but findings have generally been inconsistent. A positive association between *APOE2* with BMI and WC was found in 208 Croatian subjects (140). Similarly, a positive association between *APOE2* and BMI was reported in 4660 subjects from the Aragon Workers Health Study in Spain (141) and 94 healthy and 112 diabetic women in Turkey (142). Similarly, a greater WHR was observed in *APOE2* carriers compared to *APOE4* carriers but only in early postmenopausal Slovak women (n=129/427) (143). Moreover, the *APOE4* allele was associated with lower BMI, especially in older Caucasian individuals, while the *APOE2* allele was related with a higher BMI in a pooled analysis of seven longitudinal cohort studies (144). In contrast, in the Atherosclerosis Risk in Communities (ARIC) study, a greater BMI was associated with *APOE4* while the lowest was associated with *APOE2* allele (145). Similarly, in a case-control study which included 198 normal weight healthy and 198 obese Saudi university students, the *APOE4* allele was positively associated with BMI in obese subjects only (146). Another study investigated the association between *APOE* and WC in 164 non-diabetic first-degree relatives of people with diabetes and 962 non-diabetic people with no family history of diabetes. The authors reported that WC was significantly greater in older women with a family history of diabetes who were *APOE4* carriers compared to *APOE2* and *APOE3/E3* groups (147). Similarly, the *APOE4* allele was found to be positively associated with WC in 155 non-diabetic and 156 diabetic Iranian subjects (148). However, *APOE* and adiposity was not found to be associated in an Iranian population in a cross-sectional study included 345 men and 498 women by Zarkesh et al. (149).

In view of the impact of *APOE* genotype on CVD risk markers studies have determined the role of BMI on this relationship. Petkeviciene et al. (150) reported a lack of interaction

between *APOE2* or *APOE4* with BMI on circulating TC and LDL-C concentrations in 996 Lithuanian adults. In contrast, interactions between the *APOE2/E2* genotype and *APOE4* allele with BMI on non-HDL-C were observed in 5796 subjects with vascular disease. There was also an interaction between WC and VAT with all genotypes on non-HDL-C (15). In line with these findings, a study by Kofler et al. (16), which investigated the impact of adiposity on the association between *APOE* and CVD in 312 UK adults, reported that a lower TAG concentration in *APOE2* carriers were only evident in subjects with normal BMI and not overweight and obese BMI groups. In contrast, in 454 Chinese subjects, lower TAG concentrations were observed in *APOE3* and *APOE4* carriers compared to *APOE2* carriers in normal weight subjects (17). Moreover, higher TC, TAG and LDL-C in the *APOE4* carriers compared to *APOE3/3* group were observed in individuals with BMI≥30 kg/m^2 while there were no differences between genotypes in normal weight and overweight participants in a Mexican Amerindian population (151). Another study reported that *APOE4* carriers have lower TC and LDL-C concentrations at higher BMIs compared to *APOE3/E3* group in Amerindian population (152). In contrast, only in *APOE4* carriers, each 1% increase in android fat percentage was associated with 0.08 mmol/L increase in TAG concentration in Brazilian adults with a normal BMI but higher body fat percentage (153). In contrast, the *APOE3* allele was associated with higher TAG concentrations in Iranian subjects only with higher WC (149). Similarly, the effect of adiposity and *APOE* genotype was investigated in 359 Greek Caucasian subjects with CHD. In normal weight subjects, the *APOE4* allele was associated with higher TC levels compared with *APOE3* allele while in the overweight group, the *APOE2* allele was related to higher HDL-C concentrations compared to *APOE3* and *APOE4* genotype groups (154). Thus, the effect of BMI on the relationship between *APOE* genotype and circulating blood lipid risk markers has been reported, however, there is currently not a consensus on which genotype or BMI group is more prone to this effect. Therefore, some studies have shown an

interaction between adiposity and *APOE* on CVD risk markers, but findings are inconsistent. Further studies are needed to examine how the interaction between *APOE* genotype and adiposity impacts on CVD risk markers.

1.5 Conclusion

Obesity is a consequence of the imbalance between energy consumption and expenditure leading to either subcutaneous and/or ectopic fat (visceral) accumulation. The literature suggests that there is a complex relationship between abdominal (visceral) obesity and CVD risk mediated by inflammation and insulin resistance. The types of fat consumed in the diet have been shown to have an impact on both CVD risk markers and abdominal obesity, with SFA considered to be detrimental compared to UFAs. There is strong evidence from prospective and RCT studies that replacing SFA with PUFA reduces CVD events and that replacement with both PUFA and MUFA improve cardiometabolic health. Data predominantly from animal studies suggests that dietary SFA affects blood cholesterol concentrations by regulating intracellular cholesterol metabolism and LDL-R gene expression, however this needs to be confirmed in human studies. There is also more limited evidence that replacing dietary SFA with PUFA or MUFA has positive effects on body composition and whether an interaction exists between BMI and *APOE* genotype for CVD risk markers. Further research is urgently needed to understand the role of dietary fat composition and *APOE* gene on body fat distribution and CVD risk to reduce the morbidity and mortality caused by these diseases at the population level. Understanding the effect of body fat distribution and *APOE* genotype on the relationship between CVD risk and dietary fat composition would be beneficial to make necessary changes in public healthy guidelines to prevent obesity, CVD, and other no-communicable diseases.

1.6 AIMS AND OBJECTIVES

Aim:

The overarching aim of this PhD project is first to determine the impact of dietary SFA intake on CVD risk markers and body fat distribution, with investigating whether the impact of dietary SFA on CVD risk markers is via its effect on body fat distribution. Secondly to examine the potential mechanisms behind the effect of dietary SFA on CVD risk markers.

Objectives:

Firstly, to determine whether body composition is associated with the relationship between dietary SFA intake and LDL-C concentrations using data from the cross-sectional BODYCON study (Chapter 2).

Secondly, to determine whether the positive effect of replacing dietary SFA with UFA on fasting LDL-C is mediated via changes in body composition using data from the RISSCI-1 sequential dietary intervention study (Chapter 3).

Thirdly, to determine whether PBMC LDL-R mRNA expression in response to the level of dietary SFA intake plays a role in the LDL-C response to replacing SFA with UFA using data from the RISSCI-1 study (Chapter 4).

Lastly, to determine whether there is an interaction between *APOE* and BMI on CVD risk markers (Chapter 5).

Hypotheses:

1. People who consume higher SFA in their habitual diet have higher LDL-C concentrations and this is related to greater abdominal VAT accumulation.
2. Replacing dietary SFA with UFA reduces LDL-C concentrations and the reduction in blood

LDL-C levels are associated with the reduction in abdominal VAT accumulation.

3. Upregulation in PBMC LDL-R expression in response to replacing dietary SFA with UFA is associated with a reduction circulating LDL-C levels.
4. There is an interaction between *APOE* and BMI on CVD risk markers in healthy UK adults and differences in CVD risk markers in genotype groups will be evident in the normal BMI group only.

Chapter 2: Association between dietary saturated fat with cardiovascular disease risk markers and body composition in healthy adults-Findings from the cross-sectional BODYCON study

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Contribution towards the manuscript:

The author responsibilities were as follows- KGJ and JAL designed the study; EO, RM, SK and KGJ conducted research; MW provided advice on dietary analysis; EO analysed data; EO wrote the manuscript under the guidance of KGJ and JAL. KGJ had primary responsibility for final content. All the authors read and approved the final manuscript.

As this is the first chapter which will begin to describe the BODYCON cross sectional study (Chapters 2 and 5) the following applies for both chapters:

During the study my main responsibilities were processing the blood samples collected at screening and study visits before storing them for future analysis, conducting the adiponectin ELISA, dietary analysis on DietPlan software and collecting buffy coat samples during the study visit. These samples were used to extract DNA samples to perform *APOE* genotyping after study was completed. Moreover, I shared responsibilities with RM and KGJ for contacting, recruiting, and screening potential volunteers and for performing the study visit of eligible volunteers. DXA scans were performed by RM and KGJ. KGJ and I carried out biochemical analysis of the samples on the ILAB and RANDOX Daytona Plus Clinical chemistry analysers. Insulin was analysed by KGJ. Vitamin D concentrations were measured by the LGC group. Furthermore, I performed all the statistical analysis for this chapter and was responsible for drafting this chapter which was modified with the feedback from all authors.

Association between dietary saturated fat with cardiovascular disease risk markers and body composition in healthy adults- Findings from the cross-sectional BODYCON study

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2.1 Abstract

Background: Diets high in saturated fatty acids (SFAs) and greater abdominal obesity are both associated with raised low-density lipoprotein cholesterol (LDL-C) concentrations, an independent cardiovascular disease (CVD) risk marker. Although reducing SFA intake is a public health strategy for CVD prevention, the role of body fat distribution on the relationship between SFA and LDL-C is unclear. Therefore, our objective was to investigate whether the association between dietary SFAs and LDL-C concentrations is related to body composition.

Methods: In the BODYCON (impact of physiological and lifestyle factors on body composition) study, 409 adults (mean age 42±16y and median BMI of 23.5 (21.5-25.9) kg/m^2) underwent a measure of body composition by dual energy x-ray absorptiometry, assessment of habitual dietary intake using a 4-day weighed food diary and physical activity level using a tri-axial accelerometer. Blood pressure was measured, and a fasting blood sample was collected to determine cardiometabolic disease risk markers. Correlations between body composition, circulating risk markers and dietary macronutrients were assessed prior to multivariate regression analysis. The effect of increasing intakes of dietary SFA on outcome measures was assessed using ANCOVA after adjusting for covariates.

Results: Abdominal visceral adipose tissue (VAT) mass was moderately positively correlated with total cholesterol (TC), LDL-C, systolic blood pressure (SBP), diastolic blood pressure and HOMA-IR (r_s=0.25-0.44, $p<0.01$). In multiple regression analysis, 18.3% of the variability in LDL-C was explained by SFA intake (% total energy (TE)), abdominal VAT mass, carbohydrate%TE and fat%TE intakes. When data were stratified according to increasing SFA%TE intakes, fasting TC, LDL-C and non-high-density lipoprotein-cholesterol were higher in Q4 compared with Q2 ($p\leq0.03$). SBP was higher in Q4 versus Q3 ($p=0.01$). Android lean

mass was also higher in Q3 versus Q1 (p=0.02). Other anthropometric and CVD risk markers were not different across quartile groups.

Conclusions: Although dietary SFA was found to explain 9% of the variability in LDL-C, stratification of data according to quartiles of SFA intake did not reveal a dose-dependent relationship with LDL-C concentration. Furthermore**,** this association appeared to be independent of abdominal obesity in this cohort.

Trial registration: clinicaltrials.gov as NCT02658539. Registered 20 January 2016, https://clinicaltrials.gov/ct2/show/NCT02658539.

Keywords: body composition, abdominal obesity, dietary fat quality, SFA intake, DXA

2.2 Introduction

Diet is one of the most important modifiable risk factors for cardiovascular diseases (CVDs), with studies reporting a link between high intakes of dietary saturated fatty acids (SFAs) and elevated low-density lipoprotein-cholesterol (LDL-C), a well-documented independent risk factor for this disease (155, 156). Although many studies have investigated the effect of reducing dietary SFA intake on the fasting lipid profile, replacement with unsaturated fatty acids was found to be more beneficial compared to carbohydrates or protein (45, 157). Thus, current UK recommendations for CVD prevention are to decrease dietary SFA intake to less than 10% of total energy (TE) via replacement with polyunsaturated (PUFAs) and monounsaturated fatty acids (MUFAs) (32). However, there is also consistent evidence suggesting no beneficial effect of reducing dietary SFA intake on CVD mortality (43, 44, 158). These discrepancies between studies indicate that there may be other factors affecting this relationship.

Obesity is a rapidly growing global public health problem affecting over one third of the world's population (159, 160). An excessive accumulation of body fat is positively associated with the risk of cardiometabolic diseases such as CVD and type 2 diabetes (161). Body mass index (BMI) has been used routinely at a population level to assess adiposity and identify people with increased metabolic disease risk. However, body fat distribution is now considered to be a better indicator of chronic disease risk than BMI, with fat accumulation in the abdominal area (especially visceral adipose tissue (VAT)) associated with greater CVD risk compared with gynoid adiposity (162-165). Moderately elevated LDL-C concentrations and insulin resistance have been observed in people with increased abdominal fat accumulation (166-168). As a result, there is a considerable interest in the physiological and lifestyle characteristics that influence body fat distribution (169, 170).

Storage of body fat is influenced by non-modifiable factors such as age and sex (171), but also by modifiable lifestyle factors such as diet (172). Studies have investigated the effect of dietary fat composition on body composition, with differential associations shown between dietary SFA (positive) and PUFA/MUFA (negative) with abdominal obesity (9, 104, 107). Although the impact of dietary SFAs on LDL-C concentrations has been shown in many studies, the effect of body composition on this relationship is poorly understood. A small number of studies have reported BMI to be inversely associated with the LDL-C response to reduced SFA intake (173). As dietary SFAs are reported to influence both LDL-C concentrations and body composition, the effect of dietary SFAs on LDL-C, therefore, might be related to its effect on body fat content and distribution.

Thus, the purpose of this study was to investigate whether the impact of dietary SFA on LDL-C was associated with body composition. We hypothesized that higher SFA intakes are related to increased LDL-C concentrations due to greater fat accumulation in the abdominal area.

2.3 Methods

Subjects

Healthy men and women (n=409) aged 18-70 years were recruited from Reading and the surrounding area (UK), from 2014 through 2019 using posters, pamphlets and by contacting previous volunteers registered on the Hugh Sinclair Unit of Human Nutrition volunteer database at the University of Reading. A Medical and Lifestyle questionnaire was used to assess the suitability of interested volunteers before potentially eligible individuals were invited to attend a screening session in which they were provided with detailed information about the study before signing a consent form. All subjects were assessed after fasting overnight for 12 h. During the screening visit, blood pressure and anthropometric measurements were taken and a fasting blood

sample was collected for the measurement of fasting blood lipids (total cholesterol (TC), triacylglycerol (TAG) and high density lipoprotein cholesterol (HDL-C)), glucose, kidney and liver function markers (alkaline phosphatase, alanine aminotransferase, γ-glutamyl transferase, serum creatinine, total bilirubin and uric acid) by using the ILAB 600 clinical chemistry analyser (Werfen Ltd, Warrington,UK). To determine the haemoglobin level, a further blood sample was sent to the Royal Berkshire Hospital Pathology Department (Reading, UK). All participants whose screening measurements matched the following inclusion criteria were invited to participate in the study: BMI 18.5-39.9 kg/m^2, TC<7.8 mmol/l, TAG<2.3 mmol/l, fasting blood glucose<7.8 mmol/l, haemoglobin>115 g/l for women and 130 g/l for men. Exclusion criteria included the following: having suffered a myocardial infarction/stroke in the past 12 months, history of diabetes or other endocrine disorders, bowel disease, cholestatic liver disease, pancreatitis, cancer, being on medication for hyperlipidemia, hypertension, inflammation or hypercoagulation, being on a weight reducing diet and excessive alcohol consumption (<14 units/wk). Furthermore, due to the use of the dual energy x ray absorptiometry (DXA) to assess body composition, further exclusion criteria included arthritis or fracture deformity of spine or femur, history of bone related surgeries, radio-opaque implants or implanted medical devices. Females were also excluded if they were breast feeding, may be pregnant or planning a pregnancy in the next 12 months.

Study design

Impact of physiological and lifestyle factors on body composition (BODYCON) was a single-centered observational cross-sectional study conducted in the Hugh Sinclair Unit of Human Nutrition at the University of Reading. The NHS and University of Reading Research Ethics Committees (reference numbers 14/SC/1095 and 13/55, respectively) both gave a favorable ethical opinion for conduct. This study was carried out in accordance with the Declaration of Helsinki and was registered at www.clinicaltrials.gov (NCT02658539).

Participants attended a single study visit. For the day prior to this visit, participants were requested to abstain from strenuous exercise and consuming alcohol. A low-fat evening study meal and low-nitrate water (Buxton mineral water, Nestlé waters, UK) were provided by the researchers and participants were asked not to consume anything apart from this water after their evening meal. Before starting the study visit, a spot urine sample was collected and urine osmolarity was measured using an Osmocheck device (Vitech Scientific Ltd., UK) to ensure participants were sufficiently hydrated for the body composition measurements and asked to complete a pre-DXA scan questionnaire. Weight, waist and hip circumferences were measured, followed by clinic blood pressure. Total body composition was assessed by DXA scan before a fasting blood sample was taken to measure cardiometabolic disease risk markers. Additionally, in the few days before their visit participants were asked to complete a 4-day weighed food diary for 3 consecutive weekdays and 1 weekend day while wearing a triaxial Actigraph activity monitor (ActiGraph, Florida, US) during the same time to assess dietary intake and physical activity levels, respectively. Premenopausal women not taking oral contraceptives attended their main study visit during the same phase of their menstrual cycle (days 1-7).

Anthropometric and blood pressure measurements

Anthropometric and body composition measurements were performed with participants wearing light clothing and no shoes or metal objects. Height was measured to the nearest 1 cm using a stadiometer, facing forwards, and standing as straight as possible with their arms hanging loosely by their side and their head in the Frankfort plane. Body weight and BMI were determined by using a bioelectrical impedance analyser (Tanita BC-418, TANITA UK Ltd, Middlesex, UK) and 1 kg was automatically deducted to account for the weight of the subject's light clothing. Waist circumference (WC) was measured at the midpoint between the lowest ribs and the top of the iliac crest while hip circumference was measured at the largest circumference around the buttocks. Both measurements were taken by a trained researcher

while participants were standing straight after a gentle expiration. A non-stretch tape measure (Seca, UK) was used for both measures. The waist to hip ratio (WHR) and waist to height ratio (WHtR) were calculated as estimates of body fat distribution.

Blood pressure was measured three times using an Omron blood pressure monitor (Omron M3 digital automatic upper arm blood pressure monitor, Omron Healthcare Co UK Ltd.) and the average systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated. Pulse pressure was determined by subtracting DBP from SBP.

Visceral adiposity, fat mass and lean mas index calculations

Anthropometric indices were calculated to determine their relationship with dietary SFA and cardiometabolic disease risk markers. These included the visceral adiposity index (VAI=waist circumference/(39.68+(1.88xBMI)) x (TAG(mmol/L)/1.03) x (1.31/HDL-C(mmol/L)) for men and VAI=waist circumference/(36.58+(1.89xBMI)) x (TAG(mmol/L)/0.81) x (1.52/HDL-C(mmol/L)) for women as an indicator of visceral adipose tissue function (174)), fat mass index (FMI=fat mass(kg)/height in m^2) and lean mass index (LMI=lean mass(kg)/height in m^2) (175).

Assessment of dietary intake

Habitual dietary intake was evaluated by using a 4-day weighed diet diary. To increase accuracy, an electronic kitchen scale and a selection of food portion sizes from the Food Atlas to record meals consumed outside of home (176) were provided to the participants. Instructions on how to complete the diary were given both verbally and in written form by the researchers. For each subject, nutrient and energy intakes were calculated using Dietplan 7 (Forestfield Software) and the total dietary intakes were divided by the number of days recorded to give mean daily intakes. Data entered on Dietplan was checked by a single researcher at the end of the study. For dietary data inclusion, participants were required to complete at least 3 days of the diet diary and report feasible dietary intakes between 500 and 3500 kcal per day for women and 800 and 4000 kcal

per day for men. Individuals with dietary intakes outside of these ranges have been previously reported to be under and over reporters (177).

Physical Activity

A tri-axial accelerometer was used to measure physical activity levels (Actigraph wGT3X+, Actigraph, LLC). Participants were asked to wear the accelerometer for 4 consecutive days including 3 weekdays and 1 weekend day and keep an activity diary for data cleaning purposes. It was worn around the abdomen above their right hip bone, and they were asked to remove the device only for showering or during swimming. Device initialization, data processing and analysis were conducted using Actilife Data Analysis Software (Version 6.11.5) as previously described (178). Raw data was collected at a 30 Hz sample rate. For inclusion in the physical activity analysis, participants were required to have produced counts on their activity monitor for ≥3-d (>600 min/d of wear time) (179). Non-wear-time was defined as ≥60 min of zero activity counts (180). Data were summarized in 60-s epochs and cut-points were used to classify wear time as: sedentary behaviour (<100 counts/min), light/lifestyle physical activity (760-1951 counts/min), moderate physical activity (1952-5724 counts/min) and vigorous physical activity (≥5725 counts/min) (181). For the purposes of the data analysis, the time spent in moderate and vigorous physical activity was combined. Mean energy expenditure from physical activity (EE_{PA}) was calculated as kcal/d.

Details of the DXA procedure

Prior to the DXA scan assessment, participants changed into clothing without zips and metal buttons or a disposable hospital garment and all metal artefacts were removed. Whole body composition was measured by Lunar iDXA (GE Healthcare, UK) and two operators performed the scanning and followed the manufacturer's guidelines for volunteer positioning and for scan acquisition. Participants laid supine on the Lunar iDXA scanning table with knees and ankles

positioned together using the Lunar Velcro supports. Arms were positioned to the side of the body, with palms facing towards the body and participants were required to lie still during the total body composition scan. All scans were analysed using enCORE Software, version 15 (GE Healthcare, UK) with the advance software package CoreScan, which also estimates the mass and volume of visceral fat within the abdomen. The machine's performance was checked daily by running a quality assurance test according to the manufacturer's instructions before each scanning session.

Biochemical analysis

Blood samples collected into the serum separator and K_3EDTA blood tubes were centrifuged at 1700x g (3000 rpm) for 15 min at room temperature and 4°C, respectively before aliquoting into Eppendorf tubes and stored at -20 °C. Fasting serum lipids (non-esterified fatty acids (NEFA) (Alpha Laboratories Ltd., Hampshire, UK), TC, HDL-C and TAG), glucose, C-reactive protein (CRP), and ɣ-glutamyl transferase (GGT) were quantified in the main study visit sample by using the ILAB 600 clinical chemistry analyser with reagents from Werfen (Werfen (UK) Ltd., Warrington, UK). Plasma uric acid was measured using RX Daytona Plus clinical chemistry analyser (Randox Laboratories Ltd., County Antrim, UK) using a kit supplied by Randox. The Friedewald formula was used to estimate fasting LDL-C concentrations (182). Non-HDL-C was calculated by subtracting HDL-C from TC. ELISA kits were used to analyse serum insulin (Dako Ltd., High Wycombe, UK) and plasma adiponectin (Quantikine kit, R&D Systems, Europe Ltd.) concentrations. Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated by using the following equation: [fasting insulin (pmol/l) x fasting glucose (mmol/l)]/135 (183). Serum 25 hydroxyvitamin D_2 and 25 hydroxyvitamin D_3 was measured by the LGC group (LGC Ltd., Middlesex, UK) and summed to obtain total 25 hydroxy vitamin D (25(OH)D).

Statistical analysis:

Statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS Inc., IL, US). Data was presented as mean ± standard deviation (SD) for normally distributed variables and as median (interquartile range) for non-normally distributed variables in Tables 1 and 2. Normality was assessed using the Kolmogorov-Smirnov test and Q-Q plots. The logarithms or square root transformations were used for several outcome measures including BMI, body fat mass, abdominal VAT mass, dietary protein and trans-fat, TAG, LDL-C: HDL-C ratio, TC: HDL-C ratio, NEFA, CRP, GGT, adiponectin, insulin and HOMA-IR, steps/day, EE_{PA}, and percentage time spent performing moderate to vigorous physical activity. Parametric independent sample t tests were used for normally distributed and transformed data to determine the differences between the male and female groups. Spearman's correlations were used to analyse relationships between cardiometabolic disease risk markers with body composition measurements and dietary macronutrients in the whole group and in men and women separately (Spearman's Rho (r_s)=0-0.3 considered a weak correlation, r_s=0.3-0.7 moderate and r_s=0.7-1.0 strong). Stepwise multiple linear regression analysis was performed using P-in of 0.05 and P-out of 0.01 to establish the independent associations between LDL-C and abdominal VAT mass with the anthropometric measures, cardiometabolic disease risk markers and dietary macronutrients.

For further analysis, the study cohort with dietary data was stratified according to dietary SFA intake expressed as %TE. Subjects in Q1 were selected to be within dietary recommendations for SFA (≤10%TE). General linear model (ANCOVA) was performed to investigate the impact of increasing intakes of dietary SFAs on subject characteristics, adjusting for age and sex. Post-hoc analyses with a Bonferroni correction were used to compare differences between the SFA%TE quartile groups. Results are presented as estimated marginal

means ± SE for normally distributed and as median (interquartile range) for non-normally distributed variables in table 2.5 and $p \leq 0.05$ was considered significant.

2.4 Results

Study participants

A total of 438 healthy subjects were recruited, 29 of them dropped out between the screening and the main visit and 409 subjects (219 were women and 190 men) completed the study. The flow of participants in the study is shown in **Figure 2.1**. The cohort had a mean age of 42±16 years and median BMI of 23.5 (IQR 21.5-25.9) kg/m^2. The main characteristics of the BODYCON study participants are shown in **Table 2.1**. Men (47%) and women (53%) were approximately equally distributed and matched for age in the study population. Compared with women, men had greater BMI, body weight, WC, WHR, WHtR, SBP, and DBP ($p<0.01$ each). Women had significantly higher body fat, android fat percentage, gynoid fat percentage and fat mass ($p \leq 0.03$), whilst men had a higher lean body mass, abdominal VAT mass, and android:gynoid (A/G) percentage fat ratio ($p<0.01$ each). Moreover, men had higher fasting serum TAG, glucose, GGT and UA concentrations and TC: HDL-C ratio ($p<0.01$ for all), while women had higher HDL-C, NEFA and adiponectin concentrations ($p \leq 0.01$) (Table 2.1).

The dietary intakes (n=391) and physical activity (n=327) levels of the study participants are shown in **Table 2.2**. Within the cohort, 2 subjects were identified as under-reporters and 3 as over-reporters, with 13 further subjects excluded due to completion of <3 days of dietary intake (n=1) or did not provide a diet diary (n=12). For the mean dietary intakes, men reported greater energy intakes ($p<0.01$), but only trans-fat (%TE) intake was higher in men in terms of dietary macronutrients compared to women ($p=0.05$), while women reported higher total sugar (%TE) and n-6 PUFA (%TE) intakes compared to men ($p \leq 0.04$). Regarding physical activity levels, 82 subjects were excluded according to inclusion criteria for the

physical activity analysis. Compared with men, women had higher daily step counts and spent a greater percentage of time during the day performing light physical activity ($p \leq 0.02$). On average, men expended significantly more energy per day (approximately 100 kcal/d) performing physical activity compared with women ($p<0.01$). The percentage of time spent performing moderate to vigorous physical activity daily was not different between the sexes ($p=0.34$) (Table 2.2).

Association between body composition, cardiometabolic disease risk markers and dietary macronutrients

Correlations between body composition measurements, CVD risk markers and dietary macronutrients in the whole group are shown in **Table 2.3 and Supplementary Table 2.1** and according to sex in **Supplementary Tables 2.2 and 2.3**. In the whole group body fat mass was found to have weak positive correlations with SBP and DBP, while abdominal VAT mass and A/G fat ratio had moderate positive correlations with both SBP and DBP ($p<0.01$). In addition, inverse moderate correlations were evident between HDL-C and several adiposity measurements, including abdominal VAT mass ($p<0.01$). In contrast, moderate positive correlations were found between TAG, non-HDL-C, TC: HDL-C ratio and LDL-C: HDL-C ratio with abdominal VAT mass, android fat mass, android fat percentage and A/G fat ratio ($p<0.01$ for each). There were also weak positive correlations between LDL-C with SFA (%TE) and trans-fat (%TE) ($p<0.01$ for each). Weak correlations were found between dietary macronutrients and cardiometabolic disease risk markers, with SFA (%TE) intake positively associated with TC, LDL-C, non-HDL-C and NEFA ($p \leq 0.05$), whereas carbohydrate (%TE) intake was negatively correlated with LDL-C ($p<0.01$).

For abdominal VAT mass, moderate positive correlations were found with insulin, HOMA-IR, glucose, CRP and uric acid, while there were weak, negative correlations with adiponectin and 25-hydroxyvitamin D levels ($p \leq 0.05$). Regarding the association between diet

and body composition, we observed weak correlations. n-6 PUFA (%TE) intake was negatively correlated with abdominal VAT mass, while trans-fat (%TE) intake was positively correlated ($p<0.01$) (Table 2.3).

After stratifying the group according to sex, a few sex-specific associations were observed. Body fat mass and abdominal VAT mass were found to have weak to moderate positive correlations with both SBP and DBP in women, while only with DBP in men ($p<0.01$). In addition, inverse moderate correlations were evident between HDL-C and several adiposity measurements, including abdominal VAT mass in men ($p<0.01$), while there were weak inverse correlations between HDL-C and percentage body fat, fat mass and android fat percentage in women ($p<0.05$). Abdominal VAT mass was negatively correlated with n-6 PUFA (%TE) in men ($p<0.01$), whilst in women there was a weak inverse correlation with carbohydrate (%TE) intake ($p<0.05$) (Supplemental Table 2.3).

Stepwise multivariate regression analysis

The standardized regression coefficients, adjusted r^2 and p-values for the stepwise multivariate regression analysis are shown in **Table 2.4.** Only SFA (%TE) intake, abdominal VAT mass, total fat (%TE) and carbohydrate (%TE) intakes were found to be independently associated with fasting LDL-C, explaining 18.3% of the variability in this established CVD risk marker. Of these variables, 9% of this variability was explained by SFA (%TE) intake and 7% by abdominal VAT mass (Table 2.4).

The TC: HDL-C ratio, DBP, GGT, HOMA-IR, sex, age, HDL-C and uric acid were independently associated with abdominal VAT mass and, together, these variables explained 64% of the variability in abdominal VAT mass. This analysis showed that TC: HDL-C ratio alone explained 33% of the variability in the mass of this fat depot.

Subject characteristics according to quartiles of dietary SFA (%TE) intake

There were no significant differences in mean body weight (p=0.10) or BMI (p=0.20) across quartiles (Q) of increasing %TE from SFA (**Table 2.5**). However, android lean mass was found to be 7% higher in Q3 compared with Q1 (p=0.02). Other anthropometric measures were not different across the quartiles of SFA%TE intake.

Significant differences in several cardiometabolic disease risk markers were also evident across increasing quartiles of SFA (%TE) intake. SBP and pulse pressure were higher in Q4 compared to Q3 ($p \leq 0.01$). TC, LDL-C and non-HDL-C levels were 9%,12% and 10% higher in Q4 than Q2, respectively ($p \leq 0.05$). Regarding dietary intakes, subjects in Q4 reported higher total fat, MUFA and trans-fat (%TE) than other quartiles ($p<0.01$ each) and lower n-6 PUFA (%TE) intake than Q2 ($p<0.01$). Carbohydrate (%TE) and fiber (g/day) intakes were lowest in Q4 compared to other quartiles ($p<0.01$ each) (Table 2.5).

2.5 Discussion

The present study investigated the associations between dietary SFA intake, cardiometabolic disease risk markers and body composition to determine whether body fat distribution contributed to the relationship between SFA and LDL-C in a group of healthy adults. Although our study does not establish cause and effect relationships due to its cross-sectional nature, we observed interesting and novel associations. In particular, dietary SFA, total fat and carbohydrate intakes and abdominal VAT mass were independently associated with LDL-C and found to explain 18.3% of the variability. However, SFA intake was not related to abdominal VAT mass. Furthermore, stratification according to quartiles of dietary SFA intake did not reveal dose-dependent relationships with LDL-C, TC, non-HDL-C, blood pressure or android lean mass.

The replacement of dietary SFA with unsaturated fatty acids (n-6 PUFA and MUFA) is associated with beneficial effects on the fasting blood lipid profiles (184). In the PURE cross-sectional study, which included 104 486 men and women aged 30-70 years from 18 countries,

dietary SFA intake was positively related with LDL-C and replacing 5%TE of dietary SFA with PUFA and MUFA was associated with lower LDL-C concentrations (between 0.02-0.18 mmol/L) using a multivariable nutrient density model (185). In agreement with previous studies, we also observed an independent positive association between LDL-C and dietary SFA, with dietary SFAs explaining 9% of the variability in LDL-C response between individuals. However, after stratifying data by SFA intake, we did not observe a linear relationship between increasing SFA intakes and LDL-C, with differences only evident in TC, LDL-C and non-HDL-C concentrations between Q2 and Q4. The lack of a dose-dependent relationship between SFA intake and LDL-C may reflect the use of age and sex as co-variates in the ANCOVA analysis, which are both important non-modifiable determinants of LDL-C concentrations (186, 187). Furthermore, the association of dietary SFA with CVD risk has been proposed to be dependent on the food source and the type of individual SFA rather than the amount of the SFA. For example, although high in SFA, dairy have been reported to have neutral or positive effects on CVD risk markers (188), whereas palmitic acid has been reported to be more atherogenic than stearic acid (50). Therefore, determining total dietary SFA intake in the current study may have influenced the strength of the relationship with fasting LDL-C due to the differences in frequency of dairy product and/or individual SFA consumption within the quartile groups (188). Interestingly, n-6 PUFA intake was considerably higher in Q2 compared to Q4, which may have also influenced blood cholesterol levels. Furthermore, high intakes of plant-based MUFA are associated with lower LDL-C concentrations (59) and in this cohort those with the highest SFA intakes also had higher total fat and MUFA intakes. However, this would not necessarily represent a higher intake of plant-based MUFA-rich foods and oils, as animal products are also a rich source of both SFAs and MUFAs. Similarly, increasing trans-fat intake across quartiles of dietary SFA might be due to the major dietary sources of trans-fats being high in dietary SFA (189). Participants consuming on average 8%TE SFA (Q1) also had the highest carbohydrate

intake (54.1%TE), which exceeded the recommended intake of 45-50%TE. It is clear from literature that replacing SFA with carbohydrate can increase fasting TAG and lower HDL-C concentrations in some population sub-groups (190, 191). Moreover, Hooper et al. (45) reported no effect of replacing SFA with carbohydrate on CVD events and mortality in a systematic review and meta-analysis of 6 randomized controlled trials, while Schwab et al. (156) reported an increased risk of CVD outcomes in a systematic review of prospective cohort studies. Therefore, our contradictory results may be due to the higher carbohydrate intakes in the quartile which met the SFA dietary recommendation for CVD risk reduction (Q1). Interestingly, although Q2 consumed more carbohydrate than Q4, their fiber consumption was higher which might have positively influenced blood cholesterol concentrations (192). This could suggest that the positive association of high-fat, low SFA diets on lipid risk markers might also be dependent on other dietary macronutrients and overall dietary pattern (38).

The observation that dietary SFA intake was independently associated with the fasting LDL-C concentration may be related to the impact of dietary fatty acids on LDL particle clearance. Animal and in vitro studies have suggested that dietary SFAs increase LDL-C via a downregulation in the number and expression of the hepatic LDL receptor (LDL-R) (24, 70, 193). Although the mechanisms are still not totally understood, animal studies have provided evidence that dietary fat composition affects the LDL-R at the molecular level potentially through its effect on mRNA expression (194). A possible explanation is that dietary SFAs lower the esterification of cholesterol in the liver by inhibiting the cholesterol esterifying enzyme acyl-CoA: cholesterol acyltransferase (ACAT), leading to increased free cholesterol accumulation which then suppress the activity of transcription factors such as sterol regulatory element-binding proteins and liver X receptor, downregulating LDL-R gene expression (194-196). In contrast, a recent study showed an increase in hepatic expression of ACAT-2 in mice fed short-term with a high SFA diet and in HepG2 cells treated with 0.5 mmol/l and 1 mmol/l

palmitic acid for 14 hours (197). Furthermore, it has been argued in another study in hamsters that increased ACAT activity may result in the formation of larger cholesterol ester enriched very low-density lipoprotein (VLDL) particles which may be the reason for increased LDL-C concentrations. The authors discussed that the effect of dietary fat composition on circulating cholesterol concentration might be via increased hepatic lipoprotein secretion rather than clearance (198). Findings from an in vitro study conducted in HepG2 cells suggested that enrichment of VLDL particles with apoE following a meal rich in dietary SFA could lead to greater competition with LDL for hepatic LDL-R uptake (75). However, these findings are from cell or animal studies, so there is a need for further studies in humans to understand the mechanisms behind the association between dietary SFAs and LDL-C concentrations.

Higher intakes of dietary SFAs have been suggested to be associated with abdominal fat accumulation, increasing CVD risk (107). In contrast to some studies (9, 102, 105, 107, 109), we found no relationship between body fat distribution including abdominal VAT mass and SFA intake in this study. However, our findings are consistent with Greenfield et al. (199) who reported a lack of association between adiposity and dietary fat composition in their cross-sectional study in 334 female twins. This discrepancy between studies might be due to the difference in participant characteristics, study design or methods used for dietary and body composition assessments. Surprisingly, android lean mass was highest in Q3. This finding might be associated with their low carbohydrate, high SFA diet, which has previously been reported to increase lean mass, but this has only been observed in diets with high protein intakes (20-30%TE) (200-202). Therefore, although abdominal VAT mass explained 7% of variability in LDL-C, it was not found to be different across dietary SFA quartiles. These findings suggest that dietary SFAs and abdominal VAT may impact on LDL-C via different mechanisms.

Body fat distribution, especially abdominal VAT accumulation, has been associated with CVD risk independent of BMI, while gynoid fat is thought to be protective against

metabolic diseases (81, 93). In the current study, fasting blood lipids (TC, TAG and LDL-C) which are established CVD risk markers, were positively associated with body fat distribution measures, including abdominal VAT mass, which confirms previous studies (203). Furthermore, we found the TC: HDL-C ratio to explain the largest proportion of variability in abdominal VAT mass between individuals, which highlights the importance of body fat distribution in relation to CVD risk.

One proposed link between abdominal VAT and CVD is chronic and systemic inflammation, which may occur due to impaired adipocyte differentiation (204). People with VAT accumulation have been shown to have hypertrophic dysfunctional adipocytes which release pro-inflammatory factors. Due to the location of abdominal VAT, these pro-inflammatory factors can enter the liver via the portal vein and increase glucose production leading to insulin resistance, which plays a role in the development of CVD (205). In agreement, our study showed independent associations between CRP and HOMA-IR with abdominal VAT mass, supporting previous studies showing that increased abdominal VAT leads to development of pro-inflammatory state and insulin resistance (206). Moreover, adipocyte hypertrophy is related to decreased adiponectin levels which has been associated with increased CVD incidence (207, 208). In line with this, in our study, adiponectin levels were negatively correlated with abdominal VAT mass and were higher in women, who were shown to have lower abdominal VAT mass compared to men. Therefore, our findings lend support to the previously reported potential mechanisms for abdominal VAT mass and CVD risk.

Strengths of this study include the large sample size, the use of DXA scans to accurately measure body fat distribution and the use of detailed dietary and physical activity assessment. Moreover, compared to the results from the current National Diet and Nutrition Survey, the dietary intake of our cohort compared closely with this representative UK population (209). Several limitations need consideration. First, our study cannot investigate cause and effect

relationship due to its observational, cross-sectional design. Furthermore, dietary intake was self-reported using a 4-day weighed food diary, therefore measurement errors are inevitable. A further limitation is that participants can under and overestimate their food intake. We have tried to address this limitation by removing the under (n=2) and over (n=3) reporters from the dataset for dietary analysis. In addition, as it is not always possible to exactly match the food from volunteer's diet diary with the food composition databases available, this may have influenced the dietary analysis data. Moreover, as limited data are available for n-3 and n-6 PUFA on the current UK food composition databases, these dietary data should be interpreted with caution. Furthermore, dietary SFA was assessed as a single nutrient instead of the food matrix (e.g., dairy and red meat), type (e.g., palmitic and stearic acid) or source (e.g., animal or plant sources) of SFA, which may modify its effect on disease risk markers. Lastly, our study attracted individuals with a predominately normal BMI and higher physical activity level than the average UK population, therefore, it may be difficult to translate our results to the general population.

In conclusion, the findings from this cross-sectional study indicate that both dietary SFA (%TE) and abdominal VAT mass were important determinants of the fasting LDL-C concentration. However, the lack of dose dependent relationships between quartiles of dietary SFA intake with abdominal VAT mass and LDL-C suggests that different mechanisms of action may exist for their impact on LDL. Therefore, further studies are needed to determine the impact of the types and sources of dietary SFA, and their relationship to abdominal obesity and CVD risk.

Declarations

- ***Ethics approval and consent to participate***

The NHS and University of Reading Research Ethics Committees (reference numbers 14/SC/1095 and 13/55, respectively) both gave a favourable ethical opinion for conduct.

- ***Consent for publication***

Not applicable

- ***Availability of data and materials***

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

- ***Competing interests***

The authors declare that they have no competing interests

- ***Funding***

Not applicable

- ***Authors' contributions***

The author responsibilities were as follows- KGJ and JAL designed the study; EO, RM, SK and KGJ conducted research; MW provided advice on dietary analysis; EO analysed data; EO wrote the manuscript under the guidance of KGJ and JAL. KGJ had primary responsibility for final content. All the authors read and approved the final manuscript.

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Abbreviations used in text: ACAT, Acyl-CoA: cholesterol acyltransferase; A/G fat ratio, android to gynoid fat ratio; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DXA, dual x-ray absorptiometry; EE_{PA}, mean energy expenditure from physical activity; GGT, gamma-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low density lipoprotein cholesterol; MUFA, monounsaturated fatty acids; NEFA, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acid; SBP, systolic blood pressure; TAG, triacylglycerol; TC, total cholesterol; VAT, visceral adipose tissue; VAI, visceral adiposity index; VLDL, very low density lipoprotein; WC, waist circumference; WHR, waist to hip ratio; WHtR, waist to height ratio; 25(OH)D, 25 hydroxy vitamin D.

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Supplemental Table 2.1. Spearman's correlation coefficients (r_s) for the relationship between circulating cardiovascular disease risk markers and dietary macronutrient intakes

Supplemental Table 2.2. Spearman's correlation coefficients (r_s) for the relationship between circulating cardiovascular disease risk markers and dietary macronutrient intakes in men and women

Supplemental Table 2.3. Spearman's correlation coefficients (r_s) for the relationship between DXA body composition measurements with CVD risk factors and dietary macronutrients in men and women

Description of data:

Supplemental Table 2.1. Supplemental table showing the correlations between circulating cardiovascular disease risk markers and dietary macronutrient intakes

Supplemental Table 2.2. Supplemental tables showing the correlations between circulating cardiovascular disease risk markers and dietary macronutrient intakes in men and women

Supplemental Table 2.3. Supplemental tables showing the correlations between DXA body composition measures with CVD risk factors and dietary macronutrients in men and women

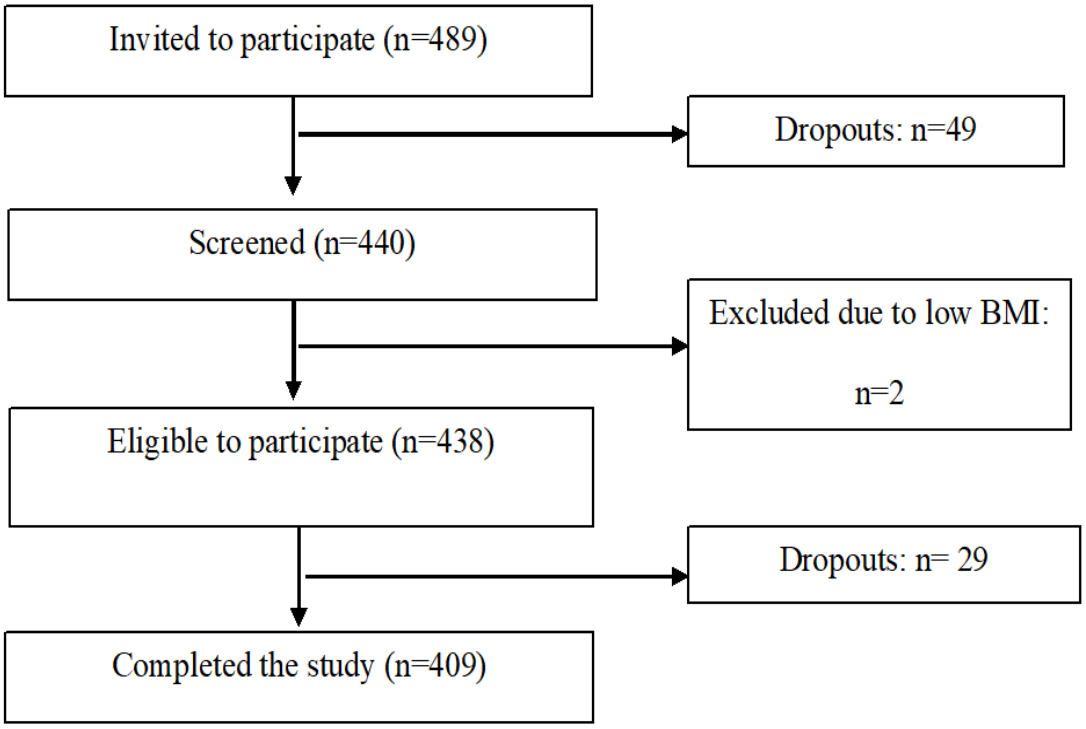

Figure 2.1. Flow chart of participants from the BODYCON study

Table 2.1 Characteristics of BODYCON study participants[3]

Outcome measures[2]	All (n=409)	Men (n=190)	Women (n=219)	P-value[1]
Age, y	42 ± 16	42 ± 15	42 ± 16	0.93
Weight, kg	70.4 ± 14.0	78.3 ± 12.2	63.5 ± 11.7	<0.01
Height, m	1.71 ± 0.01	1.78 ± 0.07	1.64 ± 0.07	<0.01
BMI, kg/m^2	23.5 (21.5-23.9)	24.2 (22.7-26.5)	22.5 (20.8-25.4)	0.01
WC, cm	83.8 ± 11.9	89.1 ± 10.3	79.2 ± 11.2	<0.01
HC, cm	101 ± 9	102 ± 9	100 ± 10	0.04
WHR	0.83 ± 0.08	0.88 ± 0.07	0.79 ± 0.08	<0.01
WHtR	0.49 ± 0.07	0.50 ± 0.06	0.48 ± 0.07	<0.01
Blood Pressure, mmHg				
Systolic	120 ± 14	124 ± 11	117 ± 15	<0.01
Diastolic	72 ± 9	74 ± 9	70 ± 9	<0.01
Pulse pressure	48 ± 11	50 ± 10	47 ± 10	<0.01
Body composition measures				
Body fat, %	28.3 ± 8.4	23.7 ± 7.2	32.3 ± 7.4	<0.01
Fat mass, kg	19.0 (14.3-25.0)	17.8 (12.9-24.9)	19.3 (15.5-25.2)	0.01
Lean mass, kg	48.4 ± 10.5	57.2 ± 7.4	40.7 ± 5.7	<0.01
Trunk fat mass, kg	10.4 ± 5.0	10.9 ± 5.2	10.0 ± 4.9	0.09
Abdominal VAT, g	393 (178-811)	691 (367-1240)	237 (99-440)	<0.01
Android fat, %	30.5 ± 12.1	29.1 ± 12.1	31.8 ± 11.8	0.03
Gynoid fat, %	32.2 ± 9.9	24.9 ± 7.0	38.7 ± 7.2	0.01
A/G fat ratio	0.96 ± 0.29	1.13 ± 0.28	0.80 ± 0.21	0.01
Body Composition Indexes				

FMI, kg/m^2	7.05 ± 2.93	6.10 ± 2.43	7.88 ± 3.09	0.01
LMI, kg/m^2	16.4 ± 2.2	18.0 ± 1.7	15.0 ± 1.5	0.01
VAI	1.01 ± 0.68	1.03 ± 0.71	1.00 ± 0.65	0.66
Biochemistry				
TC, mmol/L	5.13 ± 1.10	5.05 ± 1.18	5.20 ± 1.02	0.10
TAG, mmol/L	0.83 (0.66-1.16)	0.93 (0.69-1.39)	0.79 (0.64-1.02)	0.01
HDL-C, mmol/L	1.65 ± 0.40	1.51 ± 0.40	1.78 ± 0.36	0.01
LDL-C, mmol/L	3.03 ± 0.93	3.07 ± 1.00	2.99 ± 0.86	0.66
Non-HDL-C, mmol/L	3.48 ± 1.00	3.55 ± 1.07	3.43 ± 0.94	0.34
TC:HDL ratio	3.00 (2.63-3.76)	3.44 (2.78-4.03)	2.81 (2.56-3.29)	0.01
LDL-C:HDL-C ratio	1.76 (1.42-2.30)	2.08 (1.58-2.56)	1.60 (1.35-2.04)	0.01
Glucose, mmol/L	5.03 ± 0.48	5.13 ± 0.51	4.94 ± 0.44	0.01
Insulin, pmol/L	26.4 (17.3-39.9)	27.1 (16.9-42.5)	26.3 (18.2-37.7)	0.69
HOMA-IR	0.98 (0.07-5.30)	1.04 (0.63-1.63)	0.97 (0.62-1.41)	0.41
NEFA, µmol/L	416 (318-546)	388 (310-518)	427 (327-567)	0.01
CRP, mg/L	0.62 (0.29-1.46)	0.63 (0.31-1.43)	0.62 (0.28-1.52)	0.91
GGT, U/L	16.9 (14.0-22.7)	20.5 (16.2-27.5)	15.3 (13.2-19.0)	0.01
Uric acid, µmol/L	280 ± 68	323 ± 59	242 ± 51	0.01
Adiponectin, µg/mL	5.11 (2.48-9.07)	4.19 (2.22-6.02)	6.70 (2.93-11.38)	0.01
25-Hydroxyvitamin D, ng/mL	23.9 ± 11.3	23.4 ± 10.8	24.3 ± 11.7	0.50

[1]Data were analyzed by independent t tests and presented as mean ± SD or median (interquartile range); $p \leq 0.05$ was considered significant.

[2]Sample sizes differ as follows: Blood pressure n=406 (M:187/F:219); body composition measures n=370 (M:174/F:196); biochemistry n=405 (M:188/F:217); insulin and HOMA-IR n=272 (M:109/F:163); NEFA n=362 (M:168/F:194); CRP n=403 (M:188/F:215), GGT n=330 (M:135/F:195); UA, adiponectin and 25-hydroxyvitamin D, n=366 (M:172/F:194).

[3]Abbreviations: A/G fat ratio: android to gynoid fat ratio; BMI: body mass index; CRP: C-reactive protein; F: female; FFM: fat free mass; FMI: fat mass index; GGT: gamma-glutamyl transferase; HC: hip circumference; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; LMI: lean mass index; M: male; NEFA: non-esterified fatty acids; TAG: triacylglycerol; TC: total cholesterol, VAT: visceral adipose tissue, VAI: visceral adiposity index, WC: waist circumference; WHR: waist to hip ratio, WHtR: waist to height ratio.

Table 2.2 Dietary intake and physical activity levels of the study participants[3]

	All (n=391)	Men (n=179)	Women (n=239)	P-value[1]
Dietary Energy and Macronutrient Intake				
Energy, MJ/day	8.50 ± 2.47	9.62 ± 2.51	7.56 ± 2.00	<0.01
Total Fat, %TE	36.5 ± 8.6	34.6 ± 9.6	36.4 ± 7.8	0.82
SFA, %TE	13.0 ± 4.5	13.3 ± 5.2	12.8 ± 3.7	0.36
MUFA, %TE	13.7 ± 3.8	13.6 ± 4.1	13.8 ± 3.6	0.70
PUFA, %TE	6.17 ± 2.11	6.09 ± 2.33	6.24 ± 1.91	0.51
n-6 PUFA, %TE	5.66 ± 2.91	5.33 ± 3.03	5.93 ± 2.79	0.04
n-3 PUFA, %TE	0.86 ± 0.59	0.82 ± 0.49	0.90 ± 0.67	0.17
Trans fat, %TE	0.49 (0.34-0.68)	0.50 (0.35-0.72)	0.49 (0.33-0.63)	0.05
Protein, %TE	17.1 (14.8-20.2)	17.0 (14.5-20.4)	17.4 (15.0-19.7)	0.58
Carbohydrate, %TE	45.8 ± 10.9	45.4 ± 12.1	46.1 ± 9.8	0.52
Total Sugars, %TE	18.7 ± 6.6	17.7 ± 7.0	19.6 ± 6.0	0.01
Dietary Fibre (AOAC), g/day	24.3 ± 8.8	25.2 ± 8.9	23.5 ± 8.6	0.07
Physical activity level[2]				
Steps/day	8953 (6948-11941)	8500 (6517-10717)	9288 (7193-12024)	0.02
Energy expended (kcal/day)	254 (157-431)	324 (195-524)	224 (141-349)	<0.01
Percentage time per day spent:				
Sedentary	69.8 ± 7.3	71.1 ± 7.4	68.9 ± 7.1	0.01
Performing light PA	25.5 ± 6.8	24.3 ± 6.6	26.3 ± 6.9	0.01
Performing moderate to vigorous PA	4.2 (2.7-6.2)	4.0 (2.6-6.1)	4.4 (2.7-6.3)	0.34

[1]Differences between men and women were analyzed by independent t test and presented as mean ± SD or median (interquartile range); $p \leq 0.05$ was considered significant.
[2]Sample sizes differed as follows: Physical activity level n=327 (M:126/F:201) and steps/day n=309 (M:120/F:189). [3]Abbreviations: AOAC: Association of Official Analytical Chemist; MUFA: monounsaturated fatty acids; PA: physical activity; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; %TE: % of total energy.

Table 2.3 Spearman's correlation coefficients (r_s) for the relationship between DXA body composition measurements, with CVD risk factors and dietary macronutrients[1,2]

	Body fat, %	Fat mass, kg	Lean mass, kg	VAT, g	Android fat, kg	Android fat %	Gynoid fat %	A/G
Blood pressure, mmHg								
Systolic	-0.03	0.15**	0.33**	0.40**	0.25**	0.14**	-0.15**	0.33**
Diastolic	0.13*	0.29**	0.21**	0.44**	0.37**	0.31**	-0.003	0.39**
Pulse pressure	-0.14**	-0.02	0.25**	0.16**	0.03	-0.05	-0.19**	0.11*
Biochemistry								
TC, mmol/L	0.19**	0.17**	-0.11*	0.25**	0.23**	0.23**	0.13*	0.20**
TAG, mmol/L	0.21**	0.35**	0.18**	0.46**	0.42**	0.38**	0.06	0.43**
HDL-C, mmol/L	-0.02	-0.23**	-0.35**	-0.35**	-0.29**	-0.20**	0.11*	-0.34**
LDL-C, mmol/L	0.17**	0.20**	-0.02	0.32**	0.27**	0.25**	0.09	0.26**
Non-HDL-C, mmol/L	0.22**	0.27**	0.02	0.41**	0.36**	0.33**	0.10	0.35**
TC: HDL ratio	0.18**	0.37**	0.24**	0.55**	0.47**	0.39**	-0.001	0.50**
LDL-C: HDL-C ratio	0.16**	0.33**	0.23**	0.51**	0.43**	0.35**	-0.01	0.46**
NEFA, μmol/L	0.20**	0.12*	-0.17**	0.04	0.10*	0.15**	0.20**	-0.02
Glucose, mmol/L	0.07	0.23**	0.22**	0.41**	0.31**	0.23**	-0.05	0.35**
Insulin, pmol/L	0.35**	0.41**	0.02	0.34**	0.41**	0.42**	0.24**	0.32**
HOMA-IR	0.34**	0.42**	0.06	0.38**	0.43**	0.42**	0.21**	0.34**
CRP, mg/L	0.36**	0.41**	0.001	0.29**	0.39**	0.41**	0.27**	0.27**
GGT, U/L	-0.09	0.11*	0.35**	0.37**	0.22**	0.11	-0.22**	0.36**

Uric acid, µmol/L	-0.16**	0.13*	0.53**	0.43**	0.29**	0.13*	-0.35**	0.51**
Adiponectin, µg/mL	0.18**	0.02	-0.25**	-0.14**	-0.05	0.02	0.25**	-0.21
Total 25(OH)D, ng/mL	-0.16**	-0.14**	0.05	-0.12*	-0.08	-0.11*	-0.15**	-0.14**
Dietary Intake								
Total fat, %TE	0.02	0.01	-0.03	0.01	0.01	0.01	0.02	0.01
SFA %TE	0.04	0.06	0.01	0.08	0.06	0.04	0.03	0.04
MUFA, %TE	0.01	-0.01	-0.05	-0.02	-0.01	-0.004	0.02	-0.01
PUFA, %TE	-0.07	-0.15**	-0.09	-0.13*	-0.16**	-0.13*	-0.04	-0.14**
n-6 PUFA, %TE	-0.08	-0.16**	-0.11*	-0.17**	-0.19**	-0.15**	-0.02	-0.18**
n-3 PUFA, %TE	-0.004	-0.05	-0.08	0.01	-0.04	-0.01	0.02	-0.03
Trans fat, %TE	0.07	0.11*	0.07	0.16**	0.13**	0.11*	0.04	0.12*
Protein, %TE	-0.03	-0.003	0.07	-0.04	-0.02	-0.05	-0.03	-0.07
Carbohydrate, %TE	0.02	0.003	-0.05	-0.03	0.001	0.02	0.03	0.01
Fibre (AOAC), g/day	-0.22**	-0.14**	0.21**	-0.06	-0.13*	-0.21**	-0.21**	-0.07
Total Sugars, %TE	0.03	-0.01	-0.11*	-0.14**	-0.04	-0.04	0.07	-0.11*

[1]Data analysed by Spearman's correlations

*Significant differences at the 0.05 level

**Significant differences at the 0.01 level. [2]Abbreviations: AOAC: association of analytical chemists; A/G: android to gynoid ratio; CRP: C-reactive protein; GGT: gamma-glutamyl transferase; HDL-C: high density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment for insulin resistance; LDL-C: low density lipoprotein cholesterol; MUFA: monounsaturated fatty acids; NEFA: non-esterified fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; TC: total cholesterol; TAG: triacylglycerol; %TE: % of total energy; VAT: abdominal visceral adipose tissue; total 25(OH)D: 25-hydroxyvitamin D.

Table 2.4 Stepwise multivariate linear regression analysis exploring the relation between dietary macronutrients, body composition and biochemical variables with LDL-C and abdominal VAT[3]

Dependent variable	Independent variable	Standardized coefficient	Adjusted r^2	P-value
LDL-C[1]	SFA %TE	0.297	0.085	<0.01
	and Abdominal VAT	0.277	0.160	<0.01
	and Carbohydrate %TE	-0.157	0.172	0.013
	and Fat %TE	-0.261	0.183	0.017
Abdominal VAT[2]	TC: HDL-C	0.572	0.325	<0.01
	and DBP	0.314	0.415	<0.01
	and GGT	0.253	0.475	<0.01
	and HOMA-IR	0.240	0.518	<0.01
	and Sex (female)	-0.237	0.565	<0.01
	and Age	0.222	0.606	<0.01
	and HDL-C	-0.202	0.625	0.001
	and Uric acid	0.157	0.636	0.005

[1]Variables included in the analysis: BMI, body fat %, fat mass, abdominal VAT, android fat mass, android fat %, A/G fat ratio, WC, HC, WHR, WHtR, fat %TE, SFA %TE, trans-fat %TE, CHO %TE.

[2]Variables included in the analysis: age, sex, TC, TAG, HDL-C, LDL-C, non-HDL-C, TC: HDL-C, LDL-C: HDL-C, glucose, 25(OH)D, CRP, GGT, UA, adiponectin, insulin, HOMA-IR, SBP, DBP, PP, PUFA %TE, n-6 PUFA %TE, trans-fat %TE, total sugars %TE.

[3]Abbreviations: GGT: gamma-glutamyl transferase; HDL-C: high density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment for insulin resistance; LDL-C: low-density lipoprotein cholesterol; SFA: saturated fatty acid; TC: total cholesterol; %TE: % of total energy; VAT: visceral adipose tissue.

Table 2.5 Participant's characteristics according to quartiles of dietary saturated fatty acid (%TE) intake[3]

Characteristics[2]	**Q1 (n=78) (1.9-10.0 %TE)**	**Q2 (n=101) (10.1-11.9 %TE)**	**Q3 (n=109) (12.0-14.8 %TE)**	**Q4 (n=103) (14.9-38.7 %TE)**	**P-value[1]**
Weight, kg	68.4 ± 1.3	69.5 ± 1.2	72.5 ± 1.1	69.9 ± 1.2	0.10
BMI, kg/m^2	23.0 (20.8-25.4)	23.2 (21.4-25.5)	24.1 (22.0-27.0)	23.6 (21.5-25.7)	0.20
WC, cm	82.5 ± 1.1	83.5 ± 1.0	85.4 ± 1.0	83.3 ± 1.0	0.21
HC, cm	100 ± 1	100 ± 1	103 ± 1	101 ± 1	0.06
WHR	0.83 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.99
WHtR	0.49 ± 0.01	0.49 ± 0.01	0.50 ± 0.01	0.49 ± 0.01	0.42
Blood pressure, mmHg					
SBP	120 ± 2^{ab}	121 ± 1^{ab}	117 ± 1^{b}	123 ± 2^{a}	0.01
DBP	71 ± 1	72 ± 1	71 ± 1	72 ± 1	0.83
Pulse pressure	48 ± 1^{ab}	49 ± 1^{a}	45 ± 1^{b}	50 ± 1^{a}	<0.01
Body composition measures					
Body fat, %	28.4 ± 0.8	28.3 ± 0.7	29.1 ± 0.7	27.6 ± 0.7	0.53
Android fat, %	30.5 ± 1.4	30.8 ± 1.2	31.6 ± 1.2	29.6 ± 1.2	0.68
Gynoid fat, %	32.5 ± 0.8	32.2 ± 0.7	33.2 ± 0.7	31.6 ± 0.7	0.47
A/G fat ratio	0.94 ± 0.03	0.96 ± 0.02	0.97 ± 0.02	0.94 ± 0.02	0.79
Fat mass, kg	17.7 (12.3-25.3)	17.9 (14.5-25.0)	20.2 (16.1-25.4)	19.0 (14.3-24.0)	0.21
Lean mass, kg	46.8 ± 0.8	48.0 ± 0.7	49.2 ± 0.7	48.3 ± 0.7	0.12
Android fat mass, kg	1.63 ± 0.12	1.57 ± 0.10	1.72 ± 0.10	1.54 ± 0.10	0.58
Android lean mass, kg	3.19 ± 0.06^{a}	3.30 ± 0.05^{ab}	3.42 ± 0.05^{b}	3.28 ± 0.05^{ab}	0.02
Abdominal VAT, g	562 ± 56	582 ± 50	651 ± 48	562 ± 49	0.53
Indexes					
VAI	1.05 ± 0.08	1.02 ± 0.07	1.06 ± 0.07	0.94 ± 0.07	0.61
Biochemistry					
TC, mmol/L	5.17 ± 0.11^{ab}	4.91 ± 0.10^{a}	5.10 ± 0.09^{ab}	5.39 ± 0.09^{b}	0.01
TAG, mmol/L	0.84 (0.67-1.18)	0.82 (0.65-1.07)	0.84 (0.66-1.28)	0.84 (0.67-1.11)	0.59
HDL-C, mmol/L	1.67 ± 0.04	1.61 ± 0.04	1.61 ± 0.04	1.73 ± 0.04	0.05
LDL-C, mmol/L	3.00 ± 1.00^{ab}	2.87 ± 0.08^{a}	3.04 ± 0.08^{ab}	3.23 ± 0.08^{b}	0.03
Non-HDL, mmol/L	3.50 ± 0.10^{ab}	3.31 ± 0.09^{a}	3.49 ± 0.09^{ab}	3.66 ± 0.09^{b}	0.05

TC: HDL-C	2.95 (2.65-3.71)	2.94 (2.61-3.44)	3.03 (2.61-3.81)	3.15 (2.61-3.83)	0.61
LDL-C: HDL-C	1.69 (1.43-2.23)	1.71 (1.42-2.13)	1.82 (1.41-2.42)	1.90 (1.41-2.40)	0.50
NEFA, µmol/L	390 (327-534)	403 (294-500)	441 (310-560)	456 (340-598)	0.36
Glucose, mmol/L	4.98 ± 0.05	5.08 ± 0.04	5.01 ± 0.04	5.02 ± 0.04	0.49
CRP, mg/L	0.49 (0.25-1.29)	0.59 (0.27-1.37)	0.66 (0.38-1.54)	0.66 (0.29-1.69)	0.79
Uric acid, µmol/L	270 ± 7	275 ± 6	289 ± 6	282 ± 6	0.14
Adiponectin, µg/mL	6.03 (2.50-10.62)^{a}	4.93 (2.39-7.99)^{ab}	4.09 (1.99-8.12)^{b}	5.45 (3.11-9.87)^{ab}	0.03
Total 25-Hydroxyvitamin D, ng/mL	22.3 ± 1.3	24.3 ± 1.2	24.3 ± 1.1	24.2 ± 1.2	0.63
Insulin, pmol/L	26.0 (20.3-37.5)	26.5 (17.3-38.6)	22.4 (15.9-40.1)	28.7 (18.3-42.7)	0.61
HOMA-IR	1.00 (0.68-1.44)	1.00 (0.65-1.53)	0.85 (0.58-1.44)	1.15 (0.66-1.63)	0.60
Dietary Intake					
Energy, kcal/day	1906 ± 61	2045 ± 54	2055 ± 51	2092 ± 53	0.13
Energy, MJ/day	7.98 ± 0.26	8.56 ± 0.22	8.60 ± 0.22	8.75 ± 0.22	0.13
Total fat, %TE	28.2 ± 0.7^{a}	34.3 ± 0.6^{b}	36.5 ± 0.6^{b}	44.9 ± 0.6^{c}	<0.01
MUFA, %TE	11.0 ± 0.4^{b}	13.3 ± 0.3^{a}	13.8 ± 0.3^{a}	16.1 ± 0.3^{c}	<0.01
PUFA, %TE	5.97 ± 0.24	6.39 ± 0.21	6.04 ± 0.20	6.24 ± 0.21	0.52
n-6 PUFA, %TE	5.64 ± 0.33^{ab}	6.48 ± 0.29^{a}	5.53 ± 0.28^{ab}	5.00 ± 0.28^{b}	<0.01
n-3 PUFA, %TE	0.84 ± 0.07^{ab}	0.87 ± 0.06^{ab}	0.76 ± 0.06^{a}	0.98 ± 0.06^{b}	0.06
PUFA/SFA	0.81 ± 0.03^{a}	0.58 ± 0.03^{b}	0.46 ± 0.03^{c}	0.34 ± 0.03^{d}	<0.01
MUFA/SFA	1.44 ± 0.04^{a}	1.21 ± 0.03^{b}	1.04 ± 0.03^{c}	0.89 ± 0.03^{d}	<0.01
Trans fat, %TE	0.30 (0.23-0.40)^{a}	0.41 (0.31-0.54)^{b}	0.53 (0.43-0.68)^{c}	0.76 (0.56-0.96)^{d}	<0.01
Protein, %TE	17.6 (14.7-20.5)	18.1 (15.7-20.9)	16.5 (14.5-19.2)	16.5 (14.5-19.8)	0.44
Carbohydrate, %TE	54.1 ± 1.1^{b}	47.3 ± 0.9^{a}	46.1 ± 0.9^{a}	37.7 ± 0.9^{c}	<0.01
Fiber (AOAC), g/day	27.3 ± 1.0^{a}	26.3 ± 0.9^{ab}	23.8 ± 0.8^{b}	20.5 ± 0.8^{c}	<0.01
Total Sugars, %TE	19.6 ± 0.7	18.9 ± 0.6	19.4 ± 0.6	17.1 ± 0.6	0.03
Physical activity level					
Steps/day	9786 (7583-12573)	9153 (6883-11523)	8937 (6876-11973)	8177 (6715-11206)	0.25
Energy expended (kcal/day)	296 (164-525)	265 (162-450)	249 (146-385)	231 (144-330)	0.16
Percentage time spent per day:					
Sedentary	70.0 ± 0.9	69.7 ± 0.8	69.8 ± 0.8	70.1 ± 0.8	0.98
Performing light PA	25.0 ± 0.8	25.7 ± 0.7	25.6 ± 0.7	25.4 ± 0.7	0.89
Performing moderate to vigorous PA	4.6 (2.9-6.8)	4.2 (3.1-6.4)	4.0 (2.5-6.6)	4.0 (2.5-5.6)	0.64

[1]Data were analysed by ANCOVA with age and sex as covariates and presented as estimated mariginal means ± SE or median (interquartile range); $p \leq 0.05$ considered significant.

[2]Sample sizes differ as follows: Blood pressure, Q1 n=77, Q2 n=101, Q3 n=107, Q4 n=103; body composition measures, Q1 n=72, Q2 n=91, Q3 n=98, Q4 n=94; VAI, Q1 n=78, Q2 n=99, Q3 n=103, Q4 n=103; biochemistry, Q1 n=78, Q2 n=100, Q3 n=106, Q4 n=103; NEFA, Q1 n=72, Q2 n=89, Q3 n=92, Q4 n=94; CRP, Q1 n=77, Q2 n=99, Q3 n=106, Q4 n=103; UA, adiponectin and 25-hydroxyvitamin D, Q1 n=72, Q2 n=90, Q3 n=95, Q4 n=94; insulin and HOMA-IR, Q1 n=52, Q2 n=71, Q3 n=67, Q4 n=69; dietary intake, Q1 n=78, Q2 n=101, Q3 n=109, Q4 n=103; physical activity, Q1 n=69, Q2 n=80, Q3 n=84, Q4 n=85; steps/day, Q1 n=66, Q2 n=77, Q3 n=77, Q4 n=81.

[3]Abbreviations: AOAC: Association of Official Analytical Chemist; CRP: C-reactive protein; DBP: diastolic blood pressure; HC: hip circumference; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; MUFA: monounsaturated fatty acids; NEFA: non-esterified fatty acids; PA: physical activity; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; SBP: systolic blood pressure; TC: total cholesterol, TAG: triacylglycerol; UA; uric acid; VAI: visceral adiposity index; WC: waist circumference; WHR: waist to hip ratio; WHtR: waist to height ratio.

Supplemental Table 2.1 Spearman's correlation coefficients (r_s) for the relationship between circulating cardiovascular disease risk markers and dietary macronutrient intakes in the whole group[1,2]

	TC	LDL-C	HDL-C	TAG	Non-HDL-C	TC:HDL-C ratio	LDL-C: HDL-C ratio	NEFA	Glucose	Insulin	CRP
Fat, %TE	0.13*	0.11*	0.13*	-0.07	0.09	-0.02	-0.004	0.09	0.04	-0.04	0.03
SFA, %TE	0.20**	0.19**	0.09	-0.01	0.18**	0.06	0.08	0.13*	0.08	0.001	0.07
MUFA, %TE	0.09	0.08	0.11*	-0.07	0.06	-0.03	-0.01	0.04	-0.004	-0.03	0.01
PUFA, %TE	0.02	-0.01	0.14**	-0.08	-0.03	-0.11*	-0.10	-0.01	-0.04	-0.12*	-0.06
n-3 PUFA, %TE	0.10*	0.08	0.09	-0.02	0.06	-0.02	-0.01	0.07	0.04	0.02	-0.02
n-6 PUFA, %TE	0.01	-0.02	0.13*	-0.06	-0.04	-0.12*	-0.10*	-0.07	-0.01	-0.23**	0.004
Trans-fat, %TE	0.19**	0.19**	0.03	0.04	0.20**	0.13*	0.13*	0.11*	0.10	-0.01	0.08
Protein, %TE	0.08	0.07	0.09	-0.02	0.05	-0.003	0.01	0.03	0.03	0.05	0.08
Carbohydrate, %TE	-0.23**	-0.19**	-0.18**	0.02	-0.18**	-0.01	-0.03	-0.06	-0.12*	-0.01	-0.07

[1]Data analysed by Spearman's correlations. *Significant differences at the 0.05 level **Significant differences at the 0.01 level. [2]Abbreviations: CRP: C-reactive protein; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; MUFA: monounsaturated fatty acids; NEFA: non-esterified fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; TC: total cholesterol; TAG: triacylglycerol; %TE: % of total energy.

Supplemental Table 2.2 Spearman's correlation coefficients (r_s) for the relationship between circulating cardiovascular disease risk markers and dietary macronutrient intakes in men and women [1,2]

	TC	LDL-C	HDL-C	TAG	Non-HDL-C	TC:HDL-C ratio	LDL-C:HDL-C ratio	NEFA	Glucose	Insulin	CRP
Fat, %TE											
Women	0.14*	0.12	0.11	0.01	0.12	0.002	0.01	0.08	0.12	-0.04	0.08
Men	0.10	0.10	0.13	-0.14	0.08	-0.01	0.01	0.09	-0.03	-0.06	-0.03
SFA, %TE											
Women	0.14*	0.14*	0.07	0.09	0.14*	0.05	0.06	0.12	0.16*	0.04	0.09
Men	0.24**	0.25**	0.14	-0.11	0.22**	0.10	0.13	0.13	0.01	-0.08	0.05
MUFA, %TE											
Women	0.07	0.07	0.02	-0.01	0.06	0.03	0.03	-0.02	0.04	-0.05	0.02
Men	0.09	0.09	0.16*	-0.13	0.05	-0.05	-0.02	0.08	-0.03	-0.02	-0.01
PUFA, %TE											

Women	0.04	0.03	0.09	-0.05	0.01	-0.08	-0.06	-0.06	-0.03	-0.13	-0.01
Men	-0.03	-0.04	0.12	-0.08	-0.06	-0.11	-0.10	0.02	-0.01	-0.12	-0.12
n-3 PUFA, %TE											
Women	0.09	0.07	0.09	0.04	0.06	-0.03	-0.02	0.03	0.12	0.01	-0.02
Men	0.09	0.09	0.09	-0.06	0.07	0.003	0.02	0.11	-0.04	0.05	-0.03
n-6 PUFA, %TE											
Women	-0.04	-0.05	0.06	-0.02	-0.06	-0.09	-0.08	-0.17*	0.01	-0.21**	0.10
Men	0.03	0.03	0.12	-0.06	-0.01	-0.09	-0.06	0.01	0.04	-0.25*	-0.11
Trans-fat, %TE											
Women	0.18*	0.19**	0.01	0.03	0.20**	0.14*	0.15*	0.10	0.10	-0.02	0.07
Men	0.23**	0.21**	0.11	0.02	0.22**	0.10	0.11	0.13	0.07	-0.01	0.10

Protein, %TE											
Women	0.03	0.03	0.04	0.01	0.01	-0.01	0.01	0.11	0.06	0.06	0.10
Men	0.13	0.13	0.09	-0.03	0.11	0.03	0.04	-0.06	0.02	0.05	0.05
Carbohydrate, %TE											
Women	-0.19**	-0.17*	-0.14*	-0.06	-0.16*	-0.02	-0.04	-0.06	-0.17*	-0.05	-0.14*
Men	-0.24**	-0.21**	-0.23**	0.08	-0.19**	-0.01	-0.04	-0.05	-0.08	0.06	0.01

[1]Data analysed by Spearman's correlations

*Significant differences at the 0.05 level

**Significant differences at the 0.01 level

[2]Abbreviations: CRP: C-reactive protein; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; MUFA: monounsaturated fatty acids; NEFA: non-esterified fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; TC: total cholesterol; TAG: triacylglycerol; % TE: % of total energy.

Supplemental Table 2.3 Spearman's correlation coefficients (r_s) for the relationship between DXA body composition measurements, CVD risk factors and dietary macronutrients in men women [1,2]

	Body fat, %	Fat mass, kg	Lean mass, kg	VAT, g	Android fat, kg	Android fat %	Gynoid fat %	A/G
Blood pressure, mmHg								
Systolic								
Women	0.25**	0.29**	0.18*	0.43**	0.31**	0.27**	0.18*	0.25**
Men	0.06	0.08	0.08	0.13	0.11	0.09	-0.01	0.19*
Diastolic								
Women	0.30**	0.31**	0.15*	0.37**	0.32**	0.31**	0.24**	0.28**
Men	0.33**	0.32**	-0.04	0.41**	0.37**	0.37**	0.25**	0.40**
Pulse pressure								
Women	0.07	0.12	0.14*	0.25**	0.13	0.08	0.02	0.09
Men	-0.15	-0.11	0.14	-0.14	-0.13	-0.15	-0.16*	-0.08
Biochemistry								
TC, mmol/L								
Women	0.22**	0.16*	-0.12	0.33**	0.22**	0.22**	0.15*	0.22**
Men	0.21**	0.18*	-0.08	0.31**	0.26**	0.26**	0.15	0.31**
TAG, mmol/L								

Women	0.24**	0.25**	0.09	0.34**	0.24**	0.27**	0.15*	0.29**
Men	0.49**	0.48**	-0.01	0.48**	0.53**	0.53**	0.43**	0.46**
HDL-C, mmol/L								
Women	-0.15*	-0.17*	-0.10	-0.04	-0.13	-0.15*	-0.13	-0.13
Men	-0.37**	-0.39**	-0.04	-0.30**	-0.35**	-0.34**	-0.37**	-0.19*
LDL-C, mmol/L								
Women	0.25**	0.18*	-0.12	0.31**	0.24**	0.24**	0.20**	0.21**
Men	0.25**	0.23**	-0.09	0.34**	0.29**	0.28**	0.19*	0.31**
Non-HDL-C, mmol/L								
Women	0.30**	0.23**	-0.10	0.37**	0.29**	0.30**	0.23**	0.28**
Men	0.34**	0.32**	-0.07	0.43**	0.38**	0.38**	0.26**	0.39**
TC: HDL ratio								
Women	0.34**	0.29**	-0.03	0.33**	0.31**	0.33**	0.27**	0.31**
Men	0.52**	0.51**	-0.05	0.54**	0.54**	0.54**	0.45**	0.46**
LDL-C: HDL-C ratio								
Women	0.31**	0.26**	-0.03	0.30**	0.28**	0.29**	0.26**	0.25**
Men	0.47**	0.45**	-0.08	0.49**	0.49**	0.48**	0.41**	0.41**
NEFA, μmol/L								
Women	0.14	0.08	-0.11	0.13	0.11	0.12	0.14	0.08
Men	0.19*	0.14	-0.16*	0.10	0.14	0.16*	0.21**	0.04

Glucose, mmol/L								
Women	0.24**	0.26**	0.14	0.36**	0.28**	0.26**	0.26**	0.22**
Men	0.22**	0.23**	0.08	0.35**	0.28**	0.26**	0.19*	0.30**
Insulin, pmol/L								
Women	0.36**	0.32**	-0.02	0.28**	0.32**	0.34**	0.31**	0.31**
Men	0.51**	0.52**	0.04	0.51**	0.53**	0.53**	0.42**	0.49**
HOMA-IR								
Women	0.35**	0.32**	0.00	0.31**	0.33**	0.33**	0.30**	0.31**
Men	0.51**	0.53**	0.08	0.55**	0.55**	0.54**	0.43**	0.50**
CRP, mg/L								
Women	0.37**	0.37**	-0.001	0.27**	0.33**	0.36**	0.32**	0.31**
Men	0.45**	0.46**	0.07	0.43**	0.48**	0.47**	0.42**	0.37**
GGT, U/L								
Women	0.05	0.10	0.16*	0.17*	0.11	0.08	-0.02	0.13
Men	0.29**	0.30**	0.09	0.29**	0.33**	0.32**	0.29**	0.27**
Uric acid, µmol/L								
Women	0.20**	0.23**	0.09	0.13	0.25**	0.23**	0.15*	0.25**
Men	0.22**	0.23**	0.03	0.16*	0.22**	0.24**	0.20**	0.18*
Adiponectin, µg/mL								
Women	0.05	0.001	-0.07	-0.01	0.01	0.01	0.09	-0.06

Men	0.02	0.004	-0.07	0.01	-0.01	-0.02	0.06	-0.14
Total 25(OH)D, ng/mL								
Women	-0.19**	-0.10	0.19**	-0.02	-0.07	-0.14	-0.25**	-0.04
Men	-0.18*	-0.17*	0.06	-0.11	-0.14	-0.16*	-0.19*	-0.05
Dietary Intake								
Total fat, %TE								
Women	0.02	0.03	0.04	0.11	0.05	0.06	-0.03	0.11
Men	-0.04	-0.02	0.06	0.02	-0.01	-0.04	-0.05	-0.02
SFA %TE								
Women	0.04	0.05	0.07	0.11	0.03	0.03	0.004	0.04
Men	0.05	0.08	0.06	0.12	0.09	0.06	0.04	0.08
MUFA, %TE								
Women	0.01	0.03	0.03	0.08	0.05	0.05	-0.03	0.10
Men	-0.07	-0.04	0.04	-0.01	-0.03	-0.06	-0.08	-0.01
PUFA, %TE								
Women	-0.12	-0.12	-0.04	-0.01	-0.09	-0.07	-0.17*	0.01
Men	-0.17*	-0.18*	-0.02	-0.15*	-0.18*	-0.18*	-0.15	-0.18*
n-6 PUFA, %TE								

Women	-0.15*	-0.12	0.08	-0.06	-0.13	-0.12	-0.20**	-0.03
Men	-0.22**	-0.24**	-0.08	-0.20**	-0.23**	-0.22**	-0.17*	-0.22**
n-3 PUFA, %TE								
Women	0.05	0.004	-0.13	0.12	0.04	0.09	0.04	0.10
Men	-0.10	-0.11	0.003	-0.02	-0.10	-0.12	-0.09	-0.09
Trans fat, %TE								
Women	0.12	0.10	-0.001	0.15*	0.11	0.13	0.12	0.11
Men	0.13	0.15*	0.04	0.17*	0.16*	0.14	0.12	0.11
Protein, %TE								
Women	0.00	0.04	0.16*	0.02	0.03	-0.01	-0.02	-0.01
Men	-0.09	-0.04	0.16*	-0.09	-0.06	-0.09	-0.10	-0.08
Carbohydrate, %TE								
Women	0.01	-0.05	-0.17*	-0.14*	-0.05	-0.03	0.06	-0.07
Men	0.09	0.04	-0.15	-0.01	0.03	0.07	0.10	0.04
Fibre (AOAC), g/day								
Women	-0.26**	-0.16*	0.26**	-0.13	-0.15*	-0.25**	-0.26**	-0.20**
Men	-0.13	-0.10	0.12	-0.13	-0.12	-0.14	-0.11	-0.12
Total Sugars, %TE								
Women	-0.09	-0.06	0.05	-0.12	-0.04	-0.09	-0.12	-0.05

Men	0.03	0.01	-0.04	-0.01	0.003	-0.01	0.05	-0.02

[1]Data analysed by Spearman's correlations

*Significant differences at the 0.05 level

**Significant differences at the 0.01 level

[2]Abbreviations: AOAC: association of analytical chemists; A/G: android to gynoid ratio; CRP: C-reactive protein; GGT: gamma-glutamyl transferase; HDL-C: high density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment for insulin resistance; LDL-C: low density lipoprotein cholesterol; MUFA: monounsaturated fatty acids; NEFA: non-esterified fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; TC: total cholesterol; TAG: triacylglycerol; %TE: % of total energy; VAT: abdominal visceral adipose tissue; total 25(OH)D: 25-hydroxyvitamin D.

Chapter 3: Impact of replacing dietary saturated fatty acids with unsaturated fatty acids on cardiovascular disease risk markers and body composition

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Contribution towards the manuscript

BF, DR, BG, JAL and KGJ designed the study. EO, AK, GW, LS conducted the clinical visits. EO analysed the data and drafted the manuscript under the supervision of KGJ and JAL. All authors contributed to the final manuscript and approved final version.

As this is the first chapter which will begin to describe the RISSCI-1 study (Chapters 3 and 4) the following applies for both chapters:

My primary responsibilities were performing biochemical analysing of screening samples on the ILAB and RANDOX Daytona plus clinical chemistry analysers, isolating PBMC samples after each study visit and inputting the DXA body composition data. AK, GW, LS and I were responsible for volunteer recruitment, conducting screening and study visits. GW and I were also responsible for processing blood samples before storing them for future analysis and measuring the haemoglobin levels on haematology analyser. The analysis of study samples on the ILAB and RANDOX Daytona plus clinical chemistry analysers were performed by AK. Plasma phospholipid fatty acids were measured by the German DifE Institute in Postdam. AK prepared the volunteer dietary booklet which was used during the study visits. GW and I were responsible for ordering and preparing the snacks provided to volunteers as a part of intervention diets. LS analysed the dietary data on Nutritics. LDL subclass analysis was performed by the National Phenome Centre, Imperial College London. DXA body composition analysis was performed by KGJ and RM. I was also responsible for performing the statistical analysis and drafting the manuscript under the supervision of KGJ and JAL.

Impact of replacing dietary saturated fatty acids with unsaturated fatty acids on cardiovascular disease risk markers and body composition

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3.1 Abstract

Purpose: To determine the effect of replacing dietary saturated fat (SFA) with unsaturated fat (UFA) on fasting blood lipid profile, and relationship with measures of body composition.

Methods: In a single-blind, controlled sequential dietary intervention study healthy men aged 30-65y consumed two iso-energetic diets; a high SFA (18 % total energy (TE)) followed by a low SFA (10%TE) for 4 weeks each. Fasting blood samples were collected to determine cardiovascular disease (CVD) risk markers at each visit and participants underwent a dual energy x-ray absorptiometry scan to determine their body composition at the end of each diet (weeks 4 and 8). Paired t tests were used to compare body composition measures and CVD risk markers after the diets. To determine relationships between changes in lipid risk markers with body composition, bivariate Spearman's correlations were performed.

Results: Relative to the high SFA diet, total cholesterol (TC), triacylglycerol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels decreased after the low SFA diet ($p<0.01$). Android fat percentage reduced during the low SFA diet, while android lean mass increased ($p\leq0.02$). A positive relationship was found between changes in TC and non-HDL-C concentrations with the android:gynoid fat ratio ($r_s=0.36$-0.38, $p<0.05$) and moderate positive associations were also evident between changes in TC, LDL-C, and non-HDL-C with body fat mass (kg) ($r_s=0.31$-0.35, $p<0.05$).

Conclusion: Beneficial effects of replacement of dietary SFAs with UFAs on CVD risk markers in healthy men may be mediated, in part, via an impact of dietary fat on body fat distribution.

Clinical trial registry: NCT03270527; registration date: 2017-09-01

Keywords: dyslipidaemia, saturated fatty acids, abdominal obesity, cardiovascular disease

3.2 Introduction

Although our understanding of prevention and treatment of non-communicable diseases has progressed, cardiovascular diseases (CVDs) remain a major cause of death throughout the world (210, 211). Dyslipidaemia, characterised by elevated total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triacylglycerol (TAG), and low high-density lipoprotein cholesterol (HDL-C) concentrations, is an established risk factor for the development of this disease (212). Diet among other lifestyle factors remains to be one of the most important modifiable factors contributing to dyslipidaemia and CVD risk (213).

Numerous studies have reported the cholesterol-raising effects of diets high in saturated fatty acids (SFAs) (157). Therefore, reduction in dietary SFA intakes has been a target for CVD prevention since 1994 in the UK (32). Current Scientific Advisory Committee on Nutrition (SACN) recommendations are to reduce SFA intake to less than 10% of total energy (TE) and to replace this with unsaturated fatty acids (UFA). Findings from dietary intervention studies replacing SFAs with polyunsaturated fatty acids (PUFAs) and to a lesser extent with monounsaturated fatty acids (MUFAs) have shown beneficial effects on fasting lipid profile with reductions evident in TC and LDL-C concentrations (35, 64). However, some argue that there are no beneficial effects of replacing dietary SFAs with UFAs for CVD prevention (214).

Abnormal blood lipid profiles are commonly observed in people who are obese, and excess body fat is strongly associated with an increased risk of CVD incident and mortality (215). Recently it has been reported that body fat distribution, especially abdominal fat accumulation, is a better predictor of future disease risk than body mass index (BMI) (216). Findings from animal studies suggested that high intakes of SFAs are positively associated with abdominal obesity, while UFA have beneficial effects (217, 218). In our recent cross-sectional study, we observed both dietary SFA and abdominal obesity to be independently associated

with LDL-C, and that android lean mass and LDL-C concentrations were different across quartiles of increasing SFA intake (Chapter 2). However, the limited numbers of human studies investigating the effects of replacing dietary SFAs with PUFAs or MUFAs on body fat distribution have generated inconsistent results (9, 102, 107, 219-222). It has been reported that an increased BMI is inversely associated with the fasting lipid response to dietary fat manipulation (173). Moreover, a recent meta-analysis concluded that replacement of dietary SFAs with UFAs may marginally improve blood lipid profiles in metabolically healthy adults with increased BMI, waist circumference or waist to hip ratio (223). Thus, the discrepancy between studies investigating the effects of dietary fat manipulation on blood lipids might be, in part, due to adiposity, especially abdominal obesity. Therefore, changes in body composition in response to dietary fat composition may represent an important mechanism underlying the impact of dietary fat intake on blood lipid profiles.

In this study, we examined the effect of replacing dietary SFAs with UFAs at the level recommended for CVD prevention on blood lipid risk markers and determined whether this effect was linked with an impact on body composition. We hypothesised that replacing dietary SFAs with UFAs is beneficial for fasting lipid risk markers and related to a reduction in abdominal fat mass.

3.3 Methods

Subjects

A subset of healthy men aged 30-65 years from the Reading Imperial Surrey Saturated fat Cholesterol Intervention (RISSCI-1) study (n=41/109) were included in this analysis to determine the effect of replacing dietary SFA with UFAs on CVD risk markers and body composition (Figure 3.1). Details of the RISSCI-1 study have been previously published (Appendix I). Briefly, participants in this subgroup analysis were recruited from Reading and

the surrounding areas using posters, online advertisements on social media, by organising recruitment events in the town centre or in supermarkets and by contacting previous volunteers registered on the Hugh Sinclair clinical unit volunteer database. A medical and lifestyle questionnaire was used to assess eligibility of interested volunteers, followed by a screening visit. Inclusion criteria were healthy men with a BMI of 19-32 kg/m^2, without diagnosed CVD; fasting TAG <2.3mmol/l, fasting TC<7.5 mmol/l, non-smokers, not diabetic (fasting glucose <7.0 mmol/l) or suffering from other endocrine disorders, not on medication for hyperlipidaemia, no history of alcohol abuse or anaemia. Exclusion criteria included medical history of diabetes, heart disease, kidney, bowel or liver diseases, cancer, or hormone abnormalities. Taking certain types of medication (e.g. drugs for high blood pressure, high blood fats, inflammatory conditions and depression), or dietary supplements which can affect lipid metabolism or gut microbiota (e.g. cholesterol lowering spreads, fish oil, probiotics, prebiotics and natural laxatives), antibiotics in the last 3 months, drinking more than 14 units of alcohol per week, being on a weight reducing diet, unwillingness to consume butter/spreads, oil, dairy products and snack foods for the duration of the study, travelling frequently for work and participating in another intervention study. Furthermore, due to the use of dual energy x ray absorptiometry (DXA) participants with arthritis or fracture deformity of spine or femur, history of bone related surgeries, radio-opaque implants or implanted medical devices were excluded. All volunteers were given a unique code number throughout the study and provided verbal and written informed consent.

Study Design

RISSCI-1 was an 8-wk, single-blind, sequential dietary intervention study whereby subjects followed 2 isoenergetic diets, each of 4 weeks duration in the same order. The sequential study design was used due to inherent difficulties during fat manipulation studies for volunteers to return their habitual diet during washout periods. The two iso-energetic intervention diets were

high SFA (18%TE), low UFA (15%TE) and low SFA (10%TE), high UFA (24%TE). Participants attended 3 study visits: baseline (week 0), at the end of diet 1 (week 4) and end of diet 2 (week 8). After the baseline visit, participants were given SFA-rich foods, and the instructions on how to incorporate these within their diet were given both orally and in written form. At the end of high SFA diet, they were provided with low SFA foods and advice.

This study was carried out in accordance with the principles of the Declaration of Helsinki and registered at www.clinicaltrials.gov (NCT03270527). The University of Reading Research Ethics Committee gave favourable ethical opinions for conduct (11/05/17 UREC reference number: 17/29). The measurement of body composition using DXA scan was approved by the NHS Research Ethics Committee (27/05/14 NHS reference number: 14/SC/1095).

Dietary Intervention

The details of the dietary intervention have been published elsewhere (Appendix I). Briefly, a dietary exchange model was developed based on previous studies conducted by the researchers to achieve the target dietary fatty acids in the study population (224). Participants replaced their habitual dietary fat sources with either high SFA or UFA oils, spreads, and snacks while they were advised to maintain their habitual diet apart from the dietary fat modification. To achieve this, commercially available sweet and savoury snacks, cooking oils and spreads were provided to the subjects free of charge. Spreads (Flora buttery, Upfield Europe B.V.) and vegetable oils (KTC Edibles Ltd., West Midlands) were used for the low SFA diet, while butter (Wyke Farms Ltd., Somerset) for the high SFA diet. Compliance was evaluated with 4-day weighed food diaries (baseline, week 4 and 8) and tick sheets which were assessed every 2 weeks by a researcher. For each subject, dietary nutrient and energy intakes were calculated using Nutritics software (Research edition, V5.09) and the intakes were expressed as a mean daily intake.

Additionally, body weight was monitored weekly by the participants at home with bathroom scales, and a change of more than 1 kg was followed up by a researcher.

Study visits

Visits took place in the Hugh Sinclair Unit of Human Nutrition at the University of Reading at baseline (week 0), weeks 4 and 8. Alcohol, caffeine, and strenuous exercise were avoided 24h before each visit. Participants were provided with a low-fat evening meal and a bottle of low-nitrate mineral water (Buxton, Nestlé waters UK) (due to other measurements in the study e.g., vascular function) and advised to fast for 12 h, only drinking the mineral water during this time. On the study day anthropometric measurements were performed and blood samples were collected by a trained researcher. Participants also underwent a DXA scan on the study visits at weeks 4 and 8.

Anthropometric measures

Anthropometric measures were performed after an overnight fast at each study visit while volunteers were wearing light clothing. Height was measured using a stadiometer without shoes, with the participant facing forwards standing as straight as possible with their arms hanging loosely and their head in the Frankfort plane Body weight was measured and BMI calculated using a Tanita scale (Tanita BC-418, TANITA UK Ltd, Middlesex, UK). Waist circumference was measured halfway between the top of the iliac crest and lower ribs and hip circumference at the largest circumference of the hip bone. Both measurements were performed to the nearest 0.1 cm manually using a non-stretch tape measure (Seca, UK). Waist to hip ratio and waist to heigh ratio were calculated from waist circumference, hip circumference, and height in cm as estimates of body fat distribution.

Body composition indices

The visceral adiposity index (VAI) was calculated as VAI=waist

circumference/(39.68+(1.88xBMI)) x (TAG/1.03) x (1.31/HDL-C) (174) as an indicator of visceral adipose tissue function. Waist circumference was expressed in cm, TAG and HDL-C in mmol/l (225). Fat mass index was calculated following formula FMI=fat mass(kg)/height in m^2 and lean mass index was calculated as LMI= lean mass(kg)/height in m^2.

DXA scan measurement of total body composition

Prior to the DXA scan, participants eligibility for a scan was assessed using a questionnaire and their hydration levels were checked using a spot urine sample. Researchers ensured that participants were sufficiently hydrated (defined as a reading below 600 mOsmol/kgH2O on Osmocheck refractometer), as it may cause underestimation of fat free mass. Participants were asked to remove any clothes with metal zips, buttons, and jewellery. Lunar iDXA was used to assess whole body composition by two trained researchers and the manufacturer's guidelines for positioning and scan acquisition were followed. enCORE software version 15 (GE Healthcare, UK) with the advance software package CoreScan was used to analyse the scan. Each scan lasted between 5 to 12 minutes depending on the scan mode which was assigned based on the BMI of the participant by the enCORE software (version 15, GE Healthcare).

Biochemical analysis

Blood samples were collected into serum separator and K_3EDTA tubes after an overnight fast at each visit and centrifuged at 1700 x g (3000 rpm) for 15 min at room temperature and 4^{o}C, respectively. Serum samples were then stored at -20^{o}C and plasma samples at -80^{o}C. Fasting blood lipids (TC, HDL-C, non-esterified fatty acids (NEFA), TAG), glucose and high sensitivity C-reactive protein (hs-CRP) were measured in the serum samples using the ILAB 600 (Werfen UK Ltd., Warrington UK) and Daytona Plus (Randox Laboratories Limited, Crumlin, UK) clinical chemistry analyser. The Friedewald equation was used to estimate fasting LDL-C (182). Non-HDL-C was calculated by subtracting HDL-C from TC. Plasma

phospholipid fatty acids were measured by a modified method using extraction with tert-butyl methyl ether/methanol, solid phase separation, hydrolysis and methylation with trimethyl sulfonium hydroxide, and gas-chromatography with a flame ionization detector by the German DifE Institute in Postdam. Using the fatty acid measurements, the sums of total SFA, UFA, PUFA and MUFA were calculated and expressed as % total fatty acids.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS Incl., IL, USA). Normality was assessed using the Kolmogorov-Smirnov test and Q-Q plots. hs-CRP, NEFA, total fat %TE and PUFA%TE were log transformed prior to analysis. Descriptive statistical results were presented as mean ± standard deviation (SD) for normally distributed variables and as median (interquartile range) for non-normally distributed data. Paired t tests were used to assess the differences between participant anthropometric measurements, biochemical profile, and body composition measurements after the high and low SFA diets. Dietary macronutrient intakes and plasma fatty acid profiles during the high and low SFA diets were also compared by paired t tests. Results were presented as mean ± SD and $p<0.05$ was considered significant. Changes in body composition measures were correlated with changes in blood lipids, dietary fatty acids, and plasma fatty acid profiles after the two intervention diets by Spearman's correlations.

3.4 Results

The baseline characteristics of the subgroup of the RISSCI-1 study participants are shown in **Table 3.1.** Based on their baseline 4-day weighed food diary, this subgroup of the RISSCI-1 study consumed higher SFA than the current UK recommendations.

The macronutrient composition for the high and low SFA diets is shown in **Table 3.2**. Although energy intake was 5.5 % higher during the high SFA diet compared to low SFA diet

(p=0.05), there was no difference in the total fat %TE intake between diets. Dietary fatty acid targets were met with increases of MUFA%TE, total PUFA%TE, n-3 and n-6 PUFA%TE and decrease in SFA%TE intakes during the low SFA diet ($p<0.01$ for each). Energy intake from trans-fat was 14.7 kcal higher during the high SFA diet compared to the low SFA diet ($p<0.01$).

Body composition and CVD risk markers after the high SFA and low SFA diets are shown in **Table 3.3.** There were no significant changes in body weight (p=0.75) and BMI (p=0.89) between diets. Although there was a trend towards a decrease in total body fat percentage after the low SFA diet compared with high SFA, it was not statistically significant (p=0.09). Android lean mass was found to increase by 1.06% after the low SFA diet (p=0.02) while there was a 2% decrease in android fat percentage (p=0.01). TC, TAG, HDL-C, LDL-C, and non-HDL-C concentrations were on average 10-15% lower after the low SFA diet compared to the high SFA diet, respectively ($p<0.01$). TC:HDL-C and LDL-C:HDL-C ratios were both lower after the low SFA diet compared with high SFA diet ($p\leq0.03$). Although systolic and diastolic blood pressure were not different between the two diets, there was a trend for lower diastolic blood pressure after the low SFA diet (p=0.07). Pulse pressure was found to be higher after the low SFA diet compared to the high SFA diet (p=0.02) .

Spearman's correlation coefficients between changes in anthropometric and body composition measures and changes in CVD risk markers in response to reducing dietary SFAs are presented in **supplemental table 3.1**. The change in android lean mass was negatively correlated with the change in HDL-C ($r_s=0.43$, $p<0.01$) whilst the change in lean body mass was positively associated with the TC:HDL-C ratio ($r_s=0.32$, $p<0.05$). Moderate positive associations were found between the change in body fat mass and changes in TC and LDL-C ($r_s=0.35$ and rs=0.32 respectively, $p<0.05$). The change in android:gynoid (A/G) fat ratio was also found to be positively correlated with changes in TC and non-HDL-C concentrations

(r_s=0.38 and rs=0.36 respectively, p<0.05). Changes in anthropometric and other body composition measures were not correlated with changes in any of the other CVD risk markers.

Correlation coefficients between changes in anthropometric and body composition measures and changes in dietary fatty acids or plasma phospholipid fatty acids are shown in supplemental tables 2 and 3**,** respectively. For dietary fats, the change in gynoid fat percentage was found to be negatively associated with changes in total fat, SFA and MUFA intakes (r_s=-0.43 p<0.01, r_s=-0.35 p<0.05, r_s=-0.31 p<0.05, respectively).

For plasma phospholipid fatty acids, the change in android lean mass was negatively correlated with the change in palmitic acid (C16:0) and palmitoleic acid (C16:1 n-7) (r_s=-0.38, r_s=-0.36, p<0.05, respectively) whereas it was positively correlated with changes in stearic acid (C18:0) and linoleic acid (C18:2 n-6) (r_s=0.41p<0.01 and r_s=0.38 p<0.05, respectively). In contrast, the change in gynoid fat percentage was positively correlated with the change in palmitic acid (C16:0) (r_s=0.36, p<0.05) while it was negatively correlated with change in stearic acid (C18:0) (r_s=-0.38, p<0.05). Furthermore, the change in A/G fat ratio was positively associated with the change in eicosapentaenoic acid (C20:5 n-3) (r_s=0.41, p<0.01) (**supplemental table 3.3**).

3.5 Discussion

It is well recognised that dietary fat intake can modulate the fasting lipid profile, but little is known about the effects on body fat distribution and its relationship to changes in the CVD lipid risk markers. Using a sequential dietary intervention in the current study, we found that replacing SFAs with UFAs at the level recommended for CVD prevention had beneficial effects on the lipid profile, android lean mass, and android fat percentage. Changes in some body composition measures (android lean mass, body lean mass, body fat mass and A/G fat ratio) in

response to dietary SFA replacement were also found to be correlated with several lipid risk markers.

The effects of substituting dietary SFAs with PUFAs or MUFAs on blood lipids have been examined in many dietary intervention studies and there is strong and consistent evidence from these studies showing a reduction in TC and LDL-C concentrations (35, 64, 226). In agreement with these previous studies, reductions in TC and LDL-C concentrations were evident in our study after replacing dietary 10%TE SFA with UFA. Recently, non-HDL-C has been recognised as an important CVD risk marker as it reflects all circulating atherogenic lipoproteins (227). A decrease in non-HDL-C concentration was also observed in our participants after reducing dietary SFA and increasing UFA intakes. This is consistent with the evidence from a cross-over study which showed a significantly lower non-HDL-C concentration after an 8-week walnut-enriched diet (39%TE fat (12.3%TE SFA,11%TE MUFA, 14.1%TE PUFA), 15%TE protein, 44%TE carbohydrate) compared to the iso-caloric Western-type control diet (33%TE fat (14.4%TE SFA, 11%TE MUFA, 4.6%TE PUFA) 16%TE protein, 49%TE carbohydrate) in older men and women. However, in this study it could be argued that the findings were mostly due to an increase in PUFA intake or a reduction in carbohydrates rather than a reduction in SFA as there was only a small decrease in SFA%TE intake in walnut-enriched diet group (228). Our results are also consistent with some of the previous studies which found a reduction in HDL-C concentrations after replacing dietary SFA with PUFA or MUFA (49, 229). The possible mechanism behind this is thought to be the suppressive effect of UFAs on cholesterol transporters (ATP binding cassette subfamily G member 1 and subfamily A member 1), which play an important role in transferring cholesterol to HDL (230). In addition, previous studies reported that TC:HDL-C and LDL-C:HDL-C ratios are better predictors of CVD risk than TC and LDL-C alone (231). In our study, in line with some of the previous studies decreases in TC: HDL-C and LDL-C: HDL-C ratios were evident

(157, 190). Moreover, two recent meta-analysis and systematic reviews concluded that there is no effect of reducing dietary SFA on TAG (45, 156), however, we found 14% reduction in TAG concentration when participants followed a low SFA diet. This is in agreement with a cross-over study which reported a 14% reduction in TAG concentration during the MUFA-rich diet (8.8%TE SFA, 20.3%TE MUFA, 3.5%TE PUFA) compared to the SFA-rich diet (20.8%TE SFA, 9.6%TE MUFA, 2.7%TE PUFA) in healthy men and women (232). In our study, there was no significant difference in NEFA concentrations between the low SFA and high SFA diets supporting a previous study which reported a lack of effect after replacing dietary SFAs with PUFAs in a group of healthy and obese participants (9). Therefore, our study is in line with the literature reporting the beneficial effect of replacing dietary SFA with UFA on several blood lipid risk markers. These beneficial effects on blood lipid changes could have a positive impact on reducing CVD risk.

It is well-established that the circulating cholesterol concentration is determined by both lipoprotein production and clearance (24). Findings from animal and in vitro studies suggest that dietary SFA downregulate LDL receptor (LDL-R) gene expression and activity while UFAs have the opposite effect (71). It has been proposed that dietary SFAs increase intracellular free cholesterol concentrations by inhibiting acyl-CoA:cholesterol acyltransferase which suppresses LDL-R gene expression through inhibiting the maturation of sterol regulatory element-binding protein-2 (195, 233). Moreover, diets high in SFA have been associated with smaller and larger LDL particles and these particles have been shown to have a reduced affinity for the LDL-R than intermediate sized particles (73, 74, 234). It has also been proposed that uptake of LDL particles by LDL-R can be affected by competition with TAG rich lipoproteins enriched in apoE with meals rich in SFA associated with a higher particle apoE content (75). However, the latter mechanism has been derived from cell studies, therefore further research is needed in humans to determine the impact of dietary fat composition on LDL clearance.

Findings from animal studies suggest that diets high in SFAs are also associated with greater adiposity when compared to high UFA diet (235). However, studies in humans investigating the effect of replacing dietary SFAs with UFAs on body composition are limited. Piers et al. (102) in their randomised cross over study showed a differential effect of dietary fat composition on trunk fat mass measured by DXA in 8 overweight or obese men, with a decrease of 0.8 kg found following a high MUFA diet (SFA 11%, MUFA 22.3%, PUFA 7%) compared with an increase after 4 weeks on a high SFA diet (SFA 24.4%, MUFA 12.5%, PUFA 3%). Using magnetic resonance imaging, a reduction in the abdominal subcutaneous area was evident when dietary SFA were replaced with n-6 PUFA in women with and without obesity in a cross-over study where subjects with obesity (n=5), type 2 diabetes mellitus (n=6) and those that were non-obese (n=6) followed SFA and PUFA rich diets for 5 weeks each (9). In another dietary intervention study, 39 healthy participants were fed with muffins containing either linoleic acid-rich sunflower oil or SFA-rich palm oil for 7 weeks and their body composition was assessed by magnetic resonance imaging and air-displacement plethysmography. While total body weight gain was the same for each group, the PUFA group gained more lean mass than the SFA group which showed a greater increase in total body fat (107). Moreover, in a cross-sectional study where DXA scans were performed to analyse body composition, a negative association between dietary SFA and fat free mass and positive association between PUFA/SFA ratio and fat free mass were found in young and older women (236). These findings are in line with our study which found a decrease in android fat percentage and an increase in android lean mass without a significant change in BMI or WC when dietary SFA was replaced with UFA. Therefore, in our study, positive effects of replacing dietary SFA with UFA on blood lipids were accompanied by beneficial changes in android lean mass and android fat percentage. Moreover, although changes in android lean mass in response to replacing dietary SFA with UFA was not found to be associated with changes in dietary fat intakes, it was associated

positively with changes in plasma stearic acid and inversely associated with plasma palmitic acid. These results are similar to those reported by Shen et al. (237) who showed an increase in lean body mass when athymic nude mice were fed a stearic acid enriched diet. It has also been indicated that palmitic acid increase accumulation of toxic lipid intermediates in muscle cells which leads to inhibition of protein synthesis, thus having a negative effect on muscle mass (238).

The potential mechanisms behind the positive effects of replacing SFAs with UFAs on body composition has been investigated in several animal and in vitro studies (239, 240). Dietary fatty acid structure including degree of unsaturation and number of carbon atoms has been considered to play an important role in metabolic response. Initially, it has been suggested diets rich in PUFA, especially n-3 PUFAs affect adipose tissue metabolism via impacting on transcription factors which are involved in adipocyte differentiation, adipogenesis and lipogenesis. It has been shown that they reduce sterol regulatory element-binding protein-1c mRNA level and inhibit its maturation, act as an antagonist for the liver x receptor and plays a role in activation of peroxisome proliferator-activated receptors (241-244). Secondly, the impact of dietary fat composition on energy expenditure have also been investigated (243). Both MUFAs and n-3 PUFAs have been reported to increase fatty acid oxidation rates within the liver and n-6 PUFAs to produce greater diet-induced thermogenesis compared to long chain SFAs in animal studies (244). In other words, because long chain SFAs oxidise slower, they are more likely to be stored in the body compared to UFAs. Studies in humans which used indirect calorimetry and stable isotope labelled fatty acids to examine the effect of diet on fatty acid oxidation have also indicated that higher levels of long chain PUFAs and MUFAs increase β-oxidation and diet-induced thermogenesis (245-249). Thus, these results might indicate that diets rich in UFA cause less fat accumulation, explaining the potential positive effects of dietary UFAs in comparison to SFAs on body fat composition (110). However, it should be noted that

these results are from acute feeding studies in humans, and it is not clear that if replacing SFAs with UFAs is sufficient to increase oxidation rates to decrease body fat mass in the long term (58, 102). Moreover, although the mechanisms behind the differential effect of SFA and UFA on lean mass remains to be determined, it may involve chronic low-grade inflammation and insulin resistance. Dietary SFA increase inflammation and insulin resistance while PUFA have the opposite effect (250), with higher levels of circulating inflammatory cytokines and insulin resistance found to be associated with skeletal muscle loss (251-253). Furthermore, findings from animal studies suggest that diets rich in PUFA reduce protein oxidation, promote cell differentiation and growth (243). Dietary PUFA also prevent accumulation of lipotoxic intermediates (such as ceramides and diacylglycerol) which are involved in skeletal muscle atrophy and they down-regulate the Akt/mammalian target of rapamycin pathway (mTOR), the main regulator of cell growth and protein synthesis (243).

In order to better understand the relationship between SFA, blood lipids and body composition, changes in body composition measures with blood lipids in response to lower SFA intake were correlated. Interestingly, we observed a negative correlation between changes in HDL-C concentration and android lean mass. Although this result seems unexpected, an inverse association between HDL-C and fat free mass has been previously reported in healthy adults in a cross-sectional study. The authors concluded the link between decreased HDL-C and increased BMI might be due to non-adipose tissue instead of visceral fat mass (254). A possible explanation is that overexpression of muscle lipoprotein lipase mediates increased HDL clearance and lead to reduction in HDL-C concentrations (255). A more recent longitudinal study also reported that 1 kg/m^2 increase in fat free mass index was associated with 0.06 mmol/l decrease in HDL-C concentrations in middle aged men (256). The author suggested that this inverse relationship might be as a result of an increase in fat free mass without a decrease in fat mass. In agreement with this, we also observed a small reduction in android fat percentage

along with a greater increase in android lean mass in response to the low SFA diet. Moreover, there was a positive correlation between changes in TC and LDL-C with the change in body fat mass in response to reducing SFA intake. This is in line with a recent randomised controlled study which reported that blood lipid response to replacing dietary SFA with PUFA differed between normal-weight and obese participants (257). Positive associations between changes in TC and non-HDL-C with change in A/G fat ratio were also evident. Hence, the positive effect of replacing dietary SFA with UFA on fasting blood lipids may be, in part, associated with its effect on body fat distribution.

There are several strengths to this study. First of all, it is a controlled intervention study which investigated the cause-and-effect relationships. Secondly, the iso-energetic dietary fat exchange model allowed successful manipulation of participants' dietary fat intake, without changing other macronutrients or impacting on body weight. In addition, DXA was used to assess total body composition, providing an accurate measurement of body fat distribution, including estimation of abdominal visceral adipose tissue. Limitations included the challenges associated with assessing dietary compliance within a free-living population. However, the compliance was monitored throughout the study using 4-day diet diaries and self-completed tick sheets. Although there was a small, 134 kcal (0.5MJ) decrease in the total energy intake when SFAs were replaced with UFAs, no difference in the dietary fat intake was seen, and dietary fat composition targets were broadly met. Moreover, plasma phospholipid fatty acids, which correlate with short to medium intake of dietary fatty acids, verified the targeted dietary changes (303). In addition, only men were included in the study, therefore the findings may not be transferable to a female population. Moreover, the 4-week intervention period may not be sufficient for observing significant changes in body composition. Lastly, the sequential dietary intervention design may also be considered as a limitation.

In conclusion, replacement of dietary SFA with UFA was found to have a beneficial effect on the fasting lipid profile, abdominal obesity and android lean mass in healthy men and changes in the fasting lipid profile was found to be associated with changes in A/G fat ratio, body lean mass, android lean mass and body fat mass. Our results might indicate that change in fasting blood lipids in response to dietary fat intake may be, in part, due to changes in body fat distribution. However, the mechanisms behind the effect of dietary fat composition on body composition and impact on CVD risk markers needs to be investigated further in mechanistic studies.

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Declarations

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Conflict of interest

The authors have no conflict of interest. JAL sits on the UK Government’s Scientific Advisory Committee for Nutrition (SACN) and was on SACN’s Saturated Fats and Health

working group. JAL chaired, and KGJ was an expert on the ILSI Committee on Saturated fatty acids and cardiovascular risk.

Code availability

Not applicable.

Author's contributions

BF, DR, BG, JAL and KGJ designed the study. EO, AK, GW, LS conducted the clinical visits. EO analysed the data and drafted the manuscript under the supervision of KGJ and JAL. All authors contributed to the final manuscript and approved final version.

Ethics approval

This study was approved by The University of Reading Research Ethics Committee (11/05/17 UREC reference number: 17/29). The measurement of body composition using DXA scan was approved by the NHS Research Ethics Committee (27/05/14 NHS reference number: 14/SC/1095).

Consent to participate

Written and oral informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Table 3.1 Baseline characteristics of the study participants

	All	Range
Age, y	51±10	32-66
Weight, kg	78.7±11.5	59.3-112.9
BMI, kg/m^2	24.9±3.3	19.1-33.3
WC, cm	93.1±9.2	77.5-113.8
HC, cm	104.1±7.5	93.0-127.0
WHR	0.89±0.07	0.77-1.03
WHtR	0.53±0.06	0.43-0.67
Blood pressure, mmHg		
systolic	117±11	100-145
diastolic	79±8	63-96
pulse pressure	38±8	26-71
Biochemistry		
TC, mmol/l	5.09±0.95	3.59-7.48
TAG, mmol/l	1.09±0.47	0.34-2.33
HDL-C, mmol/l	1.47±0.34	0.99-2.51
LDL-C, mmol/l	3.11±0.82	1.80-5.32
Non-HDL-C, mmol/l	3.65±0.89	2.01-5.69
TC: HDL-C ratio	3.57±0.78	2.11-5.17
LDL-C: HDL-C ratio	2.21±0.66	0.99-3.61
NEFA, mmol/l	0.41(0.32-0.57)	0.20-1.12
Insulin		
Glucose, mmol/l	5.23±0.53	4.44-6.70
CRP, mg/l	0.94(0.48-1.95)	0.10-6.09
Dietary Intake		
Energy, MJ	9.3±2.2	5.2-13.8
Energy, kcal	2224±536	1238-3275
Total fat, %TE	35.2(30.6-40.2)	18.4-76.1
SFA, %TE	12.6±4.1	3.4-28.5
MUFA, %TE	13.3±3.8	5.3-26.7
PUFA, %TE	5.5(4.4-6.5)	1.9-12.9
n-3 PUFA, %TE	0.8±0.4	0.1-1.9
n-6 PUFA, %TE	4.6±2.0	1.8-10.8
Trans fat, %TE	0.43±0.21	0.02-0.87
Protein, %TE	15.8±2.8	9.5-21.6
Carbohydrate, %TE	45.1±9.9	8.1-72.1
Fibre (AOAC), g	26.1±9.5	11.9-47.5
Free Sugars, %TE	7.6±4.7	0.8-19.3

[a]Data presented as mean ± SD and median (interquartile range). [b]Abbreviations: AOAC: association of official analytical chemists; BMI: body mass index; CRP: C-reactive protein; HC: hip circumference; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; MUFA: monounsaturated fatty acids; NEFA: non-esterified fatty acids; SFA: saturated fatty acids; TAG: triacylglycerol; TC: total cholesterol; PUFA: polyunsaturated fatty acids; WC: waist circumference; WHR: waist to hip ratio; WHtR: waist to height ratio. [c]Sample size n=41 apart from WC, HC, WHR, WHtR n=40 and blood pressure n=37.

Table 3.2 Daily energy and macronutrient intakes of study participants during the high SFA and low SFA diets

	High SFA diet	Low SFA diet	P-value
Energy, kcal	2418±514	2284±495	**0.05**
Energy, MJ	10.1±2.1	9.6±2.1	**0.05**
Total fat, %TE	37.5 (34.3-40.6)	36.7 (32.1-41.0)	0.54
SFA, %TE	19.0±3.7	8.7±2.3	**P<0.01**
MUFA, %TE	11.1±3.3	12.2±2.9	**P<0.01**
PUFA, %TE	3.3 (2.9-4.0)	10.5 (8.8-12.3)	**P<0.01**
n-3 PUFA, %TE	0.6±0.4	1.4±0.6	**P<0.01**
n-6 PUFA, %TE	2.3±0.9	9.4±3.5	**P<0.01**
Trans fat, %TE	0.8±0.3	0.2±0.2	**P<0.01**
Protein, %TE	15.8±2.3	15.7±3.0	0.90
Carbohydrate, %TE	43.0±7.7	43.7±8.5	0.46
Fibre (AOAC), g	25.8±11.0	27.3±11.9	0.18
Free sugars, %TE	4.85±3.25	5.24±3.36	0.44

[a]Data was presented as mean ± SD for normally distributes data and as median (IQR) for non-normally distributed data.

[b] Data was analysed by paired t-tests using transformed data. $p<0.05$ is considered as significant.

[c]Abbreviations: AOAC: association of official analytical chemists; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA saturated fatty acids; TE: total energy.

[d]Sample size n=41

Table 3.3 Body composition and CVD risk markers after the high SFA and low SFA diets

	High SFA diet	Low SFA diet	P-value
Anthropometric measurements			
Weight, kg	78.6±11.9	78.6±11.9	0.75
BMI, kg/m^2	24.9±3.8	24.9±3.4	0.89
WC, cm	91.9±9.4	91.8±9.5	0.77
HC, cm	103.3±7.9	102.7±6.0	0.28
WHR	0.89±0.07	0.89±0.07	0.50
WHtR	0.52±0.06	0.52±0.06	0.79
Body composition measurements			
Body fat, %	25.9±7.0	25.7±7.2	0.09
Fat mass, kg	20.9±7.9	20.8±8.2	0.22
Lean mass, kg	55.2±6.7	55.3±6.8	0.35
Android mass, kg	5.81±1.15	5.82±1.12	0.36
Android lean, kg	3.76±0.51	3.80±0.49	**0.02**
Android fat, kg	1.99±1.04	1.97±1.06	0.20
Android fat, %	32.9±12.0	32.3±12.3	**0.01**
Gynoid fat, %	27.1±6.5	26.8±6.5	0.12
Abdominal VAT, kg	1.1±0.7	1.1±0.8	0.89
A/G fat ratio	1.19±0.26	1.17±0.28	0.20
Indexes			
FMI, kg/m^2	6.7±2.6	6.6±2.7	0.24
LMI, kg/m^2	17.4±1.3	17.4±1.3	0.39
VAI	1.11±0.13	1.05±0.67	0.14
CVD risk markers			
SBP, mmHg	119±11	121±11	0.21

DBP, mmHg	82±8	79±8	0.07
pulse pressure, mmHg	38±8	41±8	**0.02**
TC, mmol/l	5.36±0.91	4.64±0.80	**P<0.01**
TAG, mmol/l	1.15±0.56	0.99±0.47	**P<0.01**
HDL-C, mmol/	1.55±0.35	1.39±0.30	**P<0.01**
LDL-C, mmol/l	3.29±0.85	2.81±0.71	**P<0.01**
Non-HDL-C, mmol/l	3.81±0.86	3.26±0.78	**P<0.01**
TC: HDL-C ratio	3.58±0.78	3.45±0.77	**0.01**
LDL-C: HDL-C ratio	2.20±0.66	2.10±0.65	**0.03**
NEFA, mmol/l	0.37(0.27-0.52)	0.38(0.28-0.55)	0.50
Glucose, mmol/l	5.23±0.47	5.24±0.56	0.93
CRP, mg/l	0.90(0.46-1.62)	0.75(0.34-2.09)	0.32

[a]Data was analysed by paired t tests using transformed data for not normally distributed variables.

[b]Data was presented as mean ± SD for normally distributed and as median (IQR) for not normally distributed data. P<0.05 is considered as significant.

[c]Abbreviations: A/G: android to gynoid fat ratio; BMI: body mass index; CRP: C-reactive protein; CVD: cardiovascular disease; DBP: diastolic blood pressure; FMI: fat mass index; HC: hip circumference; HDL-C: high density lipoprotein cholesterol; LMI: lean mass index; LDL-C: low density lipoprotein cholesterol; NEFA: non-esterified fatty acids; SBP: systolic blood pressure; SFA: saturated fatty acids; TAG: triacylglycerol; TC: total cholesterol; VAT: visceral adipose tissue; VAI: visceral adiposity index; WC: waist circumference; WHR: waist to hip ratio; WHtR: waist to height ratio.

[d]Sample size n=41 apart from WHR, WHtR, WC, HC n=39 and SBP, DBP and pulse pressure n=35

Supplemental Table 3.1 Correlation between change in body composition and changes in CVD risk markers (V3-V2) in response to replacing dietary SFA with UFA

	TC, mmol/l	TAG, mmol/l	HDL-C, mmol/l	LDL-C, mmol/l	Non-HDL-C, mmol/l	TC: HDL-C	LDL-C: HDL-C	NEFA, mmol/l	Glucose, mmol/l	CRP, mg/l	SBP, mmHg	DBP, mmHg	PP, mmHg
Anthropometric measures													
Weight, kg	0.30	0.17	0.12	0.25	0.29	0.20	0.25	-0.25	-0.10	-0.27	0.02	-0.17	0.13
BMI, kg/m^2	0.30	0.17	0.08	0.26	0.28	0.16	0.19	-0.29	-0.08	-0.30	0.04	-0.22	0.18
WC, cm	0.17	0.04	0.29	0.12	0.16	-0.002	-0.03	-0.18	0.010	-0.22	0.32	0.18	0.10
WHR	0.25	0.08	0.26	0.19	0.24	0.10	0.08	-0.17	0.04	-0.24	0.33	0.05	0.17
WHtR	0.003	0.03	0.12	-0.01	0.03	0.04	-0.01	-0.19	-0.07	-0.28	0.31	0.07	0.11
Body composition													
Body fat, %	0.30	-0.16	0.30	0.25	0.24	-0.06	0.02	-0.01	-0.05	0.02	0.07	0.25	-0.24
Fat mass, kg	0.35*	-0.05	0.23	0.32*	0.31*	0.07	0.17	-0.22	-0.11	-0.05	0.11	0.15	-0.14

Lean mass, kg	0.07	0.17	-0.18	0.12	0.13	0.32*	0.29	-0.32*	-0.09	-0.08	0.07	-0.19	0.22
Abdominal VAT, kg	0.26	0.12	0.10	0.25	0.29	0.17	0.20	-0.15	-0.09	0.03	-0.19	-0.02	-0.21
Android fat, kg	0.22	0.14	0.04	0.18	0.23	0.20	0.21	-0.21	-0.12	0.14	-0.15	-0.04	-0.20
Android lean, kg	-0.19	-0.001	-0.43**	-0.10	-0.12	0.09	0.09	-0.11	0.26	0.09	0.12	-0.04	0.17
Android fat, %	0.30	0.08	0.24	0.25	0.27	0.13	0.15	-0.12	-0.21	0.10	-0.14	0.04	-0.23
Gynoid fat, %	-0.15	-0.14	-0.02	-0.13	-0.17	-0.25	-0.17	0.20	0.05	-0.26	-0.02	0.08	-0.10
A/G fat ratio	0.38*	0.12	0.31	0.32	0.36*	0.21	0.23	-0.20	-0.20	0.16	-0.05	0.02	-0.12

[a]Data was analysed by Spearman's correlation

[b]**Significant at 0.01 level

[c]*Significant at 0.05 level

[d]Abbreviations: A/G fat ratio: android to gynoid fat ratio; BMI: body mass index; CRP: C-reactive protein; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; NEFA: non-esterified fatty acids; PP: pulse pressure; SBP: systolic blood pressure; TAG: triacylglycerol; TC: total cholesterol; VAT: visceral adipose tissue; WC: waist circumference; WHR: waist to hip ratio; WHtR; waist to height ratio.

[e]Sample size all n=41, for WHR, WHtR and VAI n=39

Supplemental Table 3.2 Correlations between changes in dietary fatty acids and changes in the anthropometric and body composition measures

	Total fat %TE	SFA% TE	MUFA %TE	PUFA %TE	n-3PUFA %TE	n-6 PUFA %TE	Trans-fat %TE
Anthropometric measures							
Weight, kg	-0.07	0.14	-0.28	-0.28	0.02	-0.29	-0.05
BMI, kg/m^2	-0.08	0.13	-0.23	-0.31	0.07	-0.33*	-0.03
WC, cm	0.01	0.14	0.16	-0.20	0.08	-0.25	0.13
WHR	-0.03	0.08	0.12	-0.22	-0.05	-0.27	0.19
WHtR	-0.10	0.10	0.05	-0.24	0.11	-0.29	0.04
Body composition							
Body fat, %	-0.20	-0.18	-0.02	0.002	-0.06	-0.04	-0.05
Fat mass, kg	-0.26	-0.17	-0.12	-0.08	-0.04	-0.13	-0.06
Lean mass, kg	0.20	0.28	0.01	-0.17	0.11	-0.14	-0.02
Abdominal VAT, kg	-0.02	0.02	0.07	-0.01	-0.10	-0.03	0.08
VAI	0.00	0.12	-0.09	-0.28	-0.06	-0.18	0.33*
Android fat, kg	0.01	0.04	0.09	-0.05	-0.22	-0.09	0.22
Android lean, kg	0.06	-0.12	-0.07	0.03	0.08	0.12	-0.11
Android fat, %	-0.04	0.06	0.06	-0.05	-0.26	-0.11	0.16
Gynoid fat, %	-0.43**	-0.35*	-0.31*	0.03	-0.14	0.02	-0.08
A/G fat ratio	0.24	0.24	0.24	0.004	0.02	-0.04	0.07

[a]Data was analysed by Spearman's correlation. [b]**Significant at 0.01 level. [c]*Significant at 0.05 level. [d]Abbreviations: A/G fat ratio: android to gynoid fat ratio; BMI: body mass index; CRP: C-reactive protein; DBP: diastolic blood pressure; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids; TE: total energy; VAT: visceral adipose tissue; VAI: visceral adiposity index; WC: waist circumference; WHR: waist to hip ratio; WHtR; waist to height ratio. [e]Sample size n=41, for WC, WHR, WHtR n=39.

Supplemental Table 3.3 Changes in plasma phospholipid fatty acids and the changes in anthropometric and body composition measures

	Plasma total SFA	Plasma total MUFA	Plasma total PUFA	C14:0 (MC)	C16:0 (PA)	C18:0 (SA)	C18:2 n-6 (LA)	C20:4 n-6 (AA)	C20:5 n-3 (EPA)	C22:6 n-3 (DHA)	C16:1 n-7 (POA)	C18:1 n-7 (VA)	C18:1 n-9 (OA)
Anthropometric measures													
Weight, kg	-0.07	-0.06	0.11	0.11	-0.16	0.15	0.08	-0.02	0.09	0.09	0.08	0.07	-0.06
BMI, kg/m^2	-0.07	-0.04	0.08	0.11	-0.10	0.07	0.10	-0.04	0.07	0.14	0.05	0.02	-0.03
WC, cm	-0.06	-0.14	0.16	0.19	-0.07	0.04	0.05	0.09	0.23	-0.04	-0.06	-0.05	-0.10
WHR	0.15	0.01	-0.07	0.37*	0.18	-0.07	-0.06	0.02	0.27	-0.02	-0.04	-0.16	0.06
WHtR	-0.03	-0.15	0.14	0.15	-0.10	0.07	0.04	0.12	0.19	0.03	-0.07	-0.04	-0.10
Body composition													
Body fat, %	0.31*	-0.17	-0.05	0.02	0.24	-0.07	-0.07	-0.11	0.10	0.08	-0.01	-0.06	-0.16
Fat mass, kg	0.24	-0.21	0.03	0.03	0.10	0.07	0.01	-0.11	0.11	-0.09	-0.06	-0.03	-0.18
Lean mass, kg	-0.22	-0.06	0.17	0.12	-0.33*	0.28	0.22	0.02	-0.03	-0.08	-0.09	0.09	-0.05
Abdominal VAT, kg	0.09	-0.38*	0.24	-0.07	0.001	0.09	0.07	-0.07	0.16	-0.03	-0.01	-0.12	-0.33*
VAI	-0.003	0.07	-0.08	-0.04	-0.18	0.23	-0.12	-0.28	0.19	-0.05	0.32*	-0.28	0.09
Android fat, kg	0.18	-0.23	0.10	0.003	0.03	0.06	-0.02	-0.15	0.22	0.04	-0.06	-0.01	-0.22

Android lean, kg	-0.31*	-0.11	0.33*	-0.02	-0.38*	0.41**	0.38*	0.02	-0.26	-0.10	-0.36*	0.12	-0.17
Android fat, %	0.25	-0.16	-0.03	0.06	0.14	-0.04	-0.08	-0.19	0.22	0.06	0.07	-0.11	-0.12
Gynoid fat, %	0.27	0.20	-0.31	0.04	0.36*	-0.38*	-0.05	-0.11	-0.27	0.08	0.04	0.02	0.20
A/G fat ratio	0.12	-0.35*	0.23	0.03	-0.09	0.29	-0.05	-0.05	0.41**	0.01	-0.002	-0.14	-0.30

[a]Data was analysed by Spearman's correlation

[b]**Significant at 0.01 level

[c]*Significant at 0.05 level

[d]Abbreviations: AA: arachidonic acid; A/G fat ratio: android to gynoid fat ratio; BMI: body mass index; DHA: docosahexaenoic acid; EPA: Eicosapentaenoic acid; LA: linoleic acid; MC: myristic acid; MUFA: monounsaturated fatty acids; OA: oleic acid; PA: palmitic acid; PUFA: polyunsaturated fatty acids; POA: palmitoleic acid; SA: stearic acid; SFA: saturated fatty acids; VA: vaccenic acid; VAT: visceral adipose tissue; VAI: visceral adiposity index; WC: waist circumference; WHR: waist to hip ratio; WHtR; waist to height ratio.

[e]Sample size all n=41, for WC, WHR, WHtR, VAI n=39

Supplemental Table 3.4 Plasma phospholipid fatty acid profile during the high SFA and low SFA diets

Fatty acid profiles	**High SFA diet**	**Low SFA diet**	**p-value**
Total SFA (%)	46.4±0.9	45.3±1.2	**P<0.01**
C14:0 (myristic acid)	0.6±0.1	0.5±0.1	**P<0.01**
C15:0 (pentadecylic acid)	0.3±0.1	0.2±0.1	**P<0.01**
C16:0 (palmitic acid)	30.6±1.1	29.1±1.2	**P<0.01**
C17:0 (margaric acid)	0.4±0.1	0.4±0.1	**0.01**
C18:0 (stearic acid)	14.3±1.0	14.9±1.0	**P<0.01**
Total UFA (%)	53.6±0.9	54.7±1.2	**P<0.01**
Total PUFA (%)	40.8±1.8	42.4±1.8	**0.004**
C18:2 n-6 (linoleic acid)	21.0±2.6	23.2±2.8	**P<0.01**
C18:3 n-3 (α-linolenic acid)	0.2±0.1	0.2±0.1	0.99
C20:4 n-6 (Arachidonic acid AA)	9.5±1.4	10.0±1.9	**0.003**
C20:5 n-3 (Eicosapentaenoic acid-EPA)	1.2±0.5	0.9±0.3	**P<0.01**
C22:6 n-3 (Docosahexaenoic acid DHA)	3.0±0.9	2.9±0.8	0.06
Total MUFA (%)	12.9±1.5	12.3±1.3	**P<0.01**
C16 :1 n-7 (Palmitoleic acid)	0.6±0.3	0.4±0.2	**P<0.01**
C18 :1 n-7 (cis-vaccenic acid)	1.4±0.2	1.5±0.3	**P<0.01**
C18 :1 n-9 (oleic acid)	10.4±1.3	9.9±1.1	**0.01**

[a]Data was analysed by paired t test, present as mean ± SD. P<0.05 is considered as significant.

[b]Sample size n=41

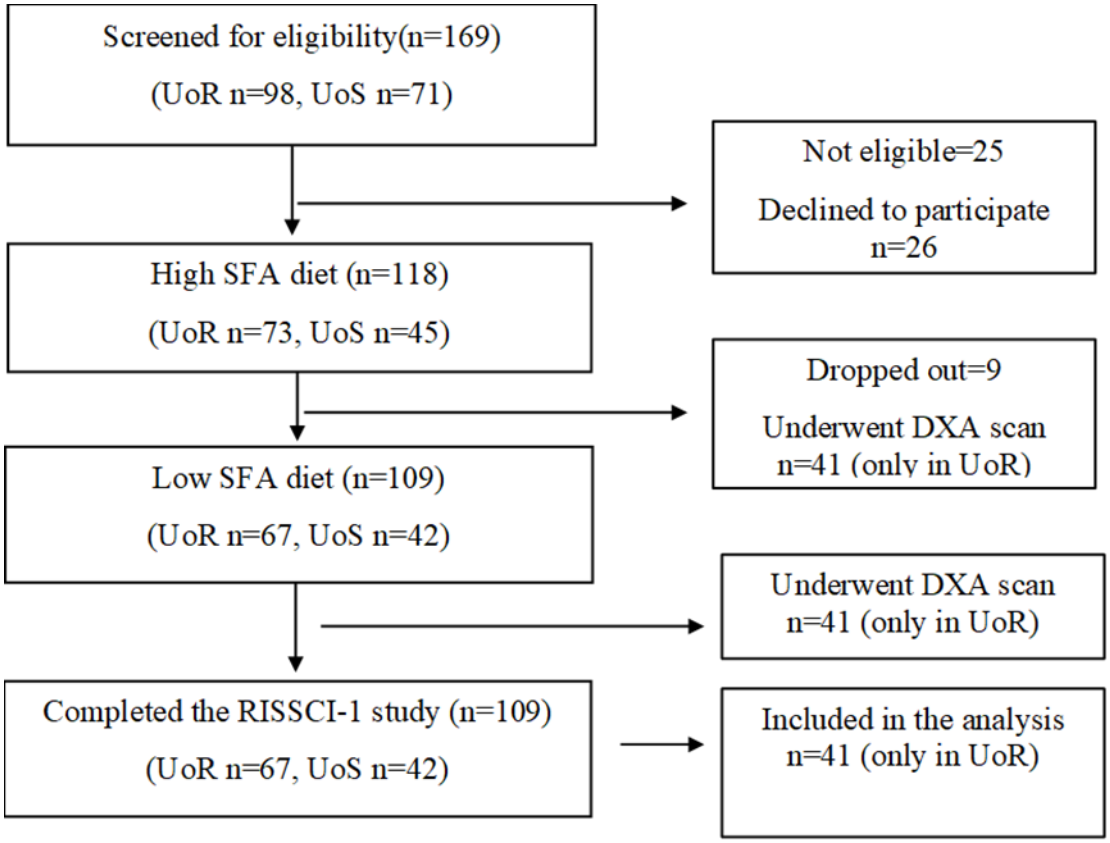

Figure 3.1 Flow chart of participants from the RISSCI study

Chapter 4: Impact of replacing dietary saturated fatty acids with unsaturated fatty acids on genes regulating cholesterol metabolism in peripheral blood mononuclear cells-Findings from the RISSCI-1 study

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Contribution towards the manuscript

BF, DR, BG, JAL and KGJ designed the study. EO, AK, GW, LS conducted the clinical visits. EO performed PBMC isolation, RNA extraction, cDNA synthesis and gene expression assays under the supervision of KGJ. EO analysed the data and drafted the manuscript under the supervision of KGJ and JAL. All authors contributed to the final manuscript and approved final version.

Impact of replacing dietary saturated fatty acids with unsaturated fatty acids on genes regulating cholesterol metabolism in peripheral blood mononuclear cells-Findings from the RISSCI-1 study

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Abbreviations: ABCG1 ATP binding cassette subfamily G member 1, CVD cardiovascular disease, HDL-C high density lipoprotein cholesterol, LDL-R LDL receptor, NEFA non-esterified fatty acids, NR1H3 nuclear receptor subfamily 1 group H member 3, PBMC peripheral blood mononuclear cells**,** SFA saturated fatty acids, SREBF1 sterol regulatory element binding transcription factor 1, TAG triacylglycerol, TC total cholesterol, UFA unsaturated fatty acids,

Keywords: LDL receptor, LDL cholesterol**,** saturated fat

4.1 Abstract

Scope: There is considerable interest in factors which explain the variability in the LDL-cholesterol response to dietary fat intake. Animal studies suggest that dietary fat composition can modulate hepatic LDL-receptor gene expression but studies in humans are limited.

Methods and Results: Healthy men (n=58) aged 30-65y followed an isoenergetic high saturated fat (SFA, 18% total energy (TE)) and then low SFA (10%TE) diet for 4 weeks each. Lipid risk markers and gene expression in peripheral blood mononuclear cells (PBMC) were analysed at weeks 0, 4 and 8. Reductions in total, LDL-cholesterol and large and intermediate LDL particle number and composition in response to reducing dietary SFA were evident along with an upregulation in the PBMC LDL-receptor, NR1H3, and ABCG1 mRNA expression ($p \leq 0.04$). To determine mechanisms underlying the inter-individual variability in LDL-C response, subjects were then classified as responders and non-responders to dietary SFA change. Although reductions in lipids were predominately observed in the responder group, only the non-responder group showed an upregulation in NR1H3 and ABCG1 mRNA gene expression in response to the low SFA diet ($p \leq 0.01$).

Conclusion: Our results are in agreement with animal and in vitro studies suggesting a role of dietary fat composition on intracellular cholesterol regulation, and potentially explains the effect of dietary SFA on blood cholesterol levels

Clinical trial registry: NCT03270527; registration date: 2017-09-01

4.2 Introduction

Dietary fat intake is an important modulator of LDL-cholesterol (LDL-C), an independent risk factor for cardiovascular disease (CVD) (59, 226, 258). Current public health recommendations are to reduce saturated fatty acid (SFA) intake to less than 10% of total energy (TE) with guidance to replace with polyunsaturated (PUFAs) and monounsaturated fatty acids (MUFAs) for chronic disease prevention (32). However, significant variation in LDL-C concentration (range from -40% to +20%) has been observed in response to change in dietary SFA intake in randomised controlled trials (35, 64). Determinants of the variation in cholesterol response to diet are not clear, although in response to dietary fat intake both responder and non-responder groups have been identified. As an example, Kirwan et al. (259) identified age as a potential determinant of the responsiveness to personalised nutrition advice based on change in total cholesterol (TC) concentration. Thus, inter-individual variation in LDL-C response to dietary fat manipulation may be dependent on both non-modifiable (e.g., age) and modifiable (such as habitual dietary intake) CVD risk factors.

The differential effects of dietary fat composition on circulating LDL-C concentrations reported in animal and in vitro studies have been linked to their role in the hepatic cholesterol regulation (260). Peripheral blood mononuclear cells (PBMC, a subset of immune cells) have been shown to reflect the gene expression profiles of liver cells (77, 261) and since they circulate within the bloodstream, are exposed to the environmental factors such as dietary changes (67, 262). Therefore PBMCs are being increasingly used to determine the underlying mechanisms of nutrient intakes on intracellular cholesterol regulation (76). Although studies in humans are limited, an upregulation of PBMC LDL-R mRNA expression after replacing 6.5%TE SFA with PUFAs for 8 weeks were observed in subjects with moderate hypercholesterolemia (79). Furthermore, the variability in LDL-C in response to a low carbohydrate, high fat diet was attributed to differences in PBMC LDL-R mRNA gene

expression between the dietary responder and non-responders in normal weight, young adults (263). However, little is known about the variability in response to dietary fat recommendations for CVD prevention and the relationship with established CVD risk markers. Therefore, the aim of this paper was firstly to investigate the effect of replacing dietary SFA with PUFA and MUFA on the expression of the LDL-R gene and other selected genes associated with cholesterol metabolism in circulating PBMCs. A secondary explorative analysis was performed to identify determinants of the LDL-C response to the level of dietary SFA intake by stratifying the group according to responders and non-responders to change in dietary SFA intake in relation to the LDL-C levels. We hypothesized that replacing dietary SFA with unsaturated fatty acids (PUFA and MUFA) would upregulate the LDL-R mRNA expression in PBMC, particularly in the responder group and that changes in gene expression are related to changes in serum LDL-C concentration.

4.3 Methods

Subjects

A subset of healthy men aged 30-65 years from the Reading Imperial Surrey Saturated fat Cholesterol Intervention (RISSCI-1) study were included in this analysis. Details of the subject group and study design have been described previously (Chapter 3). Briefly, subjects were included if they were healthy, a non-smoker, had a BMI between 19-32 kg/m^2 and fasting triacylglycerol (TAG) concentrations <2.3 mmol/l, total cholesterol (TC) <7.5 mmol/l and glucose <6.5 mmol/l. Participants were excluded in cases of medical history of diabetes, heart disease, kidney, bowel or liver disease, cancer or hormone abnormalities, taking medication for high blood pressure, high blood fats, inflammatory conditions and depression, antibiotic use in the last 3 months, drinking>14 units of alcohol per week, being on a weight reducing diet,

unwillingness to consume study products, travel frequently or participating in another intervention study.

Study design

RISSCI-1 was a single blind sequential dietary intervention study conducted in the Hugh Sinclair Unit of Human Nutrition at the University of Reading. Details of the diets have been described previously (Appendix 1). Briefly, participants followed a high SFA (18%TE), low unsaturated fatty acids (UFA, 15%TE) diet followed by a low SFA (10%TE), high UFA (24%TE) diet for 4 weeks each without a wash-out period between the diets. The diets were high fat (35%TE), iso-energetic and only differed in fatty acid profile. To achieve the fatty acid targets, a food exchange model was developed based on the previous studies (224) and participants were asked to replace their habitual dietary sources with high SFA or UFA oils, spreads and snacks. Participants attended 3 study visits, at baseline (week 0) and at the end of each intervention diet (week 4 and 8). The day before each study visit, participants were asked to refrain from alcohol and strenuous exercise and consume a low-fat evening meal provided by the researchers. Study visits took place after an overnight fast. On each study visit anthropometric and blood pressure measurements were performed and fasting blood samples were collected to determine blood lipid profiles, proprotein convertase subtilisin/kexin type 9 (PCSK9) levels, LDL subclasses and to isolate PBMCs. Compliance to diets was assessed by 4-day weighed food diaries and self-reported tick sheets. Participants mean daily nutrient and energy intakes were calculated using Nutritics software (Research edition, V5.09).

This study was conducted according to the Declaration of Helsinki and the University of Reading Research Ethics Committees (11/05/17 UREC reference number: 17/29) gave a favourable opinion for conduct. This study was registered at www.clinicaltrials.gov (NCT03270527).

Anthropometric measurements

Body weight, height, waist circumference and hip circumference were measured at baseline and BMI, waist to hip ratio and waist to height ratio were calculated.

Biochemistry

A fasting blood sample was collected into serum separator tube at baseline and each study visit and centrifuged at 1700 x g (3000 rpm) for 15 mins room temperature before storing at -20^{o}C. Fasting blood lipids (TC, high-density lipoprotein cholesterol (HDL-C), non-esterified fatty acids (NEFA) and TAG) were measured in serum samples using the ILAB 600 clinical chemistry analyser (Werfen UK Ltd., Warrington UK) and Daytona Plus (Randox Laboratories Limited, Crumlin, UK). LDL-C was estimated using the Friedewald equation (182). Non-HDL was calculated using the following formula: TC- HDL-C. Plasma PCSK9 concentrations were measured by ELISA (R&D Systems Europe Limited).

ApoB, apoA-I and LDL subfraction analysis

Plasma apoB, apoA-1, LDL subclass particle size, number and lipid composition were determined by ^{1}H-NMR high throughput metabolomics at the National Phenome Centre, Imperial College London and according to published protocols (264). Blood samples were collected in lithium heparin blood tubes and centrifuged at 1700 x g for 15 mins before the plasma was collected and stored at -80°C. Prior to analysis samples were centrifuged at 12000 x g at 4°C for 5 min. Plasma supernatants (350 µL) were combined with 350 µL of disodium phosphate buffer containing trisodium phosphate (TSP), vortexed and then transferred to 5 mm NMR tubes. Quality control samples were also measured which were prepared by pooling equal parts of each study sample. Experiments were performed on a Bruker Advance III HD 600 MHz spectrometer operating at 14.1 T. Three separate experiments were performed on each plasma sample; standard 1D spectra using 1D-NOESY presat pulse sequence, relaxation edited spin-

echo using the 1D-Carr-Purcell-Meiboom-Gill (CPMG) presat pulse sequence and pseudo-2D spectra using a *J*-resolved sequence. The Bruker IVDr B.I. LISA method (Bruker BioSpin 08/2019 T165319) was used to quantify lipoprotein main class, their particle size and density (LDL-1 to LDL-6). LDL-1 (1.019-1.031 kg/L) reflects large LDL particles while LDL-6 (1.044-1.063 kg/L) small dense particles. The intermediate LDL was grouped as sum of LDL-2 (1.031-1.034 kg/L), LDL-3 (1.034-1.037 kg/L) and LDL-4 (1.037-1.044 kg/L) (265).

PBMC isolation, cDNA synthesis and gene expression

A fasting blood sample was collected into a BD Vacutainer cell preparation tube (BD Biosciences, UK) at baseline and each study visit. Following collection, the blood was centrifuged at 3000 rpm (1700 x g) for 20 min at room temperature to separate the red and white blood cell layers. The plasma containing the white blood cells was transferred to a 15 ml Sterilin tube and the volume made up to 15 ml by adding Dulbecco's PBS without calcium and magnesium (Merck Life Science UK Ltd, Dorset, UK). After washing the cells by inverting 5 times, the tube was centrifuged at 1200 rpm (280 x g) for 20 min at room temperature. The supernatant was aspirated, and the tube was vortexed gently before adding 10 ml of PBS and centrifuging for a further 15 min at 1200 rpm (280 x g). The remaining supernatant was aspirated and RLT buffer (Qiagen, UK) containing 1% mercaptoethanol was added to lyse the cell pellet prior to storage at -80°C until the RNA extraction was performed. Total RNA was isolated using a RNeasy mini kit (Qiagen) according to manufacturer's instructions after the cell lysate had been passed through a shredder column and then stored at -80°C. RNA quality and quantity were assessed with a Nanodrop 1000 spectrophotometer (Nanodrop ND-1000 Thermo Fisher Scientific). The OD at 260 and 280 nm was used as an indicator for RNA purity with a 260:280 ratio of 2.0 considered as pure RNA. cDNA samples were then synthesised from 1.2 µg total RNA using SuperScript IV VILO Mastermix (Thermo Fisher Scientific, Winsford, UK)) and incubated at 25°C for 10 mins (reaction volume=20 µl) followed by 50°C for 10 mins

and 85°C for 5 mins. Samples were diluted 1:10 with UltraPure RNAse/DNAse free distilled water (Invitrogen) and stored at -20°C until further analysis.

Gene expression from cDNA samples (5ng/µl) were performed to determine the gene expression of reference and target genes using real time RT-PCR (QuantStudio 3, Thermo Fisher Scientific) with TaqMan gene expression assays (Applied Biosystems) using the normal cycling parameters. Expression of each target gene (LDL-R, sterol regulatory element binding transcription factor 1 (SREBF1), nuclear receptor subfamily 1 group H member 3 (NR1H3) and ATP binding cassette subfamily G member 1 (ABCG1)) was normalised to the reference genes cytochrome c1 (CYC1) and ATP synthase subunit β (ATP5B). These genes were identified as the best reference genes out of a possible 9 candidates (Succinate dehydrogenase complex flavoprotein subunit A (SDHA), ubiquitin C (UBC), Actin Beta (ACTB), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), DNA Topoisomerase I (TOP1), 18S ribosomal RNA (18S), Ribosomal protein L13a (RPL13A), ATP5B and CYC1) as they had the least variation between samples using human geNorm reference gene selection kit with double-dye hydrolysis probe and qbase+ software according to manufacturer's protocol (Primerdesign Ltd, Camberley, UK). The fold change in mRNA expression relative to the baseline visit for each diet was calculated by ΔΔCt method expressed as $2^{-\Delta\Delta Ct}$ (266). Briefly, C_t values of each target gene was normalised to the C_t value of the average of the two reference genes ($\Delta Ct = C_{t\ target} - C_{t\ reference}$) and the relative change calculated to the baseline visit for each diet ($\Delta\Delta Ct = \Delta Ct_{\ end\ of\ diet} - \Delta Ct_{\ baseline\ visit\ for\ the\ diet}$).

Statistics

Statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS Incl., IL, USA). Normality was assessed by the Kolmogorov-Smirnov test and Q-Q plots. The fold change in mRNA expression for the selected genes after the high and low SFA diets were compared by a general linear model after adjusting for age, baseline BMI and baseline LDL-C

concentration. Changes in fasting blood lipids, PCSK9, apoB/apoA1 ratio and LDL subclass particle size and composition relative to the baseline for each diet (High SFA diet-Baseline (V2-V1)) and (Low SFA diet-High SFA diet (V3-V2)) were compared by a general linear model after adjusting for age, BMI and concentration of the outcome of interest from the pre-intervention study visit. Spearman's correlations were used to analyse relationships between the fold change in gene mRNA expression after the low SFA diet ($2^{-\Delta\Delta Ct}$) and changes in circulating CVD risk markers (V3-V2).

To assess the determinants of inter-individual response in LDL-C to the level of dietary SFA intake, subjects were identified as responders and non-responders. To classify subjects, the actual change in LDL-C concentrations (low SFA to high SFA diets (V3-V2)) were calculated and subjects were ranked to allow the responders (top 20%) and non-responders (bottom 20%) to be identified. The fold change in mRNA expression for the selected genes after the high and low SFA diets were compared by a general linear model after adjusting for age, baseline BMI and baseline LDL-C concentration. Changes in fasting blood lipids, PCSK9, LDL subclass particle size and apoB/apoA1 ratio (V2-V1 and V3-V2) in responder and non-responder groups were compared by a general linear model with adjustment for age, baseline BMI and baseline concentration of the outcome of interest. Spearman's correlations were used to analyse relationships between the fold change in gene mRNA expression after the low SFA diet ($2^{-\Delta\Delta Ct}$) and changes in CVD risk markers prior to stepwise multiple linear regression analysis to establish the independent associations between change in LDL-C with anthropometric measures, CVD risk markers and dietary macronutrients in responder and non-responder groups separately. Results were presented as estimated marginal means ± SE and $p \leq 0.05$ was considered significant.

4.4 Results

Participant characteristics from the pre-intervention study visit and changes in anthropometric measures and fasting blood lipids during the high and low SFA diets are shown in **Table 4.1**. The subjects included in this analysis of the RISSCI study (n=58/109) had a mean age of 47±2 y and BMI of 25.2 ± 0.5 kg/m^2. Participant self-reported mean macronutrient and energy intakes are shown in Supplementary Table 1. Energy intake was 0.6 MJ greater during the high SFA diet compared to the low SFA diet (p=0.02). %TE from MUFA, PUFA, n-3 PUFA and n-6 PUFA were significantly higher during the low SFA diet while SFA%TE and trans-fat %TE were lower compared to the high SFA diet (p<0.01 for each). Other macronutrients were not different between the diets (**Supplementary Table 4.1**).

Fasting TC, LDL-C, TAG, non-HDL-C and NEFA concentrations and TC: HDL-C, LDL-C: HDL-C and apoB/apoA1 ratios were 4%-15% lower after the low SFA diet compared to high SFA diet (p≤0.05). There was no significant difference in PCSK9 concentrations after the high and low SFA diets. NMR analysis revealed a significant reduction in the number (apoB) and lipid composition (cholesterol ester, free cholesterol and phospholipids) of the particles within the large and intermediate LDL subclasses after the low compared with the high SFA diets (p<0.01). The TAG concentration was also lower within the intermediate LDL subclass after the low SFA diet (p<0.01). There were no significant differences in the number or lipid composition of the particles within the small dense LDL subclass (LDL-6) after the high and low SFA diets.

The fold change in the PBMC mRNA gene expression in response to the high and low SFA diets are shown in **Figure 4.1**. Relative to the high SFA diet, there was a significant upregulation in the LDL-R (p=0.04), NR1H3 (p=0.01) and ABCG1 (p=0.001) mRNA gene expression after the low SFA diet (p=0.04). Moreover, there was a non-significant trend for an

increase in the relative mRNA expression of SREBF1 gene after the low SFA diet (p=0.08). Positive correlations were identified between the fold change in LDL-R with fold changes in SREBF1 and ABCG1 mRNA expressions after the low SFA diet (r=0.65, p=0.01 and r=0.33, p=0.05). In addition, the change in NR1H3 mRNA expression was correlated with changes in SREBF1 (r=0.29, p=0.05) and ABCG1 (r=0.31, p=0.05) mRNA expressions (**supplemental Table 4.2**). However, relative to the high SFA diet, there were no significant correlations between the fold change in LDL-R, SREBF1, ABCG1 and NR1H3 mRNA expressions and changes in circulating CVD risk factors after the low SFA diet (**supplemental Table 4.3**).

To examine the determinants of the LDL-C response to dietary fat intervention, the RISSCI study cohort was stratified according to the change in LDL-C concentrations to identify the responder (n=12) and non-responder (n=13) groups. The subject characteristics within these sub-groups are shown in **Table 4.2**. Age, anthropometric and body composition measures did not differ significantly between the two groups prior to the start of the dietary intervention (pre-intervention visit). However, the fasting LDL-C concentration was found to be 19% lower in non-responder compared to responder group (p=0.05), while there were no significant differences in any other CVD risk markers between these subgroups. Dietary intakes were similar in the two groups prior to the start of the dietary intervention apart from the total energy intake in which the non-responder group reported a 19% lower intake (p=0.02).

The fold change in the mRNA expression of genes, changes in blood lipids and LDL subclasses relative to the baseline for each diet in responder and non-responder groups are shown in **Table 4.3**. In the responder group, changes in TC, LDL-C, HDL-C, non-HDL-C concentrations, and TC: HDL-C, LDL-C: HDL-C and apoB/apoA1 ratios were significantly different after the low SFA diet compared to the high SFA diet (p<0.01). In contrast, only the change in HDL-C concentration was significantly different between diets in the non-responder

group, with a non-significant trend for an increase in the LDL-C: HDL-C ratio after the low SFA diet ($p=0.08$).

The number (apoB) and concentrations of cholesterol ester and phospholipids within the large and intermediate LDL subclasses were lower after the low SFA diet than high SFA diet in the responder group ($p\leq0.04$). The TAG and free cholesterol concentration of intermediate LDL was also lower after the low SFA diet in responder group ($p<0.01$). In the non-responder group, the number (apoB) within the large and small LDL subclasses were lower after the low SFA compared to high SFA diet ($p\leq0.04$). Changes in concentrations of cholesterol ester, free cholesterol phospholipids and TAG in the small dense LDL subclass were also lower after the low SFA diet compared to high SFA diet ($p=0.01$). In the intermediate LDL subclass, only the TAG concentration was lower after the low SFA diet compared with after the high SFA diet ($p\leq0.01$). While there were no significant differences within the responder group, the PBMC mRNA expression of the NR1H3 and ABCG1 genes were upregulated after the low SFA diet in non-responders ($p\leq0.01$).

To investigate whether changes in gene expressions were associated with changes in serum lipids after the diets in responder and non-responder groups, bivariate Spearman's correlations were performed. There were no significant correlations between LDL-R mRNA expression and CVD risk markers in responder and non-responder groups. However, there was a strong negative correlation between change in PCSK9 with change in NR1H3 mRNA expression in the non-responder group ($r=-0.72$, $p=0.01$). In the responder group there were positive moderate correlations between the change in NR1H3 mRNA gene expression and changes in concentrations of cholesterol ester, free cholesterol, phospholipids in large and intermediate LDL subclasses ($r=0.63$-0.68, $p=0.05$). Moreover, in this group the change in TAG concentration within particles in the large LDL subclass was inversely associated with the change in NR1H3 mRNA expression ($r=-0.61$, $p=0.05$). In the non-responder group, the particle

number (apoB-100) and the concentrations of cholesterol ester and phospholipids in large and intermediate LDL subclasses were negatively associated with changes in LDL-R and SREBF1 mRNA gene expressions (r=-0.74 to -0.60, p≤0.05) (**Supplemental Table 4.4**).

Multivariate regression analysis

The standardized regression coefficients, adjusted r^2 and p-value for the stepwise multivariate regression analysis are shown in **Table 4.4.** Only changes in pulse pressure, apoB/apoA1 ratio and protein%TE were found to be independently associated with change in LDL-C in the responder group, explaining 96.8% of the variability of LDL-C.

The fold change in LDL-R mRNA expression, change in WC, HC and large LDL cholesterol ester concentration were independently associated with change in LDL-C concentration in the non-responder group and these variables explained 100% of the variability in LDL-C. Of these variables, 98% of this variability was explained by fold change in LDL-R mRNA expression.

4.5 Discussion

It is well known that variability in the blood cholesterol response to dietary SFA intake exists but mechanisms underlying these differences between individuals are unclear. In this sequential dietary intervention study, we observed an increase in the expression of genes involved in hepatic cholesterol regulation after the low SFA diet, which was accompanied by reductions in fasting TC, LDL-C, and number and lipid composition of particles within the large and intermediate LDL subclasses. However, when subjects were categorised as responders and non-responders according to their LDL-C response, the upregulation in LDL-R mRNA expression after the low SFA diet was found to be an important determinant of the change in LDL-C concentration in the non-responder group only. Moreover, we observed novel associations between the change in NR1H3 mRNA expression and changes in lipid composition of particles within the large and intermediate LDL subclasses after the low SFA diet in the responder group.

In the current study replacing 10%TE SFA with PUFA/MUFA had a beneficial effect on blood lipids and was accompanied by an upregulation of the LDL-R, NR1H3 and ABCG1 mRNA gene expressions in PBMCs. As expected we observed a concordant upregulation of the mRNA expression of the LDL-R and NR1H3, a protein which is highly expressed in the liver and acts as a cholesterol sensor (267). Increased intracellular cholesterol concentrations have been shown to upregulate NR1H3 and its target genes SREBF1 and ABCG1 (268). In line with this, we observed the relative change in NR1H3 mRNA gene expression in response to replacing dietary SFA with PUFA/MUFA to be positively correlated with changes in SREBF1 and ABCG1. Moreover, in vitro studies have reported dietary n-3 and n-6 PUFA to reduce SREBF1 mRNA gene expression and protein abundance (269, 270). However, in our study there was a non-significant trend towards an upregulation of the mRNA expression of SREBF1 after the low SFA, high UFA (mixture of n-6 PUFA and MUFA) diet relative to the high SFA diet. ABCG1 plays an important role in hepatic cholesterol regulation by mediating reverse cholesterol transport transferring cellular phospholipids and free cholesterol to nascent HDL (271). In line with the literature there was an upregulation in ABCG1 mRNA expression along with the increase in NR1H3 mRNA expression after replacing dietary SFA with PUFA/MUFA (268). Thus, our findings are in line with a recent study which reported an increase in LDL-R, NR1H3 and ABCG1 gene expression after replacing 6.5%TE dietary SFA with n-6 PUFA for 8 weeks in healthy subjects with moderate hypercholesterolemia (79). However, the lack of a relationship between the relative change in gene expression and the change in LDL-C concentration could be due to the measurement of only mRNA gene expression in the current study which might not reflect actual protein levels.

Beneficial effects of replacing dietary SFA with n-6 PUFA/MUFA on LDL-C concentrations have been shown previously (35, 184), although findings are inconsistent (43). The discrepancies between studies were explained by inter-individual response of LDL-C

concentrations to dietary SFA in some dietary fat intervention studies (35, 64). In agreement with this, in the current study we also observed a variation in the LDL-C response of between -39% to +19% to dietary SFA intake. Therefore, to further explore the determinants of the intra-individual variability, we stratified the group into responder and non-responders. Although greater benefits of changing from a high to a low SFA diet were evident on blood lipids in the responder group, these did not translate to changes in the PBMC gene expression. Moreover, although there was an upregulation of NR1H3 mRNA gene expression in the non-responder group, no corresponding significant change in LDL-R mRNA gene expression was observed relative to the low SFA diet. It should be noted that baseline LDL-C concentration in the responder group was 19% higher than the non-responders. Interestingly, in the multivariate regression analysis, baseline LDL-C was not found to be an important determinant of change in LDL-C, with independent positive associations with changes in body fat distribution (WC and HC), large LDL-C lipid composition and relative change in LDL-R mRNA expression evident in non-responders. Further work is needed to understand the determinants of the variability in LDL-C to dietary SFA intake.

A novelty of this study was determination of LDL subclass analysis in relation to dietary SFA intake and mRNA gene expression. We observed that replacing dietary SFA with PUFA/MUFA reduced the particle number (apoB100 concentration) and composition (cholesterol, free cholesterol, phospholipids and TAG) of large and intermediate LDL subclasses. In agreement with these results, Ulven et al. (79) also reported a decrease in large and medium LDL particle concentrations when 6.5%TE SFA was replaced with n-6 PUFA for 8 weeks. However, in contrast to our findings a decrease in the number of small dense LDL particles was also evident in the subjects with moderate hypercholesterolaemia. Similarly, compared to a high SFA diet (20%TE), following a high MUFA diet (20%TE) for 8 weeks reduced phospholipid concentrations within large, intermediate and small LDL subclasses

whereas free cholesterol was only found to be lower in only large and intermediate LDL subclasses in subjects at risk of metabolic syndrome (272). Therefore, short term replacement of dietary SFA with PUFA/MUFA appears to have a greater impact on the number and composition of larger LDL particles compared to the more atherogenic, small dense LDL in healthy men. Although we did not observe a change in small LDL particles after the low compared to high SFA diet, as mentioned earlier studies have reported a decrease in small LDL particles in response to reducing dietary SFA intakes. This discrepancy could be due to the methodological differences such as the variety of techniques used to determine LDL subclasses. For example, in a study, small LDL concentration decreased after following a low (38%TE, 8%TE SFA) compared to high SFA diet (38%TE, 15%TESFA) for 3 weeks, however LDL subclasses were grouped as LDL-I to LDL-IV using density gradient ultracentrifugation (305). In addition, associations between the relative change in the NR1H3 mRNA gene expression and changes in particle number and composition of large and intermediate LDL subclasses were observed in responder group, suggesting a potential molecular locus for the effect of dietary SFA on LDL-C.

The strengths of this study include design, relatively large sample size and the use of dietary fat exchange model which allowed us to successfully manipulate participants dietary fat intake, although, the exploratory analysis of the determinants in the responders and non-responders, resulted in relatively small sample sizes. Limitations include difficulty of assessing dietary compliance and only men were recruited thus, this study cannot be generalised to the whole population. In addition, using a candidate gene expression approach, that did not consider all genes/transcription factors involved in cholesterol regulation or fatty acid metabolism which could have impacted on the LDL-C response, could be considered as a limitation.

In conclusion, replacing dietary SFAs with UFAs upregulated the expression of LDL-R, NR1H3 and ABCG1 genes in PBMCs, with co-ordinated reductions in TC and LDL-C concentrations. These findings are in line with previous studies suggesting the role of dietary fat composition on LDL-R and intracellular cholesterol regulation. However, the lack of a significant upregulation in the LDL-R mRNA expression in the responder group suggests that other physiological or lifestyle factors may impact on the inter-individual response to dietary SFA intake. Further work is needed to identify the important determinants of responsiveness of CVD risk markers to current dietary fat recommendations.

Author contributions:

BF, DR, BG, JAL and KGJ designed the study. EO, AK, GW, LS conducted the clinical visits. EO performed PBMC isolation, RNA extraction, cDNA synthesis and gene expression assays under the supervision of KGJ. EO analysed the data and drafted the manuscript under the supervision of KGJ and JAL. All authors contributed to the final manuscript and approved final version.

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Conflict of interest:

The authors have no conflict of interest. JAL sits on the UK Government's Scientific Advisory Committee for Nutrition (SACN) and was on SACN's Saturated Fats and Health working group. JAL chaired, and KGJ was an expert on the ILSI Committee on Saturated fatty acids and cardiovascular risk.

Table 4.1. Changes in anthropometric measures, circulating CVD risk markers, the number and lipid composition of particles within the LDL subclasses during the high and low SFA diets

	Pre-intervention (V1)	Change during the high SFA diet (V2-V1)	Change during the low SFA diet (V3-V2)	p-value
Age, y	47±2	-	-	-
Weight, kg	79.3±2.0	0.02±0.12	-0.08±0.12	0.57
BMI, kg/m^2	25.2±0.5	0.01±0.04	-0.03±0.04	0.47
WC, cm	92.5±1.3	-0.24±0.30	-0.25±0.30	0.97
HC, cm	103±1	-0.15±0.23	0.04±0.23	0.56
WHR	0.90±0.01	-0.00±0.00	-0.00±0.00	0.58
Blood pressure, mmHg				
SBP	117±2	1.0 ±1.0	1.0±1.0	0.72
DBP	78±1	2.0±1.0	-1.0±1.0	0.03
PP	39±1	-1.0±1.0	2.0±1.0	0.08
TC, mmol/l	4.96±0.11	0.33±0.06	-0.70±0.06	P<0.01
LDL-C, mmol/l	2.97±0.10	0.20±0.05	-0.47±0.05	P<0.01
HDL-C, mmol/l	1.48±0.05	0.09±0.02	-0.16±0.02	P<0.01
TAG, mmol/l	1.11±0.07	0.06±0.04	-0.12±0.04	P<0.01
NEFA, mmol/l	0.48±0.03	-0.05±0.03	0.05±0.03	0.01
TC: HDL-C ratio	3.48±0.10	0.06±0.04	-0.15±0.04	P<0.01
LDL-C: HDL-C	2.11±0.09	0.05±0.04	-0.14±0.04	P<0.01
PCSK9, ng/ml	189±7	4.10±3.79	2.36±3.79	0.75
ApoB/apoA1 ratio	0.54±0.01	0.01±0.01	-0.02±0.01	0.05
LDL subclasses				
Large (LDL-1), mg/dL				
ApoB-100	10.6±0.4	0.25±0.26	-1.08±0.26	<0.01
CE	21.1±0.8	0.48±0.53	-2.49±0.53	<0.01
FC	6.26±0.24	0.10±0.16	-0.66±0.16	<0.01
PL	12.1±0.4	0.26±0.27	-1.26±0.27	<0.01
TAG	3.68±0.18	0.12±0.16	-0.23±0.16	0.12
Intermediate (LDL-2 to 5), mg/dL				
ApoB-100	38.5±1.8	2.42±1.10	-5.82±0.15	<0.01
CE	66.7±3.2	4.26±2.04	-10.7±2.09	<0.01
FC	19.5±0.9	0.92±0.57	-2.35±0.58	<0.01
PL	36.8±1.6	2.13±0.98	-5.35±1.04	<0.01
TAG	6.02±0.32	0.60±0.23	-1.16±0.24	<0.01
Small (LDL-6), mg/dL				
ApoB-100	13.8±0.8	0.55±0.64	-0.81±0.64	0.14
CE	17.9±1.0	0.70±0.83	-1.13±0.83	0.12
FC	5.13±0.23	0.05±0.20	-0.17±0.20	0.44
PL	10.4±0.47	0.32±0.41	-0.59±0.41	0.12
TAG	3.13±0.16	0.14±0.14	-0.19±0.14	0.11

Data was analysed by ANCOVA, age, baseline BMI, baseline of the outcome of interest as covariates and presented as estimated marginal means ± SE, $p<0.05$ considered as significant. Sample size as follows WC, HC, WHR, n=55, BP n=43 PCSK9 n=56, apoB/apoA1 and LDL subclass analysis, n=54
Abbreviations: Apo: apolipoprotein, BMI: body mass index, CE: cholesterol, DBP: diastolic blood pressure, FC: free cholesterol, HDL-C: high-density lipoprotein cholesterol, HC: hip circumference, LDL-C: low-density lipoprotein cholesterol, NEFA: non-esterified fatty acids, PL: phospholipid, PP: pulse pressure, PCSK9: Proprotein convertase subtilisin/kexin type 9, SBP: systolic blood pressure, TAG: triacylglycerol TC: total cholesterol, WC: waist circumference, WHR: waist to hip ratio

Figure 4.1. Fold change in gene expression after the high SFA and low SFA diets relative to the baseline visit for each diet which was arbitrarily set at 1 Data was given as ΔΔCt and normalised for reference genes and baseline for each diet. Data was analysed by ANCOVA, age, baseline BMI and LDL-C concentration added as covariates. Values represent estimated marginal means ± SE. *p=0.03, **p=0.03 ***p=0.001, n=57 for SREBF1, NR1H3, ABCG1 n=58 for LDL-R

Abbreviations: ABCG1: ATP Binding Cassette Subfamily G Member; LDL-R: low-density lipoprotein receptor; NR1H3: Nuclear Receptor Subfamily 1 Group H Member 3; SFA: saturated fatty acid; SREBF1: Sterol Regulatory Element Binding Transcription Factor 1

Table 4.2. Baseline characteristics of the subjects assigned to the responder and non-responder groups

Variable	**Responders (n=12)**	**Non-responders (n=13)**	
	Mean ± SE	Mean ± SE	p-value
Weight, kg	73.3±3.7	83.0±3.7	0.08
Height, cm	1.75±0.03	1.79±0.03	0.36
BMI, kg/m^2	24.0±1.1	26.0±1.1	0.20
WC, cm	88.1±3.1	93.6±3.1	0.22
HC, cm	101±2	104±2	0.39
WHR	0.87±0.02	0.90±0.02	0.30
SBP, mmHg	119±3	117±4	0.56
DBP, mmHg	76±3	81±3	0.22
PP, mmHg	43±3	36±3	0.07
TC, mmol/l	5.25±0.26	4.68±0.25	0.12
LDL-C, mmol/l	3.23±0.21	2.62±0.20	0.05
HDL-C, mmol/l	1.56±0.12	1.46±0.11	0.55
TAG, mmol/l	1.01±0.17	1.30±0.16	0.21
PCSK9, ng/ml	198±13	188±13	0.59
Dietary intake			
Energy kcal	2470±139	2004±125	0.02
Fat %TE	38.5±3.3	37.3±3.05	0.79
SFA %TE	13.6±1.5	13.0±1.4	0.78
MUFA %TE	14.0±1.4	14.5±1.2	0.81
PUFA %TE	6.13±0.84	6.33±0.77	0.86
n-3 PUFA %TE	0.78±0.17	0.98±0.15	0.39
n-6 PUFA %TE	5.03±0.73	5.17±0.68	0.89
Trans-fat %TE	0.55±0.09	0.43±0.08	0.34
Protein %TE	15.9±0.9	16.2±0.8	0.83
Carbohydrate %TE	41.9±3.6	43.9±3.3	0.68

Data was analysed by ANOVA and presented as mean ± SE.Sample size: WC, HC, WHR, responders n=12, non-responders n=12. BP responders n=9 non-responders n=8, Diet responders n= 11, non-responders n=13 PCSK9 responder n=11, non-responders n=12.Abbreviations: BMI: body mass index, DBP: diastolic blood pressure, HC: hip circumference, HDL-C: high density lipoprotein

cholesterol, LDL-C: low density lipoprotein cholesterol, MUFA: monounsaturated fatty acids, SBP: systolic blood pressure, SFA: saturated fatty acids, TAG: triacylglycerol, TC: total cholesterol, PP: pulse pressure, PUFA: polyunsaturated fatty acids, WC: waist circumference, WHR: waist to hip circumference

Table 4.3. Changes in the relative LDL-R, SREBF1, NR1H3 and ABCG1 mRNA gene expression and blood lipids during the high and low SFA diets in the responder and non-responder groups

	RESPONDERS (n=12)			NON-RESPONDERS (n=13)		
	Change during the high SFA diet (V2-V1)	Change during the low SFA diet (V3-V2)	p-value	Change during the high SFA diet (V2-V1)	Change during the low SFA diet (V3-V2)	p-value
	Mean ± SE	Mean ± SE		Mean ± SE	Mean ± SE	
Fold change in target gene						
LDL-R	1.13±0.23	1.25±0.23	0.70	0.97±0.11	1.19±0.11	0.17
SREBF1	1.05±0.17	1.11±0.17	0.80	0.89±0.07	1.06±0.07	0.11
NR1H3	0.97±0.16	1.18±0.16	0.36	0.91±0.04	1.07±0.04	0.01
ABCG1	0.93±0.25	1.34±0.25	0.26	0.79±0.10	1.27±0.10	<0.01
CVD risk markers						
TC, mmol/l	0.69±0.09	-1.35±0.09	<0.01	0.04±0.11	-0.16±011	0.19
HDL-C, mmol/l	0.16±0.05	-0.27±0.05	<0.01	0.05±0.04	-0.11±0.04	0.01

TAG, mmol/l	-0.04±0.08	-0.09±0.08	0.67	0.07±0.13	-0.23±0.13	0.10
LDL-C, mmol/l	0.55±0.07	-1.04±0.07	<0.01	-0.04±0.09	0.05±0.09	0.47
Non-HDL, mmol/l	0.53±0.08	-1.08±0.08	<0.01	-0.01±0.10	-0.05±0.10	0.77
NEFA, mmol/l	-0.11±0.04	0.03±0.04	0.03	-0.05±0.06	0.07±0.06	0.21
TC: HDL-C	0.10±0.10	-0.32±0.10	0.01	-0.01±0.09	0.06±0.09	0.54
LDL-C: HDL-C	0.14±0.08	-0.35±0.08	0.001	-0.05±0.07	0.15±0.07	0.08
PCSK9, ng/ml	8.07±8.13	2.60±8.13	0.64	10.9±9.3	-7.97±9.32	0.17
apoB/apoA1 ratio	0.04±0.02	-0.08±0.02	<0.01	0.03±0.02	-0.00±0.02	0.28
Large (LDL-1), mg/dL						
ApoB-100	0.77±0.68	-1.59±0.68	0.03	1.04±0.61	-0.91±0.61	0.04
CE	1.34±1.53	-3.47±1.53	0.04	1.96±1.16	-1.32±0.16	0.06
FC	0.33±0.44	-0.87±0.44	0.07	0.59±0.38	-0.36±0.38	0.09
PL	0.79±0.75	-1.70±0.75	0.03	0.91±0.50	-0.84±0.60	0.06
TAG	0.41±0.32	-0.29±0.32	0.14	0.27±0.37	-0.77±0.37	0.07

Intermediate (LDL2-5), mg/dL						
ApoB-100	4.37±2.74	-9.81±2.74	<0.01	4.19±2.27	-0.30±2.27	0.18
CE	7.35±5.31	-16.8±5.3	0.01	9.79±4.31	0.33±4.31	0.14
FC	1.83±1.46	-3.34±1.46	0.02	2.69±1.21	0.32±1.21	0.18
PL	3.69±2.53	-8.29±2.53	<0.01	5.18±2.27	-0.18±2.27	0.11
TAG	1.31±0.38	-2.03±0.38	<0.01	1.52±0.54	-1.66±0.54	<0.01
Small (LDL-6), mg/dL						
ApoB-100	0.68±1.97	-2.81±1.97	0.23	2.62±1.22	-2.20±1.22	0.01
CE	0.61±2.64	-3.51±2.64	0.29	3.45±1.47	-2.50±1.47	0.01
FC	-0.03±0.64	-0.65±0.64	0.50	0.73±0.38	-0.36±0.38	0.06
PL	0.19±1.30	-1.70±1.30	0.32	1.68±0.75	-1.28±0.75	0.01
TAG	0.06±0.45	-0.37±0.45	0.51	0.46±0.29	-0.76±0.29	0.01

Data was analysed by a general linear model, age baseline BMI and baseline LDL-C as covariates for the relative gene expression data and age, baseline BMI and baseline of the outcome of interest for biochemistry data. Sample size PCSK9 responders n=11 non-responders n=12, LDL subclasses and apoB/apoA1 non-responders/ responders n=11. Abbreviations: Apo: apolipoprotein, ABCG1: ATP Binding Cassette Subfamily G Member, CE: cholesterol, FC: free cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, LDL-R: low-density lipoprotein receptor NEFA: non-esterified fatty acids, NR1H3: Nuclear Receptor Subfamily 1 Group H Member 3, PL: phospholipid, SFA: saturated fatty acids, SREBF1: Sterol Regulatory Element Binding Transcription Factor 1, TAG: triacylglycerol, TC: total cholesterol, PCSK9: proprotein convertase subtilisin/kexin type 9.

Table 4.4. Multivariate linear regression analysis exploring the relationship between dietary macronutrients, body composition, CVD risk markers and gene expression with change in LDL-C (V3-V2)

Dependent variable (responders, n=12)	**Independent variable**	**Standardized coefficient**	**Adjusted r^2**	**P-value**
LDL-C (V3-V2)	PP (V3-V2)	-0.791	0.573	0.01
	and ApoB/apoA1 ratio (V3-V2)	0.571	0.922	<0.01
	and Protein %TE (V3-V2)	0.333	0.960	<0.01
Dependent variable (non-responders, n=13)	**Independent variable**	**Standardized coefficient**	**Adjusted r^2**	**P-value**
LDL-C (V3-V2)	LDL-RV3	-0.992	0.978	0.001
	and WC (V3-V2)	-0.154	1.000	<0.01
	and HC (V3-V2)	-0.028	1.000	<0.01
	and LDL-1 CE (V3-V2)	0.002	-	-

Variables included in the analysis for change in LDL-C: age, change (V3-V2) in weight, BMI, WC, HC, SBP, DBP, PP, energy intake, fat %TE, SFA%TE, MUFA%TE, PUFA%TE, trans-fat %TE, protein %TE, CHO %TE, PCSK9, LDL subclass particle size. The fold change in LDL-R, SREBF1, ABCG1, NR1H3 after the low SFA diet and pre-intervention LDL-C.

Abbreviations: Apo: apolipoprotein, HC: hip circumference, LDL-C: low density lipoprotein cholesterol, LDL-R: LDL receptor, PP: pulse pressure, WC: waist circumference

Supplementary Table 4.1. Dietary intake on high SFA and low SFA diets in the whole group

	High SFA diet	**Low SFA diet**	**p-value**
Energy, kcal	2445±74	2303±71	0.02
Energy, MJ	10.3±0.3	9.7±0.3	0.02
Fat, %TE	38.3±0.9	38.4±1.0	0.95
SFA, %TE	19.0±0.5	9.0±0.3	P<0.01
MUFA, %TE	11.4±2.8	13.2±3.2	P<0.01
PUFA, %TE	3.8±0.2	11.2±0.5	P<0.01
n-3 PUFA, %TE	0.7±0.1	1.3±0.1	P<0.01
n-6 PUFA, %TE	2.6±0.1	9.5±0.4	P<0.01
Trans-fat, %TE	0.74±0.03	0.20±0.02	P<0.01
Protein, %TE	15.6±0.4	15.9±0.4	0.55
Carbohydrate, %TE	42.6±1.0	43.0±1.1	0.67
Fibre (AOAC), g	24.4±1.3	25.4±1.5	0.34
Free sugars, %TE	5.1±0.6	5.1±0.5	0.91

Data was analysed by paired t tests

Data was presented as mean ± SE, $p<0.05$ considered as significant, n=57

Abbreviations: AOAC: association of official analytical chemists, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, TE: total energy.

Supplementary Table 4.2. Correlations between changes in LDLR, SREBF1, NR1H3 and ABCG1 gene expressions in the whole group

	LDL-R	SREBF1	NR1H3	ABCG1
LDL-R	-	0.65**	0.17	0.33*
SREBF1	0.65**	-	0.29*	0.72**
NR1H3	0.17	0.29*	-	0.31*
ABCG1	0.33*	0.72**	0.31*	-

Data was analysed using Spearmans correlations

**Significant at 0.01 level

*Significant at 0.05 level

Abbreviations: ABCG1: ATP Binding Cassette Subfamily G Member; LDL-R: low-density lipoprotein receptor; NR1H3: Nuclear Receptor Subfamily 1 Group H Member 3; SREBF1: Sterol Regulatory Element Binding Transcription Factor 1.

Supplementary Table 4.3. Correlation between changes in circulating CVD risk markers and changes in LDL-R, SREBF1, NR1H3 and ABCG1 gene expression after dietary fat manipulation

	LDL-R	SREBF1	NR1H3	ABCG1
TC, mmol/L	0.06	0.09	0.01	0.12
LDL-C, mmol/L	-0.003	0.07	0.03	0.11
HDL-C, mmol/L	0.08	0.12	0.10	0.13
Non-HDL-C, mmol/L	0.04	0.08	-0.02	0.10
TAG, mmol/L	0.14	0.05	-0.22	-0.12
TC:HDL-C	-0.06	0.14	-0.05	0.12
LDL-C:HDL-C	-0.12	0.08	-0.04	0.12
PSCK9, ng/ml	0.07	0.03	-0.26	-0.14

Data was analysed using Spearmans correlations.

**Significant at 0.01 level

*Significant at 0.05 level

Abbreviations: ABCG1: ATP Binding Cassette Subfamily G Member; HDL-C: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; LDL-R: low-density lipoprotein receptor; NR1H3: Nuclear Receptor Subfamily 1 Group H Member 3; SREBF1: Sterol Regulatory Element Binding Transcription Factor 1; TAG: triacylglycerol; TC: total cholesterol.

Supplementary Table 4.4. Correlation between changes in blood lipids and changes in LDL-R, SREBF1, NR1H3 and ABCG1 gene expression after dietary fat manipulation in responder and non-responder groups

	RESPONDERS				NONRESPONDERS			
	LDL-R	SREBF1	NR1H3	ABCG1	LDL-R	SREBF1	NR1H3	ABCG1
TC, mmol/L	-0.02	-0.17	0.14	-0.02	-0.12	0.05	-0.48	0.13
LDL-C, mmol/L	0.07	0.05	0.03	-0.05	0.03	0.08	0.32	-0.37
HDL-C, mmol/L	-0.06	-0.09	0.29	0.11	-0.11	0.07	-0.40	-0.09
Non-HDL-C, mmol/L	0.18	0.05	-0.20	-0.04	-0.03	0.04	-0.14	0.24
TAG, mmol/L	0.20	-0.04	-0.32	-0.14	-0.08	-0.07	-0.14	0.20
TC:HDL-C ratio	0.10	0.06	-0.23	-0.13	0.07	0.26	0.34	0.37
LDL-C:HDL-C ratio	0.11	0.18	-0.17	-0.08	0.14	0.23	0.43	0.30
PCSK9, ng/ml	-0.15	0.12	0.11	-0.06	0.15	-0.02	-0.72**	0.31
ApoB/apoA1 ratio	0.28	0.43	0.15	0.37	-0.31	0.43	-0.44	0.18
Large (LDL-1), mg/dL								
ApoB-100	0.12	0.05	0.55	0.26	-0.70*	0.04	-0.34	0.03
CE	0.13	0.06	0.68*	0.24	-0.67*	0.15	-0.45	0.23

FC	0.01	-0.09	0.63*	0.11	-0.55	0.07	-0.22	0.12
PL	0.08	-0.04	0.66*	0.15	-0.74**	0.07	-0.39	0.17
TAG	0.23	0.17	-0.61*	0.23	-0.48	0.15	0.18	0.08
Intermediate, mg/dL								
ApoB-100	-0.13	-0.08	0.60*	-0.34	-0.26	-0.60*	-0.08	-0.15
CE	-0.23	-0.20	0.65*	-0.33	-0.20	-0.67*	-0.06	-0.23
FC	-0.35	-0.32	0.68*	-0.46	-0.26	-0.47	-0.11	-0.18
PL	-0.23	-0.20	0.65*	-0.38	-0.24	-0.72**	-0.08	-0.29
TAG	0.20	0.01	-0.56	-0.27	-0.35	-0.55	-0.39	0.000
Small (LDL-6), mg/dL								
ApoB-100	0.11	0.16	0.35	0.11	0.28	0.15	0.55	-0.06
CE	0.03	0.10	0.40	0.06	0.25	0.08	0.48	-0.12
FC	-0.08	-0.10	0.51	-0.07	0.13	0.000	0.43	-0.13
PL	0.01	0.06	0.42	0.04	0.23	-0.03	0.49	-0.17
TAG	0.13	0.20	0.28	0.11	0.30	-0.24	0.31	-0.29

Data was analysed using Spearmans correlations. **Significant at 0.01 level. *Significant at 0.05 level. Abbreviations: Apo: apolipoprotein, ABCG1: ATP Binding Cassette Subfamily G Member, CE: cholesterol, FC: free cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, LDL-R: low-density lipoprotein receptor NEFA: non-esterified fatty acids, NR1H3: Nuclear Receptor Subfamily 1 Group H Member 3, PL: phospholipid, SFA: saturated fatty acids, SREBF1: Sterol Regulatory Element Binding Transcription Factor 1, TAG: triacylglycerol, TC: total cholesterol, PCSK9: proprotein convertase subtilisin/kexin type 9.

Supplementary Table 4.5. List of genes analysed in peripheral blood mononuclear cells.

Gene symbol	Full name	Function	Assay ID
LDL-R	LDL receptor	Lipoprotein metabolism	Hs01092524_m1
SREBF1	Sterol regulatory element binding transcription factor 1	Transcription factor targeting lipid genes	Hs01088691_m1
NR1H3	Nuclear receptor subfamily 1 group H member 3	Transcription factor targeting lipid genes	Hs00172885_m1
ABCG1	ATP-binding cassette subfamily G member 1	Reverse cholesterol transport	Hs00245154_m1

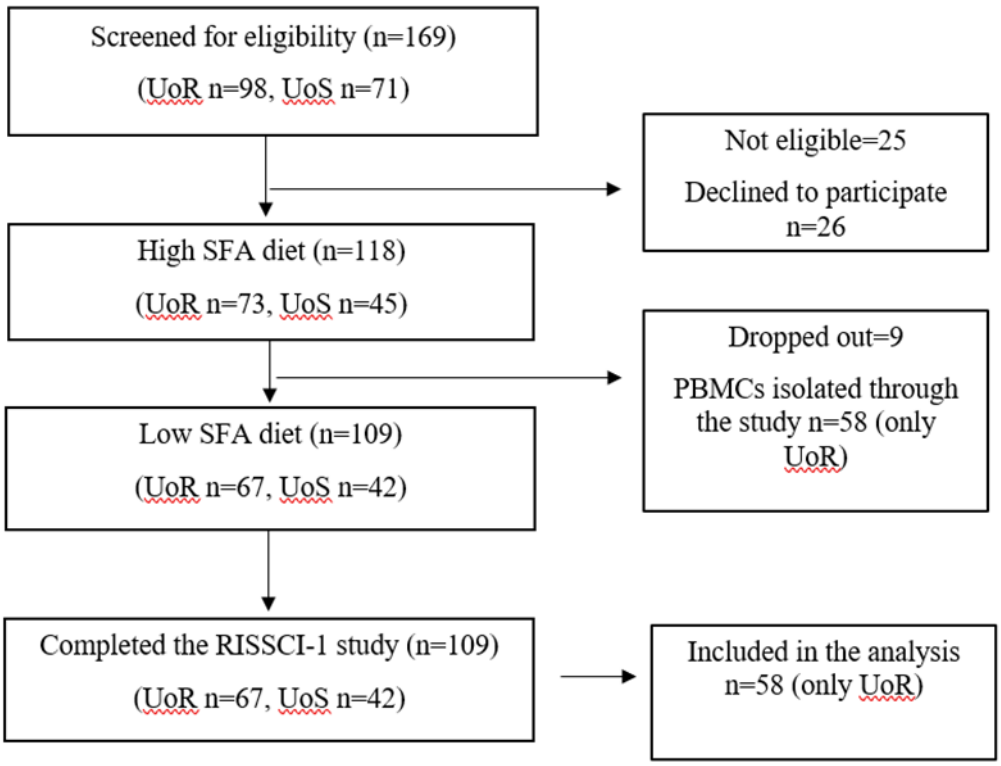

Figure 4.2 Flow chart of participants from the RISSCI study

Chapter 5: Association between *APOE* genotype with body composition and cardiovascular disease risk markers is modulated by BMI in healthy adults: Findings from the BODYCON study

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Contribution towards the manuscript

The author responsibilities were as follows- KGJ and JAL designed the study; EO, RM, NL and KGJ conducted research; NL, EO, KGJ carried out DNA extraction and genotyping of the samples; EO analysed data and wrote the manuscript under the guidance of KGJ and JAL. KGJ had primary responsibility for final content. All the authors read and approved the final manuscript.

Association between *APOE* genotype with body composition and cardiovascular disease risk markers is modulated by BMI in healthy adults: Findings from the BODYCON study

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Running title: *APOE*, BMI and cardiovascular disease risk markers

Abbreviations in the text

Apo, apolipoprotein; A/G ratio, android to gynoid ratio; BMI, body mass index; CVD, cardiovascular disease; CRP, C-reactive protein, DXA, dual energy x-ray absorptiometry; HDL-C, high-density lipoprotein cholesterol; LDL-C low-density lipoprotein cholesterol; LDL-R, LDL receptor; NEFA, non-esterified fatty acids; TAG, triacylglycerol; TC, total cholesterol; VAT, visceral adipose tissue.

5.1 Abstract

Background: The association between *APOLIPOPROTEIN (APO)E* genotype and cardiovascular disease (CVD) risk is well studied. Although some studies suggest a BMI x *APOE* interaction on CVD risk markers, the extent of this relationship remains unclear.

Aim: To assess the association between *APOE* genotype with body composition and CVD risk markers, with further examination of the role of BMI on this relationship.

Methods: In the cross-sectional observational BODYCON study 360 healthy men and women, with a mean age of 42±1 y and body mass index (BMI) of 24.1±0.2 kg/m^2 were recruited. A fasting blood sample was collected to measure CVD risk markers and body composition was measured using dual energy x-ray absorptiometry. Physical activity level and habitual dietary intake were also assessed using a tri-axial accelerometer and a 4-day weighed food diary, respectively. Participants were genotyped retrospectively for *APOE* (rs429358 and rs7412).

Results: The *APOE2/E3* group had lower fasting total (TC), low-density lipoprotein (LDL-C) and non-high density lipoprotein cholesterol (HDL-C) concentrations compared to *APOE4* carriers and *APOE3/E3* group ($p \leq 0.01$). *APOE* x BMI interactions on body weight and android fat mass were observed ($p \leq 0.01$). When the group were stratified into normal weight and overweight/obese subgroups based on BMI, lean body mass was higher in *APOE4* carriers compared to the *APOE3/E3* participants in the normal weight BMI group ($p=0.02$), while in the overweight/obese BMI group, the android to gynoid fat ratio was greater in the *APOE3/E3* group compared to *APOE4* carriers ($p=0.04$). Differences in lipid concentrations were only evident between the *APOE2/E3* and other genotype groups within the normal weight BMI subgroup ($p \leq 0.04$). This finding was associated with a lower dietary fibre and trans-fat intake in the *APOE2/E3* participants compared with *APOE4* carriers, and a lower carbohydrate intake relative to the *APOE3/E3* group.

Conclusion: Our findings confirm previous reports that BMI modulates the effect of *APOE* on CVD risk markers and suggest novel interactions on body composition, with diet a potential modulator of this relationship.

This trial was registered at clinicaltrials.gov as NCT02658539.

Keywords: *APOE*, body composition, fasting blood lipids, BMI

5.2 Introduction

The *APOLIPOPROTEIN (APO)E* gene is one of the most widely studied in relation to cardiovascular disease (CVD) risk due to the association with circulating blood lipids. It encodes the multifunctional apoE apoprotein which represents an important ligand for the receptor-mediated uptake of triacylglycerol (TAG)-rich lipoproteins and their remnants from the circulation (112). The *APOE2*, *APOE3* and *APOE4* alleles have different affinities for the low-density lipoprotein (LDL) receptor (LDL-R) which impacts on cholesterol homeostasis and blood lipid profile (273-275). It has been well documented that *APOE* gene accounts for 7% of the variance in cholesterol in Caucasians (276). Although several studies have reported elevated total cholesterol (TC) and LDL cholesterol (LDL-C) concentrations in *APOE4* carriers and lower concentrations in *APOE2* carriers compared to the wild-type *APOE3/E3* group (117, 120), these relationships have not been reported by others (122, 147, 153, 277, 278). These inconsistencies between studies have been attributed to the metabolic status and adiposity of the study populations suggesting that other factors such as body mass index (BMI) may impact on the relationship between *APOE* genotype and chronic disease risk (122, 153, 278).

It is well-known that obesity is an independent risk factor for CVD (279). Animal studies have shown deficiency of *APOE* was protective against obesity (129, 131) and suggested a differential effect of the apoE alleles on the ability of the body to store fat, with *APOE3* mice having a higher body weight than *APOE4* mice on a Western type diet (132, 133, 135). Moreover, increased visceral adipose tissue (VAT) accumulation, which is associated with increased CVD risk (162, 163), was reported in *APOE3* compared to *APOE4* mice (133, 136, 137). Therefore, a possible explanation for the inconsistent results on association between *APOE* genotype and blood cholesterol concentrations might be dependent on adiposity. In

agreement, several studies have reported the relationship between *APOE* and blood lipid risk markers to differ depending on BMI but the mechanisms underlying this association are unclear. Lower TAG concentration in *APOE2* carriers compared with *APOE3/E3* group and *APOE4* carriers was reported to be evident only in the UK adults with a normal BMI (16), whereas in Mexican Amerindian population, differences in TC, LDL-C and TAG among *APOE4* carriers and *APOE3/E3* genotype were only found in obese subjects (BMI≥30 kg/m^2) (151). However, the limited human studies conducted to date have failed to identify which apoE allele is more prone to obesity and whether an interaction exists between *APOE* and adiposity on CVD risk markers (16, 150, 151, 280) (281). In addition, APOE genotype may have an impact on food preferences which can affect body composition, however the evidence is limited. In the Australian Imaging, Biomarkers and Lifestyle study of ageing APOE4 carriers were found to have lower protein intakes than non-APOE4 carriers (302).

Therefore, this paper aims to investigate the association between *APOE* genotype with body composition and CVD risk markers, with further examination of the role of BMI on this relationship.

5.3 Methods

Subjects

A total of 360 healthy men and women aged 18-70 y from the impact of physiological and lifestyle factors on body composition (BODYCON) study were included in the present analysis. Details of the study design have been described previously (Chapter 2). Briefly, participants were recruited from Reading and the surrounding areas and inclusion criteria were body mass index (BMI) 18.5-39.9 kg/m^2, TC<7.8 mmol/l, TAG<2.3 mmol/l, fasting blood glucose<7.8 mmol/l, haemoglobin>115 g/l for women and 130 g/l for men. Exclusion criteria were having suffered a myocardial infarction/stroke in the past 12 months, history of diabetes or other

endocrine disorders, bowel disease, cholestatic liver disease, pancreatitis, cancer, arthritis or fracture deformity of spine or femur, history of bone related surgeries, radio-opaque implants or implanted medical devices, breastfeeding, being pregnant or planning pregnancy in the next 12 months, being on medication for hyperlipidemia, hypertension, inflammation or hypercoagulation, being on a weight reducing diet and excessive alcohol consumption (<14 units/wk).

Study design

The BODYCON study is an observational cross-sectional study conducted in the Hugh Sinclair Unit of Human Nutrition at the University of Reading. The main outcomes of the BODYCON study have been described previously (Chapter 2). Briefly, participants attended a single study visit in which a fasting blood sample was collected, and anthropometric measurements were taken. Participants also underwent a dual energy x-ray absorptiometry (DXA) scan to assess their total body composition. The NHS and University of Reading Research Ethics Committees both gave a favourable ethical opinion for the conduct of the BODYCON study (NHS reference number:14/SC/1095 and UREC reference numbers: 17/29 and 13/55). Participants were only included in the analysis dataset if written consent was obtained for the retrospective genotyping for *APOE*. The BODYCON study was carried out in accordance with the principles of the Declaration of Helsinki and registered at www.clinicaltrials.gov (NCT02658539).

Anthropometric measurements

Anthropometric measures were performed with participants wearing light clothing and no shoes. Height was measured by a stadiometer. Body weight was measured and BMI was calculated using a Tanita BC-418 scale (TANITA UK Ltd, Middlesex, UK). Waist and hip circumferences were measured using a non-stretch tape measure. As estimates of body fat distribution, the waist to hip ratio (WHR) and waist to height ratio (WHtR) were calculated. To

assess the body composition, DXA scan was performed by trained researchers and described elsewhere (Chapter 2). Briefly, prior to the scan participants were required to wear clothes without metal fastenings, buttons or zips, and all metal artefacts were removed. For the total body composition scan, participants lay still on Lunar iDXA scanner bed with Velcro straps around their knees and ankles. All scans were analysed using enCORE Software, version 15 (GE Healthcare) with the advance software package CoreScan, which also estimates the mass and volume of VAT within the abdomen. Fat mass index (FMI) and lean mass index (LMI) were calculated as FMI= fat mass(kg)/height in m^2 and LMI=lean mass(kg)/height in m^2.

Dietary intakes and Physical activity

Habitual dietary intake was assessed using a 4-day weighed diet diary. Dietary data was analysed using DietPlan 7 software (Forestfield, Horsham, UK) and dietary intakes were averaged. Physical activity levels were measured using a tri-axial accelerometer (Actigraph wGT3X+, Actigraph, LLC). Participants were asked to wear the accelerometer directly above the right iliac crest during sleeping and waking hours (except for during water-based activities) for four days, including three weekdays and one weekend day during the same time that dietary intake was assessed. Device initialization, data processing and analysis were conducted using Actilife Data Analysis Software (Version 6.11.5).

Biochemical analysis

Fasting blood samples collected into the serum separator and K3EDTA blood tubes were centrifuged at 1700 x g (3000 rpm) for 15 min at room temperature and 4°C, respectively before aliquoting into Eppendorf tubes and stored at -20 °C and -80°C, respectively. Fasting lipids (TC, high-density lipoprotein cholesterol (HDL-C), non-esterified fatty acids (NEFA), TAG), glucose and high sensitivity C-reactive protein (hs-CRP)) were quantified in the serum sample by using the ILAB 600 (Werfen (UK) Ltd., Warrington UK) and RX Daytona Plus (Randox

Laboratories Limited, Crumlin, UK) clinical chemistry analysers. The Friedewald equation was used to estimate fasting LDL-C concentrations and non-HDL-C was calculated by subtracting HDL-C from TC. Plasma uric acid was measured using Daytona Plus clinical chemistry analyser (Randox Laboratories Ltd., County Antrim UK). ELISA kits were used to analyse serum insulin (Dako UK Ltd and Crystal Chem, Inc., USA) and plasma adiponectin (Quantikine kit, R&D Systems, Europe Ltd.).

DNA extraction and Genotyping

The buffy coat layer was isolated from the blood sample collected into a 9 ml EDTA blood tube prior to the extraction of DNA using a DNA blood mini kit (Qiagen Ltd., UK) according to the manufacturers protocol. DNA samples were genotyped for the single nucleotide polymorphisms (SNP) rs429358 and rs7412 with the use of TaqMan SNP genotyping assays (Thermofisher Scientific) on the QuantStudio 3 real time PCR machine (Applied Biosystems).

Statistical analysis:

Statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS Inc., IL, USA). Normality of data was checked using Kolmogrov-Smirnov test and Q-Q plots. Hardy-Weinberg equilibrium was tested by a chi-square test. To assess the effect of *APOE* genotype, a general linear model (ANCOVA) was performed using the study outcome measures as the dependent variable, genotype as a fixed factor and age and sex as covariates. To assess the effect of adiposity, a BMI x genotype interaction was added to the model. Participants were then stratified into normal and overweight/obese BMI subgroups and analysed using ANCOVA including age and sex as covariates. If a significant genotype effect was found, pairwise comparisons with a Bonferroni correction were carried out. Results are presented as estimated marginal means ± SE and $p \leq 0.05$ was considered significant.

5.4 Results

The effect of APOE genotype on body composition measures and cardiovascular disease risk markers

The main characteristics for 360 participants (187 female and 168 male) according to the *APOE* genotype is shown in **table 5.1**. The study population had an average age of 42±1 y and BMI of 24.1±0.2 kg/m^2, and n=46 participants were *APOE2/E3*, n=228 the wild type *APOE3/E3* group and n=81 *APOE4* carriers (*APOE3/E4* and *APOE4/E4*). Subjects with the *APOE2/E4* genotype (n=5) were not included in the analysis due to the small sample size and no participants with the *APOE2/E2* genotype were identified in the study cohort. The *APOE* allele distribution was found to be in Hardy-Weinberg equilibrium.

Fasting TC, LDL-C and non-HDL-C concentrations and LDL-C: HDL-C ratio in the *APOE2/E3* group were on average 9%-18% lower compared to *APOE4* carriers and 9%-16% lower compared with the *APOE3/3* group ($p \leq 0.01$) (**Table 5.1**). Anthropometric and body composition measures were not different between genotype groups. The habitual dietary intakes of participants were shown in **table 5.2.** Total dietary fibre intake was on average 4 g higher in *APOE4* carriers than *APOE2/E3* group (p=0.04). There was also an association between *APOE* genotype and total protein intake, with the *APOE3/E3* group and *APOE4* carriers consuming 3%TE and 4%TE lower than participants in the *APOE2* group, respectively ($p<0.01$). The *APOE* genotype did not affect total dietary energy or intake of other macronutrients .

Significant BMI x genotype interactions were observed for body weight and android fat mass and for the dietary intakes of total polyunsaturated fatty acids (%TE) and total protein (%TE) ($p \leq 0.03$). Genotype x BMI interactions were not found for other measures of body composition, dietary intakes, or CVD risk markers. (**Tables 5.1 and 5.2**).

Effect of APOE genotype and BMI on body composition measures and CVD risk markers

To assess the effect of the *APOE* genotype according to adiposity level, participants were split

into 2 BMI groups representing normal weight (n=232) and overweight/obese (n=128) groups. The subject characteristics, body composition and CVD risk markers according to BMI subgroups were shown in **table 5.3**.

In the normal weight BMI group, *APOE4* carriers had 3 kg higher lean mass and 240 g greater android lean mass than the wild-type *APOE3/E3* group ($p \leq 0.02$). LDL-C and non-HDL-C concentrations were 17% and 15% lower respectively in the *APOE2/E3* group compared to *APOE4* carriers and 15% and 12% lower compared to the *APOE3/E3* group ($p \leq 0.02$). The LDL-C: HDL-C ratio was also 17% lower in the *APOE2/E3* group compared to *APOE4* carriers ($p=0.04$). TC concentrations were 9% lower in the *APOE2/E3* compared with the *APOE3/3* group ($p=0.04$). In the overweight/obese BMI group, the android to gynoid fat percentage ratio was higher in the *APOE3/E3* group compared to *APOE4* carriers ($p=0.04$). Other body composition measures and CVD risk factors did not differ across the three genotype groups in this BMI subgroup .

Habitual dietary intakes are presented in **table 5.4** according to normal and overweight/obese BMI subgroups. In the normal BMI group, while dietary fibre intake was 6 g higher, trans-fat %TE was 0.15% lower in *APOE4* carriers compared to the *APOE2/E3* group ($p \leq 0.05$). The participants in the *APOE2/E3* group also had a lower dietary carbohydrate (%TE) intake compared to the *APOE3/E3* group ($p=0.01$). Moreover, in the normal BMI subgroup, the *APOE2/E3* group had the highest total protein (%TE) intake compared to the *APOE4* carriers and the *APOE3/E3* group ($p=0.01$). Dietary intakes were not different between genotype groups in the overweight/obese BMI group (Table 5.4). Physical activity levels (steps/day, energy expended performing physical activity per day, and percentage time spent performing sedentary, light, or moderate to vigorous physical activity) were not significantly different according to *APOE* genotype neither in the whole group or after stratifying according to normal and overweight/obese BMI groups (**supplemental tables 5.1 and 5.2**).

5.5 Discussion

This study examined the association between *APOE* genotype with body composition and CVD risk factors and the impact of BMI classification on this relationship. Using data from the BODYCON cross-sectional study, we found *APOE* genotype to impact on the fasting lipid profile, with differences only evident in participants with a normal BMI. Novel associations between genotype and body composition were observed, with divergent effects of *APOE* on the android to gynoid fat percentage ratio and lean body mass within the normal and overweight/obese BMI subgroups.

Several studies have reported associations between *APOE* genotype and blood lipid risk markers (282). In agreement with the previous studies (117, 120, 283), we also observed TC, LDL-C and non-HDL-C concentrations to be significantly higher in *APOE4* carriers and *APOE3/E3* group compared to the *APOE2/E3* group. However, after dividing the cohort into normal weight and overweight/obese BMI subgroups, LDL-C and non-HDL-C concentrations were only significantly higher in *APOE4* carrier and *APOE3/E3* groups compared to the *APOE2/E3* group in the normal BMI subgroup. Our findings support those of Kofler et al. (16) who reported the lowest TAG concentration in *APOE2* carriers only in participants with normal BMI in the FINGEN study where 312 participants living in the UK were prospectively genotyped for *APOE*. In agreement with this, Kolovou et al. (154) observed the *APOE4* allele to be associated with higher TC levels compared with *APOE3* allele in normal-weight coronary heart disease patients based in Greece. Therefore, our data shows that the effect of *APOE* on CVD risk markers may be dependent on their BMI. This could imply that negative effects of high BMI could mask the effect of *APOE* genotype on the fasting lipid profile. Although the

mechanism of this interaction is not yet understood, it is known that dyslipidaemia is associated with a higher BMI (284). This is in line with our findings that the detrimental effect of an increased BMI outweighs the positive effect of *APOE2* allele on blood lipid risk markers. It should be noted that *APOE4* carriers consumed more dietary fibre and less trans-fat compared to *APOE2* carriers. Furthermore, our study included healthy subjects with a higher-than-average physical activity level. This may have impacted on the fasting lipid profile observed within the genotype groups as exercise has been shown to favourably affect cholesterol and TAG levels (301). Thus, further studies are needed to draw a conclusion.

The effect of *APOE* genotype on body composition has been investigated in animals and a small number of human studies. Arbones-Mainar et al. (133) reported greater increases in abdominal VAT accumulation and body weight after a high fat western type diet (21%TE fat) in *APOE3* mice compared to *APOE4*. In the current study, our participants consumed on average a high fat diet (37%TE) and abdominal VAT was not different between *APOE* genotype groups. However, findings from human studies investigating the association between *APOE* genotype and body composition are inconsistent. Positive associations between *APOE2* allele with waist circumference and BMI have been reported in 230 Croatian subjects aged 20-85 y and in 4660 Caucasian middle-aged men (140, 141). Another study in 290 children aged 8 years reported lower BMI, trunk fat mass and waist circumference in *APOE4* carriers compared to non-*APOE4* carriers (*APOE3/E3*, *APOE2/E2* and *APOE2/E3*) (285). In contrast, in a case-control study including 198 normal weight healthy and 198 obese Saudi university students, the *APOE4* allele was positively associated with BMI in overweight and obese subjects (BMI>25kg/m^2) (146).These discrepancies between studies might be influenced by the participants sex, age, ethnicity and/or habitual diet, thus further studies are needed to confirm these findings.

In the current study, we found genotype x BMI interactions on body weight and android fat mass. After stratifying the cohort according to BMI, there were no differences in body weight between *APOE* genotypes in either the normal or overweight/obese BMI groups. However, we observed *APOE4* carriers with a normal BMI to have higher lean body mass and android lean mass compared to the wild-type *APOE3/E3* group. Therefore, this might provide a possible explanation for the lower body fat and VAT mass accumulation in the *APOE4* carriers compared to the *APOE3/E3* group in animal studies (132, 137). The mechanisms behind the relationship between the *APOE4* allele and increased lean body mass is not clear although animal studies have suggested that adiponectin may play a role. In *APOE4* mice, a greater increase in adiponectin levels were observed compared to *APOE3* mice on an obesogenic diet (286) and the protective role of adiponectin against muscle loss and muscle growth have been described in some studies (287). In addition, an association between appendicular lean mass and circulating adiponectin was reported in postmenopausal women (288). However, in this study adiponectin concentrations were not different between the genotype groups, therefore this potential mechanism needs to be examined in further studies. Moreover, in the overweight/obese BMI subgroup *APOE4* carriers had a lower android to gynoid fat percentage ratio suggesting a difference in body fat distribution compared to the wild-type group which had similar dietary intakes and physical activity levels. These findings are interesting since it is well-known that abdominal obesity is associated with dyslipidaemia (168), and our findings suggest that *APOE3/E3* genotype had lower LDL-C and non-HDL-C concentrations but higher android body fat distribution compared to *APOE4* carriers. Our finding provide support to those of a previous study which reported that *APOE4* mice accumulated less VAT than *APOE3* mice after following a high fat diet for 6 months (135). The authors speculated that endoplasmic reticulum stress is a potential mechanism linking *APOE* and adiposity. Since apoE4 has a lower protein stability and is abnormally folded in the

endoplasmic reticulum, increased endoplasmic reticulum stress in *APOE4* carriers may have negative effect on adipogenesis (135). Moreover, in a study by Huebbe et al. (132) less weight gain in *APOE4* compared to *APOE3* mice on high and low-fat diets was observed and the authors reported higher expression of fatty acid-binding protein 4, carnitine palmitoyl transferase 1B and uncoupling protein in *APOE4* mice which suggested increased fatty acid oxidation in skeletal muscle in *APOE4* mice compared to the *APOE3* mice. However, as mice do not usually consume high fat diets it is difficult to translate findings from animal studies to humans. Therefore, further clarification of the association between *APOE* and body fat distribution measures and the potential mechanisms observed are needed in humans.

The use of a DXA scan, which is known to be an accurate and precise tool for body composition measurement including estimation of abdominal VAT mass, to measure body composition is one of the important strengths of this study. In addition, we included the analysis of a range of outcome measures such as physical activity, dietary intake and CVD risk markers in this cohort. Limitations include the cross-sectional study design, retrospective genotyping, and small sample size for some genotype groups, especially during the sub-group analysis according to BMI. Moreover, subjects were not stratified according to the median BMI but normal and overweight/obese sub-groups, with only 36% of this cohort having a BMI >24.9 kg/m^2. Finally, it should be noted that using BMI as a marker of adiposity to stratify the group has its own limitations since it cannot distinguish between excess body fat and muscle mass.

In summary, our results indicate an interaction between *APOE* genotype and BMI, with higher blood lipid risk marker concentrations only evident in *APOE4* carriers compared to the *APOE2/E3* group in participants with a normal BMI. Moreover, differential effects on body fat distribution and composition were observed within the BMI subgroups between the *APOE4* carriers and the wild-type *APOE3/E3* group, with diet also a potential modulator of this relationship. However, the association between *APOE* genotype, adiposity, diet and CVD risk

markers needs further investigation in humans with prospective genotyping to draw a firm conclusion.

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Statement of authors' contributions to manuscript

The author responsibilities were as follows- KGJ and JAL designed the study; EO, RM, NL and KGJ conducted research; NL, EO, KGJ carried out DNA extraction and genotyping of the samples; EO analysed data and wrote the manuscript under the guidance of KGJ and JAL. KGJ had primary responsibility for final content. All the authors read and approved the final manuscript. None of the authors have any conflict of interest.

TABLE 5.1 Participant characteristics and anthropometric measures according to *APOE* genotype[1]

	All (n=360)	*E2* carriers (n=46)	*E3/E3* (n=228)	*E4* carriers (n=81)	P[2] value	Genotype x BMI[3]
Genotype frequency (%)		12.8	63.3	22.5		
Sex, F/M	187/168	28/18	121/107	38/43		
Age (y)	42±1	45±2	41±1	44±2	0.23	
BMI (kg/m^2)	24.1±0.2	23.7±0.5	24.2±0.2	24.2±0.4	0.58	
Anthropometric measurements						
Weight (kg)	70.8±0.7	69.6±1.7	70.8±0.8	71.9±1.3	0.55	
WC (cm)	84.3±0.6	83.8±1.5	84.6±0.7	84.0±1.1	0.83	0.12
HC (cm)	101±1	100±1	101±1	102±1	0.52	0.72
WHtR	0.49±0.01	0.49±0.01	0.50±0.01	0.49±0.01	0.58	0.39
Body composition measurements						
Body fat (%)	28.2±0.4	28.1±1.0	28.4±0.5	27.9±0.8	0.83	0.60
Fat mass (kg)	20.3±0.4	19.6±1.2	20.5±0.5	20.4±0.9	0.78	0.42
Lean mass (kg)	48.5±0.6	48.0±1.0	48.4±0.4	49.4±0.7	0.36	0.21
Abdominal VAT (g)	599±31	561±70	622±32	569±53	0.57	0.70
Android fat mass (kg)	1.61±0.05	1.52±0.14	1.65±0.07	1.57±0.11	0.63	**P<0.01**
Android lean mass (kg)	3.32±0.04	3.27±0.07	3.30±0.0.3	3.43±0.05	0.06	0.39
Android fat (%)	30.5±0.6	30.4±1.7	30.9±0.8	29.6±1.3	0.65	0.74
Gynoid fat (%)	32.1±0.5	31.8±1.0	32.2±0.5	32.1±0.8	0.94	0.42

A/G fat % ratio	0.96±0.02	0.98±0.03	0.97±0.02	0.92±0.03	0.26	0.87
CVD risk markers						
Blood pressure (mmHg)						
Systolic	120±1	118±2	121±1	119±1	0.35	0.11
Diastolic	72±1	69±1	73±1	71±1	0.04	0.96
Pulse pressure	48±1	49±2	48±1	48±1	0.96	0.06
TC (mmol/l)	5.16±0.06	4.77±0.14[b]	5.22±0.06[a]	5.25±0.10[a]	**0.01**	0.90
TAG (mmol/l)	0.98±0.03	1.02±0.07	0.96±0.03	1.00±0.05	0.63	0.80
HDL-C (mmol/l)	1.65±0.02	1.68±0.05	1.66±0.02	1.61±0.04	0.42	0.97
LDL-C (mmol/l)	3.05±0.05	2.63±0.12[b]	3.11±0.05[a]	3.18±0.09[a]	**P<0.01**	0.87
Non-HDL-C (mmol/l)	3.51±0.05	3.09±0.13[b]	3.56±0.06[a]	3.64±0.10[a]	**P<0.01**	0.93
TC: HDL-C ratio	3.25±0.05	3.01±0.12	3.27±0.06	3.35±0.09	0.09	0.99
LDL-C: HDL-C ratio	1.94±0.04	1.69±0.11[b]	1.97±0.05[a]	2.05±0.08[a]	**0.02**	0.99
NEFA(μmol/l)	398±12	404±32	390±14	417±24	0.61	0.19
Glucose (mmol/l)	5.04±0.03	5.00±0.07	5.04±0.03	5.03±0.05	0.88	0.47
CRP (mg/l)	1.35±0.12	1.01±0.34	1.48±0.15	1.24±0.26	0.40	1.00
Adiponectin(μg/ml)	6.55±0.29	5.28±0.76	6.69±0.35	6.58±0.57	0.24	0.74
Uric acid(μmol/l)	275±4	286±8	275±4	272±6	0.37	0.64

[1]Data was presented as estimated marginal means ± SE, p<0.05 is considered significant. *E2* carriers= *E2/E3*, *E4* carriers= *E3/E4* and *E4/E4*.
[2]Data was analysed by univariate general linear model (ANCOVA) adjusted for age and sex. [3]*APOE* genotype x BMI interaction by ANCOVA, adjusted for age and sex. Carrier code and BMI as fixed factors and variable of interest as dependent variable. [abc] significant differences (P<0.05) shown as different superscript letters. Sample sizes are as follows: for WC, HC, WHR, WHtR, all n=359, *APOE2* carriers n=46, *APOE3/E3* n=227, *APOE4* carriers n=81; for BP, all n=357, *APOE2* carriers n=46, *APOE3/E3* n=225, *APOE4* carriers n=81; for NEFA all n=355 *APOE2* carriers n=45, *APOE3/E3* n=225, *APOE4* carriers n=80; for CRP all n=359, *APOE2* carriers n=46, *APOE3/E3* n=227, *APOE4* carriers n=81; for adiponectin and uric acid all n=322 *APOE2* carriers n=42, *APOE3/E3* n=201, *APOE4* carriers n=75. Abbreviations: A/G fat % ratio android to gynoid fat % ratio, BMI body mass index, CRP C-reactive protein, FMI fat mass index, HC hip circumference, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, LMI lean mass index, NEFA non-esterified fatty acids, TC total cholesterol, TAG triacylglycerol, VAT visceral adipose tissue, WC waist circumference, WHR waist to hip ratio, WHtR waist to height ratio.

TABLE 5.2 Participants habitual dietary intake according to *APOE* genotype[1]

	All (n=360)	*E2* carriers(n=46)	*E3/E3*(n=228)	*E4* carriers(n=81)	P[2] value (genotype)	P value (BMI)	Genotype x BMI[3]
Dietary intake							
Energy intake (MJ)	8.51±0.14	8.05±0.35	8.58±0.16	8.72±0.27	0.30	0.58	0.68
Total fat (%TE)	36.6±0.5	37.5±1.3	36.3±0.6	36.6±1.0	0.67	0.12	0.77
SFA (%TE)	13.0±0.2	13.5±0.7	13.0±0.3	12.6±0.5	0.57	0.10	0.81
MUFA (%TE)	13.7±0.2	13.9±0.6	13.6±0.3	13.9±0.4	0.79	0.14	0.60
PUFA (%TE)	6.3±0.1	6.2±0.3	6.1±0.2	6.7±0.3	0.08	0.76	**0.03**
Trans fat (%TE)	0.55±0.02	0.60±0.04	0.54±0.02	0.53±0.03	0.33	0.05	0.17
Total CHO (%TE)	45.4±0.6	42.2±1.6	46.4±0.7	44.8±1.2	**0.05**	0.37	0.81
Total sugars (%TE)	18.5±0.4	17.3±1.0	18.6±0.4	19.1±0.8	0.32	0.56	0.20
Total fibre (AOAC, g)	24.6±0.5	22.5±1.4[b]	24.4±0.6[ab]	26.7±1.0[a]	**0.04**	0.94	0.35
Total protein (%TE)	18.5±0.3	21.3±0.8[b]	18.0±0.4[a]	18.7±0.6[a]	**0.01**	0.91	**0.02**

[1]Data was presented as estimated marginal means ± SE, *E2* carriers= *E2/E3, E4* carriers= *E3/E4* and *E4/E4*.
[2]Data was analysed by univariate general linear model (ANCOVA) adjusted for age and sex.
[3]APO E genotype x BMI interaction by ANCOVA, adjusted for age and sex

Abbreviations: AOAC: Association of official analytical chemists, CHO carbohydrate, SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

TABLE 5.3. Participant characteristics and anthropometric measures according to *APOE* genotype in normal and overweight/obese BMI groups

	BMI ≤24.9 kg/m² (n=232)				BMI ≥25 kg/m² (n=128)			
	*E2*carriers (n=33)	*E3/E3* (n=147)	*E4* carriers (n=48)	P value	*E2* carriers (n=13)	*E3/E3* (n=81)	*E4* carriers (n=33)	P value
Genotype frequency (%)	14.2	63.4	20.7		10.2	63.3	25.8	
Female/male	22/11	85/62	26/22		6/7	36/45	12/21	
Age (y)	43±3	40±1	44±2	0.26	50±4	44±2	44±2	0.40
Anthropometric measurements								
Weight (kg)	65.0±1.2	63.1±0.6	65.4±1.0	0.08	80.8±2.7	84.7±1.1	82.1±1.7	0.25
WC (cm)	80.1±1.1	78.3±0.5	78.0±0.9	0.28	92.4±2.4	95.6±1.0	93.6±1.5	0.32
HC (cm)	98.2±1.0	96.6±0.5	97.8±0.9	0.24	106.3±2.1	109.4±0.8	108.7±1.3	0.38
WHtR	0.47±0.01	0.46±0.01	0.45±0.01	0.11	0.54±0.02	0.55±0.01	0.55±0.01	0.52
Body composition measures								
Body fat (%)	26.5±0.9	25.6±0.4	23.8±0.8	0.06	32.3±1.6	33.3±0.7	33.9±1.0	0.70
Fat mass (kg)	17.1±0.7	16.1±0.3	15.5±0.6	0.20	26.0±2.0	28.2±0.8	28.0±1.2	0.57
Lean mass (kg)	46.1±1.0[ab]	45.2±0.5[b]	48.1±0.9[a]	**0.02**	52.4±1.7	54.0±0.7	51.8±1.1	0.20
Abdominal VAT (g)	380±44	341±21	331±36	0.66	929±138	1126±55	963±86	0.17
Android fat mass (kg)	1.21±0.12	1.11±0.06	1.01±0.10	0.42	2.25±0.23	2.61±0.09	2.44±0.15	0.27
Android lean mass (kg)	3.15±0.01[ab]	3.10±0.04[b]	3.34±0.07[a]	**0.01**	3.54±0.13	3.65±0.05	3.60±0.08	0.66
Android fat (%)	27.2±1.5	25.2±0.7	22.9±1.3	0.09	38.2±2.5	41.1±1.0	39.8±1.5	0.47
Gynoid fat (%)	30.7±1.0	30.4±0.5	28.5±0.8	0.11	35.2±1.7	35.5±0.7	37.4±1.1	0.29
A/G fat % ratio	0.90±0.03	0.85±0.02	0.82±0.03	0.18	1.14±0.05[ab]	1.19±0.02[b]	1.10±0.03[a]	**0.04**

CVD risk markers								
Blood pressure (mmHg)								
Systolic	116±2	119±1	118±2	0.67	123±3	125±1	122±2	0.48
Diastolic	68±2	71±1	70±1	0.19	73±3	76±1	73±1	0.16
Pulse pressure	49±2	48±1	48±2	0.94	49±3	49±1	49±2	0.99
TC (mmol/l)	4.70±0.17^{b}	5.14±0.08^{a}	5.19±0.14^{ab}	**0.04**	4.91±0.26	5.35±0.10	5.34±0.16	0.27
TAG (mmol/l)	0.88±0.06	0.82±0.03	0.90±0.05	0.29	1.32±0.16	1.20±0.06	1.17±0.10	0.72
HDL-C (mmol/l)	1.72±0.06	1.76±0.03	1.68±0.05	0.40	1.58±0.09	1.49±0.04	1.49±0.06	0.66
Non-HDL-C (mmol/l)	2.98±0.15^{b}	3.39±0.07^{a}	3.51±0.12^{a}	**0.02**	3.33±0.25	3.87±0.10	3.85±0.16	0.14
LDL-C (mmol/l)	2.57±0.14^{b}	3.01±0.07^{a}	3.10±0.12^{a}	**0.01**	2.73±0.23	3.28±0.09	3.32±0.14	0.07
NEFA (µmol/l)	402±39	405±19	423±33	0.88	420±56	361±22	406±34	0.41
TC: HDL-C ratio	2.83±0.11	2.99±0.05	3.14±0.09	0.07	3.42±0.29	3.77±0.11	3.68±0.18	0.52
LDL-C: HDL-C ratio	1.57±0.10^{b}	1.76±0.05^{ab}	1.89±0.08^{a}	**0.04**	1.93±0.25	2.34±0.10	2.30±0.15	0.29
Glucose (mmol/l)	5.00±0.08	4.95±0.04	5.00±0.07	0.68	5.00±0.12	5.21±0.05	5.08±0.08	0.14
CRP (mg/l)	0.83±0.43	1.31±0.20	0.88±0.36	0.42	1.47±0.56	1.78±0.23	1.80±0.35	0.87
Adiponectin (µg/ml)	5.18±0.97	7.30±0.46	6.38±0.79	0.12	5.53±1.20	5.48±0.51	6.77±0.76	0.36
Uric acid(µmol/l)	271±9	269±4	254±7	0.16	322±17	286±7	302±11	0.13

Data was presented as estimated marginal means ± SE, $p<0.05$ is considered significant *E2* carriers= *E2/E3*, *E4* carriers= *E3/E4* and *E4/E4*. Data was analysed by univariate general linear model (ANCOVA) adjusted for age and sex. ^{abc} significant differences ($P<0.05$) shown as different superscript letters. Sample sizes are as follows: WC, HC, WHR, WHtR BMI≤24.9; *APOE2* carriers n=33, *APOE3/E3* n=146, *APOE4* carriers n=48; BMI≥25.0; *APOE2* carriers n=13, *APOE3/E3* n=81, *APOE4* carriers n=33; BP BMI≤24.9; *APOE2* carriers n=33, *APOE3/E3* n=145, *APOE4* carriers n=48; BMI≥25.0; *APOE2* carriers n=13, *APOE3/E3* n=80, *APOE4* carriers n=33; NEFA BMI≤24.9 *APOE2* carriers n=33, *APOE3/E3* n=145, *APOE4* carriers n=48; BMI≥25.0 *APOE2* carriers n=12, *APOE3/E3* n=80, *APOE4* carriers n=32; CRP BMI≤24.9 *APOE2* carriers n=33, *APOE3/E3* n=147, *APOE4* carriers n=48; BMI≥25.0 *APOE2* carriers n=13, *APOE3/E3* n=80, *APOE4* carriers n=33; Adiponectin and uric acid BMI≤24.9 *APOE2* carriers n=30, *APOE3/E3* n=135, *APOE4* carriers n=45; BMI≥25.0 *APOE2* carriers n=12, *APOE3/E3* n=66, *APOE4* carriers n=30.

Abbreviations: A/G fat ratio android to gynoid fat ratio, BMI body mass index, CRP C-reactive protein, FMI fat mass index, HC hip circumference, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, LMI lean mass index, NEFA non-esterified fatty acids, TAG triacylglycerol, TC total cholesterol, VAT visceral adipose tissue, WC waist circumference, WHR waist to hip ratio, WHtR waist to height ratio.

TABLE 5.4 Participant habitual dietary intake according to *APOE* genotype in normal and overweight/obese BMI groups

	BMI <24.9 kg/m² (n=225)				BMI ≥ 25.0 kg/m² (n = 125)			
	E2 carriers (n=33)	*E3/E3* (n=143)	*E4* carriers (n =45)	P value	*E2* carriers (n=13)	*E3/E3* (n =79)	*E4* carriers (n=32)	P value
Energy intake (MJ)	7.73±0.39	8.31±0.19	8.94±0.33	0.06	8.83±0.73	9.04±0.29	8.43±0.46	0.54
Total fat (% TE)	38.7±1.5	35.9±0.7	36.1±1.3	0.24	34.8±2.4	36.9±1.0	37.4±1.6	0.67
SFA (%TE)	13.7±0.7	12.8±0.4	12.3±0.6	0.32	13.0±1.4	13.5±0.6	12.9±0.9	0.86
MUFA (% TE)	14.5±0.7	13.5±0.3	13.8±0.6	0.42	12.5±1.0	13.8±0.4	13.9±0.7	0.47
PUFA (%TE)	6.5±0.4	6.2±0.2	6.9±0.3	0.18	5.5±0.6	5.9±0.2	6.5±0.4	0.24
Trans fat (% TE)	0.62±0.05[b]	0.52±0.02[ab]	0.47±0.04[a]	**0.05**	0.55±0.10	0.57±0.04	0.61±0.06	0.77
Total CHO (% TE)	41.3±1.8[b]	47.4±0.9[a]	46.9±1.6[ab]	**0.01**	43.9±3.2	44.6±1.3	42.1±2.0	0.58
Total sugars (% TE)	17.7±1.1	19.1±0.5	19.7±1.0	0.34	16.1±2.0	17.7±0.8	18.2±1.3	0.68
Total fibre (AOAC, g)	22.6±1.6[b]	24.9±0.8[ab]	28.3±1.4[a]	**0.03**	22.2±2.4	23.4±1.0	24.4±1.5	0.71
Total protein (%TE)	20.9±0.9[b]	17.6±0.5[a]	17.5±0.8[a]	**0.01**	22.3±1.5	18.6±0.6	20.4±1.0	0.05

Data was presented as estimated marginal means ± SE, p<0.05 is considered significant *E2* carriers= *E2/E3*, *E4* carriers= *E3/E4* and *E4/E4*.
Data analysed by univariate general linear model (ANCOVA) adjusted for age and sex.
[abc] significant differences (P<0.05) shown as different superscript letters**.** Abbreviations: AOAC: Association of official analytical chemists, CHO carbohydrate, SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

Supplemental Table 5.1. Participant physical activity levels according to *APOE* genotype groups[1]

	All (n=301)	*E2* carriers (n=40)	*E3/E3* (n=186)	*E4* carriers (n=71)	P[2] value	Genotype x BMI[3]
Steps/ day	9672±216	9500±594	9850±275	9287±446	0.54	0.31
Energy expended (kcal/day)	308±13	320±35	313±16	296±26	0.82	0.57
Percentage time per day spent:						
Sedentary	69.7±0.4	71.3±1.1	69.5±0.5	69.4±0.8	0.32	0.67
Performing light PA	25.5±0.4	23.7±1.0	25.6±0.5	26.1±0.8	0.13	0.75
Performing moderate to vigorous PA	4.8±0.2	5.1±0.4	4.8±0.2	4.6±0.3	0.67	0.23

[1]Data was presented as estimated marginal means ± SE, *E2* carriers= *E2/E3*, *E4* carriers= *E3/E4* and *E4/E4*.
[2]Data was analysed by univariate general linear model (ANCOVA) adjusted for age and sex.
[3]APOE genotype x BMI interaction by ANCOVA, adjusted for age and sex

Supplemental Table 5.2. Participant physical activity measures according to *APOE* genotype in normal and overweight/obese BMI groups[1]

	BMI <24.9 kg/m² (n = 203)				BMI ≥ 25.0 kg/m² (n = 98)			
	E2 carriers (n=29)	*E3/E3* (n =127)	*E4* carriers (n = 43)	P value	*E2* carriers (n=11)	*E3/E3* (n =59)	*E4* carriers (n = 28)	P value[2]
Steps/ day	9527±724	10362±345	10001±595	0.56	9385±1007	8779±432	8142±629	0.53
Energy expended (kcal/day)	290±38	296±18	292±32	0.99	401±77	348±33	306±48	0.56
Percentage time per day spent:								
Sedentary	71.5±1.3	69.9±0.6	69.8±1.1	0.21	70.7±2.1	70.8±0.9	69.3±1.3	0.61
Performing light PA	23.4±1.2	25.9±0.6	25.5±1.0	0.17	24.3±1.8	25.1±0.8	26.8±1.1	0.38
Performing moderate to vigorous PA	5.06±0.52	5.14±0.25	4.92±0.43	0.91	4.96±0.83	4.14±0.36	3.97±0.52	0.60

[1]Data was presented as estimated marginal means ± SE, p<0.05 is considered significant *E2* carriers= *E2/E3*, *E4* carriers= *E3/E4* and *E4/E4*.
[2]Data analysed by univariate general linear model (ANCOVA) adjusted for age and sex.

Chapter 6. General discussion and future studies

6.1. General Discussion

Cardiovascular diseases (CVD) remain a leading cause of death globally (19), with dietary recommendations for disease prevention based on reducing saturated fatty acid (SFA) intakes to below 10%TE. The link between SFA and CVDs has been proposed by some to be via effects on low-density lipoprotein cholesterol (LDL-C) concentrations (184). As presented in the introduction (Chapter 1), studies have shown that reduction in dietary SFA intakes is associated with lower LDL-C concentrations. However, variability in the LDL-C lowering response to reductions in SFA intake are being increasingly recognised, suggesting that other factors play a role in the relationship between SFA with CVD risk. With the increasing prevalence of overweight and obesity in the population, body fat distribution, especially abdominal obesity, has been identified as an indicator of CVD risk and is also modified by dietary fat composition (168). Thus, body composition may be an important determinant of the inter-individual variability in LDL-C response to dietary fat intake. This chapter discusses the main findings of the studies included in this thesis and directions for future research.

The impact of SFA on lipid CVD risk markers was examined both in a cross-sectional study (Chapter 2) and a dietary intervention trial (Chapter 3). In the cross-sectional BODYCON study, we found some positive correlations between dietary SFA intakes with total and LDL-C concentrations, with dietary SFA found to be an independent predictor of LDL-C. However, a dose-dependent relationship was not evident between SFA intake and the fasting lipid profile after stratifying the group according to quartiles of SFA intake. In the UK cereal, dairy and meat products are the main contributors of SFA in the diet (32), and the source of dietary SFA has been shown to have an impact on the relationship with CVD risk (289). It should be noted that only total SFA intake was examined in this study, thus the source and amount of dietary

SFA might have had an influence on our findings. In the dietary intervention RISSCI-1 study, a dietary exchange model was used to replace dietary SFA with PUFA/MUFA without changing other components of the participant's diet. To achieve target levels, sweet and savoury snacks, cooking oils and spreads were provided and also the type and amount of dairy consumed were modified. In agreement with the current evidence, we found a significant 15% reduction (0.50 mmol/L) in LDL-C when dietary SFA was replaced with unsaturated fatty acids (PUFA and MUFA). It has been suggested that each 1 mmol/L reduction in LDL-C is associated with 22% relative risk of CVD (290). Therefore, our findings support the current dietary saturated fat recommendation for CVD prevention, however the mechanisms of action are not fully understood.

In order to identify the molecular mechanisms behind the positive effect of replacing dietary SFA with PUFA/MUFA, the expression of key genes involved in hepatic cholesterol regulation were explored in Chapter 4. Our results confirmed a previous studies showing that replacing dietary SFA with PUFA/MUFA increased the mRNA expression of LDL-R which potentially increase intracellular cholesterol levels, increasing NR1H3 (regulates genes involved in lipid metabolism) and ABCG1 (transporter protein involved in reverse cholesterol transport) mRNA expression (79). However, our results should be interpreted carefully as there was no correlation between the change in our target gene expression and changes in fasting blood lipids in men. This could be because the mRNA gene expression levels might not translate to the actual protein levels of the LDL-R on PBMC. In other words, it is possible that there is a time lag between the change in the production of the message (mRNA) and translation into the actual protein level. Another potential explanation could be the effect of non-modifiable risk markers proposed in cholesterol response to dietary fat intake such as *APOE* genotype. As presented in the introduction (Chapter 1) some studies have suggested that *APOE4* carriers have higher LDL-C concentrations and to have a greater responsiveness to dietary fat. In an in vitro

study, *APOE4* carriers were shown to have greater LDL-R binding affinity of triacylglycerol-rich lipoproteins after dietary SFA compared to *APOE3/E3* group (291). This means that greater competition of TAG-rich lipoproteins with LDL for uptake by the LDL-R would reduce uptake of LDL by liver cells and could potentially lead to higher LDL-C concentrations. Therefore, a RCT with measurement of PBMC gene expression in participants recruited according to *APOE* genotype may provide some insights into the mechanism underlying the relationship between changes in LDL-R expression and LDL-C concentrations. Moreover, increased dietary cholesterol absorption and decreased bile acid pool production has been reported in some studies after high dietary SFA intake (292, 293). In this thesis we did not examine intestinal cholesterol absorption or bile acid synthesis which may contribute to the mechanisms mediating the reduction in LDL-C concentrations after replacing dietary SFA with PUFA/MUFA. Therefore, these mechanism needs to be explored in future studies.

Obesity is another key complex disease affecting people worldwide which also contributing to CVD risk. It is defined as an excess body fat and measured widely by body mass index (BMI) at population level (159). However, as presented in the introduction (Chapter 1) the importance of body fat distribution has recently been recognised. Several studies have shown an association between abdominal obesity, especially abdominal visceral adipose tissue (VAT), and CVD risk while gynoid fat accumulation have been shown to be protective against this disease (161). Diets rich in SFAs have been shown to contribute towards abdominal fat accumulation whereas dietary PUFA/MUFA have been argued to have the opposite effect (9, 104). Therefore, it is also a question of interest that whether there is an association between dietary fat composition and body fat distribution and whether the positive effect of the reduction in dietary SFAs on LDL-C is associated with body fat distribution.

In the first instance, our question was addressed in the cross-sectional BODYCON (Chapter 2) study which used dual energy x ray absorptiometry (DXA) to accurately measure

body composition and estimate body fat distribution including abdominal VAT. The nature of observational study allowed us to examine some interesting associations. Although, dietary SFA intake was found to be the main predictor of LDL-C concentration and LDL-C was independently associated with abdominal VAT, there was no association between dietary SFA intake and abdominal VAT. The lack of association might be due to not exploring the type of SFA-rich foods consumed, observational design of the study or the effect of the other macronutrients consumed in the diet. In other words, only investigating the effect of total SFA intake may have influenced our results. Moreover, those individuals in Quartile 1 (consumed <10%TE SFA) had the higher intakes of carbohydrates which may have had an impact on these results. Contrary to our observations from the BODYCON study, in the RISSCI-1 study there was an association between the effect of dietary SFA on body composition and LDL-C. In particular, we observed an increase in android lean mass and a decrease in android fat percentage which was in agreement with previous RCTs which replaced dietary SFA with PUFA/MUFA (107). The discrepancy between our studies might be due to the difference in the study design, the inclusion of only men and the types of dietary SFA since this was not examined in our studies. It has been reported that source and type of dietary SFA consumed in the diet may play a different role in CVD risk (59). For example, while palmitic acid has been shown to increase blood cholesterol concentration, stearic acid did not show the same effect on lipid CVD risk markers (294). In addition, despite its SFA content, dairy have been shown to be negatively correlated with CVD risk (59). Therefore, only determining the relationship with total dietary SFA intake may have had an impact on our results. Moreover, interestingly, in the BODYCON study, those individuals which met the dietary recommendations for CVD risk reduction (Quartile 1) had the higher intakes of carbohydrates whereas in the RISSCI-1 study only the type of fat was substituted with no change in other dietary macronutrients. Thus, the beneficial effect of reducing dietary SFAs on body fat distribution may be only evident when

replaced with dietary n-6 PUFA/MUFA and the effect of other macronutrients and n-3 PUFA needs further examination.

To further examine the mechanisms behind the variability in LDL-C in response to reducing dietary SFA fat intake, responder and non-responders to the intervention were identified as top and bottom 20% of the group when stratified according to change in LDL-C concentration after the low SFA diet. In both responder (n=12) and non-responders (n=13) there was an increase in the mRNA LDL-R expression after replacing dietary SFA with PUFA/MUFA. However, this increase did not reach statistical significance, which might represent a power issue. Previous studies have suggested that age, BMI and PBMC LDL-R (as a surrogate marker of liver gene expression) expression could be factors behind this variability in LDL-C in response to dietary fat intake (259, 263). In our multivariate regression analysis, the change in LDL-R mRNA expression, waist and hip circumferences were found to explain the variation in LDL-C in the non-responder group. Therefore, it appears that body fat content and distribution may represent important determinants of the LDL-C response to dietary SFA intake.

Both CVDs and obesity risk have also been shown to be affected by non-modifiable risk factors such as genetic polymorphisms. In Chapter 5, the influence of the interaction between *APOE* and adiposity on CVD risk markers was explored. As presented in the introduction (Chapter 1), only a very small number of studies suggested that adiposity has an impact on the relationship between *APOE* and CVD risk. Our study adds to this growing evidence base suggesting that there is an impact of BMI on the relationship between *APOE* and CVD risk. Although the potential mechanism explaining this interaction between adiposity and *APOE* genotype on CVD risk markers is unclear, endoplasmic reticulum stress (one of the potential causes of cardiometabolic disease in obesity) have been reported as a potential mechanism linking *APOE* genotype and adipogenesis in mice (135). These findings are important at the

population level as there is a growing interest in studies investigating the effect of *APOE* on blood lipid response to diet, dietary recommendations may be personalized to specific groups in the future. However, our study shows that the role of adiposity on genotype-CVD risk relationship is another factor that needs to be understood before going further with the personalized nutrition. It should be noted that in our study retrospective genotyping generated small sample sizes for some genotypes which may have had an impact on our results.

6.2. Conclusion

In conclusion, the findings from this thesis add novel and interesting insight into the impact of dietary SFAs on CVD risk and body fat distribution in healthy adults. Our findings on the question of whether the positive effect of reducing dietary SFAs on LDL-C is related to body composition has generated inconsistent results, however this might be due to the differences in study designs (cross-sectional versus RCT) and study population (men and women versus middle-aged men). It is also possible that dietary SFA and body composition may impact on LDL-C concentration via different mechanisms. As one potential mechanism, an increase in LDL-R mRNA expression in response to replacing dietary SFA with PUFA/MUFA was observed, however, there was no association between the change in LDL-C and change in LDL-R mRNA expression. Moreover, our results showed that adiposity may mask the effect of *APOE* on CVD lipid risk markers and body composition. These findings add to growing evidence that improving dietary habits is an important strategy to reduce CVD and obesity. Currently UK adults consume 11.9%TE as SFA, which is higher than the current UK recommendations (32). Replacing 8%TE SFA (from 18%TE to 10%TE) with UFAs reduced LDL-C levels by 15% in our dietary intervention study which can reduce population CVD risk (290). However, in our cross-sectional study there was not a dose-dependent relationship between dietary SFA and LDL-C levels. It should be noted that those individuals in Quartile 1 (consumed <10%TE SFA) had the higher intakes of carbohydrates which may have had an impact on these results.

Therefore, beneficial effect of reducing dietary SFAs on CVD risk and body fat distribution may be dependent on the replacement nutrient. Moreover, source and type of dietary SFA consumed in the diet may play a different role in CVD risk. Therefore, when moving forward with public guidelines, it is important to consider the impact of the whole food on disease risk rather than total SFA intakes. Furthermore, public health campaigns could be effective in preventing these diseases, however with understanding the role of non-modifiable mechanisms behind the inter-individual response to diet such as genetic makeup, there is a need for individual behavioural change interventions. Moreover, testing our findings in other populations such as different age groups, people with CVDs or obesity and for a longer dietary intervention period may help to understand the effect of dietary SFA on adiposity and CVD.

6.3. Future studies

In the current thesis, only the relationship between total SFA intake and CVD risk and body composition was considered. However, findings from epidemiological studies have highlighted the importance of the food matrix, type and source of SFA (e.g., dairy/ red meat) may impact on the relationship between SFA and CVD risk markers (289). Dairy is one of the major sources of dietary SFA and have been shown to be inversely associated with CVD risk and body fat percentage (295, 296). In addition, a small number of studies have reported differential effects of individual SFAs (e.g., palmitic acid and stearic acid) on CVD risk markers (59). Although the current dietary recommendations are to reduce total dietary SFA below 10%TE, it is important to understand if the effect of dietary SFA on CVD or obesity risk depends on the type of SFA-rich foods to inform public health recommendations for healthy dietary patterns. Therefore, this is an area of research that needs further investigation. Moreover, in Chapter 5 although we examined the molecular mechanisms behind the positive effect of replacing dietary SFA with PUFA/MUFA, studies are needed which also measure the protein levels of the LDL-

R, NR1H3 and ABCG1 in PBMC by Western blotting to provide some further insights into the effects of dietary fat composition on hepatic cholesterol regulation.

Obesity is a multifactorial disease and the mechanisms behind linking the abdominal obesity and CVD is not totally understood. In both cross-sectional and dietary intervention design studies we reported that there is an association between abdominal obesity and CVD risk markers. Although our observational and dietary intervention studies lend support to this link, we have not explored the potential mechanisms. As discussed in the introduction (Chapter 1), there is growing evidence on the effect of inflammation associated with abdominal obesity on CVD risk markers. It has been argued that gut microbiota is, partly, responsible for pathogenesis of obesity via leading to systemic inflammation (297). Moreover, in our dietary intervention study we found that the effect of dietary SFA on LDL-C might be mediated by changes in body fat distribution Therefore, our findings highlight the importance of understanding the factors affecting body composition not only to prevent obesity but also for CVD prevention. Although obesity could be related to lifestyle and genetic prepositions, growing evidence suggests that gut microbiota has an important role in the development of obesity. Studies provide some evidence on the link between gut microbiota and abdominal obesity; however, this relationship is still not totally understood. High SFA diets have been shown to have unfavourable effect on the gut microbiota which is associated with an unhealthy cardiometabolic state (298). In particular, changes in the proportion of *Firmicutes* and *Bacteroidetes* were reported to be positively associated with obesity in humans (299, 300) and increased ratio of *Firmicutes/Bacteroidetes* after a high-fat diet were observed in mice (298). Further examination of the role of the gut microbiota in response to dietary fat intake would be of interest. The effect of dietary SFA on LDL-C may be, partly, via changes in gut microbiota, however without mechanistic studies, drawing a conclusion is not possible. Therefore, considering the findings from Chapter 2 and Chapter 3 presented in this thesis, further research

which investigates the role of gut microbiota on the relationship between dietary SFA, abdominal obesity and dyslipidaemia is warranted.

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Appendices

Appendix I: Impact of a food-based dietary fat exchange model for replacing dietary saturated with unsaturated fatty acids in healthy men on plasma phospholipids fatty acid profiles and dietary patterns.

Impact of a food-based dietary fat exchange model for replacing dietary saturated with unsaturated fatty acids in healthy men on plasma phospholipids fatty acid profiles and dietary patterns.

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Abstract

Purpose: UK guidelines recommend dietary saturated fatty acids (SFAs) should not exceed 10% total energy (%TE) for cardiovascular disease prevention, with benefits when SFA are replaced with unsaturated fatty acids (UFAs). This study aimed to assess the efficacy of a dietary exchange model using commercially available foods to replace SFAs with UFAs.

Methods: Healthy men (n=109, age 48, SD 11y) recruited to the Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1 (RISSCI-1) study followed two sequential 4-week isoenergetic moderate-fat (34%TE) diets: high-SFA (18%TE SFAs) and low-SFA (10%TE SFAs, 24%TE UFAs). Dietary intakes from 4-day weighed diet diaries were assessed by a single researcher in each study centre. Nutrient intakes were analysed using paired *t*-tests, while fasting plasma phospholipid fatty acid (PL-FA) profiles and dietary patterns were analysed using orthogonal partial least square discriminant analyses.

Results: Participants exchanged 10.2%TE (SD 4.1) SFAs for 9.7%TE (SD 3.9) UFAs between the high and low-SFA diets, with no effect on other nutrient or energy intakes. Analyses of dietary patterns confirmed successful incorporation of recommended foods from commercially available sources (e.g.

dairy products, snacks, and added oils and fats), without affecting participants' overall dietary habits. Analyses of plasma PL-FAs indicated good compliance to intervention foods of varying SFA content.

Conclusions: RISSCI-1 dietary exchange model successfully replaced dietary SFAs with UFAs in free-living healthy men using commercially available foods, and without altering their dietary patterns. Further intervention studies are required to confirm utility and feasibility of such food-based dietary fat replacement models on a population level.

Trial registration: ClinicalTrials.Gov - NCT03270527

Keywords (4-6): dietary fat composition, food-exchange model, dietary compliance, dairy biomarkers, dietary fat replacement

Declarations

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Conflicts of Interest/Competing interests

JAL is a member of the UK Government's Scientific Advisory committee on Nutrition (SACN). The other authors have no conflicts of interest or competing interests to declare.

Availability of data and material

N/A

Code availability

N/A

Authors' contribution

BAG, JAL, KGJ, BF and MDR obtained the funding and designed the study. AK, BAG, JAL, KGJ, MW, and RA developed the methodology for data collection. LS, RA, AK, EO, GW, and HA collected the data. LS performed statistical analyses. LS prepared the original draft of the manuscript and revised each version of the manuscript, under the supervision of JAL and KGJ. LS, RA, AK, EO, GW, HA, MW, BF, MDR, KGJ, BAG, and JAL contributed to the interpretation of the data, read, and approved the final manuscript.

Ethics Approval

The RISSCI-1 study was conducted according to the 2008 Declaration of Helsinki on 'Ethical principles for medical research involving human subjects' and was given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (17/29) and University of Surrey Ethics Committee (UEC/2017/41/FHMS).

Consent to Participate

Written informed consent was collected from all participants before inclusion in the study.

Consent for publication

N/A

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Abbreviations

%TE, % total energy; AOAC, Association of Analytical Chemists; BMI, body mass index; CVD, cardiovascular disease; FA, fatty acid; FAME, fatty acid methyl ester; FID, flame ionization detector; GC, gas chromatograph; LDL-C, low-density lipoprotein cholesterol; MUFAs, monounsaturated fatty acids; NDNS, National Diet and Nutrition Survey; PL, phospholipid; PUFAs, polyunsaturated fatty acids; RCT, randomised controlled trial; SACN, Scientific Advisory Committee on Nutrition; SD, standard deviation; SFAs, saturated fatty acids; TFAs, *trans* fatty acids; UFAs, unsaturated fatty acids; wt%, weight %.

Introduction

Cardiovascular diseases (CVD) cause a quarter of all deaths in the UK and represent a major burden on public health worldwide [1]. While the aetiology of CVD is multifactorial, elevated circulating low-density lipoprotein cholesterol (LDL-C) has been established as a causal risk factor for the development of atherosclerosis [2]. Evidence from epidemiological prospective cohorts, strictly controlled metabolic ward studies, and randomised controlled trials supports consistent associations between a high consumption of dietary saturated fatty acids (SFAs) and elevated serum LDL-C [3–6]. This evidence has formed the basis of public health guidelines in the UK which since 1983, have recommended dietary SFAs should not exceed 10% of total energy (%TE) intake in adults [7, 8].

To study the impact of reducing dietary SFAs on health, many previous dietary interventions replaced SFAs with mono- (MUFAs) or polyunsaturated fatty acids (PUFAs) [9]. However, these studies often used dietary fats manufactured specifically for the purpose of the intervention, which limited the translation and applicability of the findings to non-experimental, free-living people settings [10–13]. This limitation raises the importance of developing interventions based on commercially available whole-foods to improve the practicability of reducing dietary SFAs and adherence to dietary guidelines, while minimising the impact on other dietary components. In particular, since about a third of dietary SFAs is consumed from dairy foods and fat spreads in UK adults aged 19-64 y [14], the replacement of full-fat dairy and butter for lower fat or vegetable-based alternatives has been proposed as a food-based strategy to help reduce dietary SFAs in this group [15].

In parallel with developing food-based interventions, the assessment of dietary compliance beyond traditional approaches using diet diaries, or food-frequency questionnaires linked with food composition databases, would increase understanding of the impact and feasibility of dietary intervention studies in free-living individuals. Plasma phospholipid fatty acids (PL-FAs) correlate with the intake of dietary medium-term fatty acid (FA), and PL odd-chain SFAs (e.g. pentadecanoic or heptadecanoic acids) [16, 17], as such, have been used as biomarkers of dairy fat consumption [18]. The use of plasma PL-FA as an objective tool to assess dietary compliance may thus be particularly effective in the context of interventions that manipulate dietary fat using full-fat dairy foods. Furthermore, the analysis of dietary patterns can identify residual confounding from changes in dietary habits, which are not routinely assessed in dietary intervention studies.

The Reading, Imperial, Surrey, Saturated fat Cholesterol Intervention-1 ('RISSCI'-1) study was based on a tailored, dietary fat-exchange model, matched to the average diet of UK adult men. This aimed to replace dietary SFAs with unsaturated fatty acids using common, commercially available foods, while minimising impacts on dietary habits, and improving dietary compliance and reproducibility. The present study assessed the efficacy of a food-based dietary fat exchange model, that replaced dietary SFAs with MUFAs and PUFAs in free-living UK men, with endpoint measures of nutrient intake, overall dietary patterns, and plasma PL-FAs.

Methods

Study design

The RISSCI-1 study was a single-blind sequential dietary intervention study (ClinicalTrials.Gov registration No. NCT03270527). The study was given a favourable ethical opinion for conduct by the University of Reading Research Health Ethics Committee (17/29) and the University of Surrey Ethics Committee (UEC/2017/41/FHMS) and was conducted in accordance with the Declaration of Helsinki guidelines. Written informed consent was collected from all participants before inclusion in the study.

Participants

The RISSCI-1 study included healthy men aged 30 to 65 y, which were recruited from the Reading, Berkshire and Guildford, Surrey areas between 2017 and 2019. Eligible participants were required to meet the following inclusion criteria: body mass index (BMI) between 19-32 kg/m^2; fasting serum total cholesterol < 7.5 mmol/L and triacylglycerol < 2.3 mmol/L; blood pressure < 140/90 mmHg; fasting glucose < 7.0 mmol/L; haemoglobin > 130 g/L; no history of myocardial infarction, stroke, diabetes, or any other endocrine disorder in the past 12 months; no history of kidney, liver, or gastrointestinal disorder, or history of cancer; not taking any medication for hyperlipidaemia, hypertension, inflammation, or prescribed antibiotics in the last three months; not smoking; drinking ≤ 14 units of alcohol per week; participating in vigorous exercise ≤ 3 times per week; not participating or planning to participate in a weight-loss diet; not taking any dietary supplements known to influence circulating lipids or gut microbiota (e.g. plant stanols, fish oil, phytochemicals, natural laxatives, probiotics and prebiotics); not being involved in another dietary intervention study and willing to regularly consume study intervention products (butter/spreads, oils, dairy foods, snacks). Upon inclusion, participants were advised to maintain their usual physical activity levels, and to inform the researchers of any important changes to their health or medication use.

Dietary intervention and food exchange model

The replacement of dietary SFAs with MUFAs/PUFAs was based on a food exchange model which was successfully implemented in previous intervention studies at the University of Reading [11–13]. The food exchange model aimed to identify dietary sources of exchangeable fat that would not impact total energy or other macronutrient intakes. Estimated amounts of dietary exchangeable fat from oil, butter and fat spreads, dairy foods, and snacks were calculated using data from the National Diet and Nutrition Survey (NDNS) (y 1 to 4) in UK adult men aged 19-64 y [19], and the Dietary Intervention and Vascular function (DIVAS) randomised controlled trial (RCT) [12], *Table 1*. These estimates were then converted into servings of common commercially available cooking oils and fat spreads, dairy foods, and sweet and savoury snacks that participants were required to consume daily to achieve the nutrient targets in each dietary intervention period (*Table 2*).

To achieve the exchange of dietary fat, the RISSCI-1 sequential dietary intervention consisted of two, 4-week, isoenergetic, moderate-fat diets (34% TE from fat). The first intervention period was a high-SFA diet (target %TE SFA:MUFA:PUFA = 18:12:4), and the second intervention period was a low-SFA, high-MUFA/PUFA diet (target %TE SFA:MUFA:PUFA = 10:14:10), which otherwise matched macronutrient intakes from the first intervention period. All participants received the high-SFA diet for the first 4-week period, followed by the low-SFA, high-MUFA/PUFA diet for the second 4-week period without a washout period.

Implementation of intervention diets

Participants were invited to attend three study visits: at baseline upon inclusion (week 0), after completing the high-SFA diet (week 4), and low-SFA diet (week 8). At the first two study visits, participants were provided with a detailed information booklet containing instructions on how to comply with the high-SFA or low-SFA dietary guidelines, along with tailored recommendations to suit their lifestyle (e.g. meals out of the home, cooking for the family meal ideas and recipes.). Participants also received free-of-charge study food items to incorporate into their baseline diets. Supplied food items included commercially available fat spreads, cooking oils, and an assortment of sweet and savoury snacks in sufficient quantity for each 4-week dietary intervention period. Due to their shorter shelf-life, dairy foods such as milk and cheese were not supplied, and participants were instructed to purchase these foods.

To ensure compliance to dietary guidelines, each dietary intervention period was scheduled outside of major holiday periods (e.g. Christmas and Easter), and participants were required to avoid any extended periods away from their home. Participants were also asked to return any leftover study

items from the high-SFA diet before starting the low-SFA dietary intervention period. To help incorporate the study foods into their usual diet, and to assess compliance, participants were provided with daily tick sheets to be completed throughout each intervention period. Participants were also permitted to consume more than the minimum required daily servings of any study food items, if they were maintaining a stable body weight (± 1 kg from week 0). The importance of the latter was emphasised to the participants at follow-up visits at the mid-point of each dietary intervention (weeks 2 and 6). During these short visits, daily tick sheets were reviewed, and participants were supplied with any additional study food items required to complete the remainder of the intervention period. If body weight varied by greater than 1 kg from baseline or the previous study visit, participants were advised to reduce or increase their consumption of the provided snacks or other food items as appropriate.

Collection of dietary data

Participants were instructed to complete a 4-day weighed diet diary, a week before each study visit, to assess their baseline, habitual dietary intake (week -1), and during each dietary period to assess compliance to the interventions (weeks 3-4 and 7-8). Each diet diary included 3 weekdays and 1 weekend day during which participants were provided with digital scales to record the amount and description of all food items and beverages consumed. To improve the accuracy of the diet diaries, participants received additional diary templates to record all individual ingredients used in homemade recipes, along with published food portion tables to record foods consumed outside of the home [20]. Researchers assessed the completion and accuracy of the diet diary during each study visit, and requested any additional information necessary to improve data entry precision.

Paper diet diaries were analysed using Nutritics Research Edition v5.64 (Dublin, 2019) to assess foods consumed and nutrient intakes. Every item consumed was matched to its closest equivalent in the McCance and Widdowson's Composition of Foods Integrated Dataset (CoFID)[21], which was used to calculate daily dietary consumptions of total energy, and selected macro- and micro-nutrients: protein, carbohydrate, free sugars, Association of Analytical Chemists (AOAC) fibre, alcohol, total fat, SFAs, MUFAs, PUFAs, n-3 PUFAs, n-6 PUFAs, *trans* fatty acids (TFAs), cholesterol, and sodium. In addition, researchers used the NDNS Rolling Programme nutrient databank to impute missing values of n-3/n-6 PUFAs in food items contributing to at least 1 g of PUFAs in each diet diary [19]. Food items consumed (in g/d) were classified into 40 food categories (*supplementary table 1*), which were used to assess dietary patterns. In addition, the consumption of total dairy foods was further categorised into milk, cheese, cream, yogurt, dairy desserts, and butter.

Assessment of underestimation of energy consumption

Underestimation of dietary TE at baseline and during each dietary intervention periods was checked by the method proposed by Black [22]. Researchers estimated the basal metabolic rate of each participant using the Henry equations for men, based on age and body weight [23]. On the basis of a sedentary lifestyle (physical activity level score of 1.2 [12]), the lower 95% confidence limit of the Goldberg cut-off was estimated to lie between 1.13 and 1.16 to identify under-reporters of dietary TE.

Biochemistry analyses

Blood was collected into EDTA vacutainers after an overnight fast (12 hours) at baseline (week 0) and at the end of each dietary intervention period (weeks 4 and 8). After collection, vacutainers were chilled on ice for 20 min before centrifugation at 1750 g (3000 rpm) for 15 min at 4°C for the collection of plasma, which was stored at -80°C before subsequent analysis.

The extraction of fatty acids methyl esters from plasma PL was performed by researchers from the German Institute of Human Nutrition (Potsdam-Rehbruecke, Germany) using a 3-step protocol (i.e. lipid extraction, solid phase extraction and transmethylation) based on methods from Metges *et al.* [24], Kaluzny *et al*. [25], and Baylin *et al.* [26]. Briefly, plasma lipids were extracted using a tert-butyl methyl ether (MTBE)/methanol solution and PL were eluted in methanol using solid phase extraction

on aminopropyl-silica columns (Chromabond, MachereyNagel GmbH & Co. KG, Düren, Germany). Dried PL were then suspended in 200 µL of toluene and 15 µL of trimethyl sulfonium hydroxide solution (TMSH, 0.2 mol/L in methanol, Macherey-Nagel, 701 520.101) to obtain fatty acid methyl esters (FAME). FAMEs were separated using a gas chromatograph (GC) (Agilent 7890A, Agilent Technologies, Waldbronn, Germany) and flame ionization detector (FID) equipped with a 100m capillary column (HP-88, 100 m x 0.25 mm I.D., 0.2 µm film thickness, Agilent). Finally, FAMEs were identified against a standard mixture of 37 FAMEs (Supelco^{TM}) containing FAMEs of chain-length between C4-C24. In subsequent analyses, fatty acid concentrations were calculated as weight percentage of total fatty acids detected (wt%). Inter-assay coefficients of variation (n = 10) were all below 6.4% (range 0.5% to 6.4%).

Measurement of anthropometrics and physical activity levels

The evening before each study visit (weeks 0, 4, and 8), participants were asked to consume a supplied, low-fat meal (< 1.46 MJ and < 7 g total fat content) with low-nitrate water (Buxton Mineral Water, Nestlé Waters, Buxton, UK) and to fast overnight for at least 12 hours consuming only the low-nitrate water provided. On the morning of the study visit, researchers recorded height (to the nearest 0.1 cm), body weight (to the nearest 0.1 kg), and calculated the BMI of each participant using a wall-mounted stadiometer and a Tanita BC-418 (Reading) or Tanita BC420MA (Surrey) digital scale (Tanita Europe), respectively. An allowance of 1 kg was included for light clothing when assessing body weight, and the digital scale was operated under the "standard body type" setting. Physical activity habits were assessed through the participants' completion of the International long version of the Physical Activity Questionnaire (IPAQ), and physical activity levels were classified into three categories (i.e. "Low", "Moderate", and "High") using the IPAQ guidelines for categorisation [27].

Power calculations and statistical analyses

A required sample size of 92 participants was estimated for the detection of a 0.16 mmol/L (SD 0.54) decrease in fasting LDL-C concentrations (primary outcome) between the high- and low-SFA diets, as observed in the DIVAS parallel RCT [28], with an 80% statistical power and a 5% significance level. After accounting for a 15% dropout rate, this increased to a total of 106 participants. A sample size of 106 participants was also adequate for the investigation of PL-FA responses to the interventions. In this study, the successful replacement of dietary SFAs with MUFAs/PUFAs was expected to decrease the abundance of total SFAs in plasma PL-FAs by an estimated 0.46 % of area of total PL-FAs (SD 0.8) [12], leading to a required sample size of 30 participants (i.e. n = 26 participants for a detection with an 80% statistical power and a 5% significance levels, and n = 4 participants to allow for a 15% dropout).

Since the RISSCI-1 dietary intervention was isoenergetic, the stability of BMI throughout the intervention was assessed using a linear mixed model which included age (continuous, y), study visit (week 0, week 4, or week 8), and study centre (University of Reading, University of Surrey) as fixed effects, and participants as a random effect. Daily average nutrient intakes from 4-day diet diaries and plasma PL-FA concentrations were compared between the high-SFA diet (week 4) and the low-SFA diet (week 8) using paired *t*-tests. All variables were checked for normality and log-transformed if necessary. In the case of alcohol consumption, *t*-tests were performed on alcohol consumers only and non-consumers were excluded from statistical analyses.

Furthermore, food categories and plasma PL-FA concentrations during the high-SFA and low-SFA diet were analysed using orthogonal partial least square discriminant analyses (OPLS-DA) to identify dietary patterns and circulating FA profiles in response to the RISSCI-1 dietary intervention [29, 30]. All variables were mean-centred and divided by their standard deviation (SD). Statistical significance of the OPLS-DA models was tested using internal cross-validation permutation tests (n=1000 permutations), and goodness of fit and predictive accuracy were assessed using the R^2Y and Q^2 values,

respectively. For the interpretation of the models, variable loadings scaled as correlations towards the predictive model (p(corr)) were used to identify the variables that contributed the most to the discrimination of dietary patterns or plasma PL-FA profiles between the high-SFA and the low-SFA diets.

In further analyses, a constraint-based feature selection algorithm was used to identify plasma PL-FAs associated with dairy fat consumption [31]. This method is based on a forward-backward feature selection approach and aims to reduce the dimension of a given dataset by providing multiple statistically equivalent subsets of features with maximised predictive accuracy. In prospective analyses, plasma PL-FA concentrations were calculated as changes between the high-SFA diet (week 4), which was enriched in full-fat dairy foods, and baseline (week 0). In addition, cross-sectional analyses aimed to identify predictors of baseline dairy fat consumption among baseline concentrations of plasma PL-FAs. In both approaches, selected predictors among plasma PL-FAs were fitted in multiple linear regression models with adjustments for age (y), BMI (kg/m^2), baseline dairy fat consumption (g/d, in prospective models only), and energy intakes at baseline (kcal/d). Predictive R^2 coefficients were used to assess the predictive accuracy of multiple linear regression models. Finally, we conducted exploratory analyses to assess the cross-sectional Pearson correlations between the selected predictors among plasma PL-FAs and the baseline (week 0) consumptions of dairy fat, total dairy foods, and specific dairy food groups (i.e. milk, butter, cheese, yogurts, and cream).

All statistical analyses were conducted in R (version 4.0.4), except from OPLS-DA models which were fitted in MetaboAnalyst version 5.0 [32].

Results

The flowchart of participants included in the RISSCI-1 study is presented in *Figure 1*. A total of n=118 participants were enrolled to follow the first dietary intervention period (i.e. high-SFA diet), including n=9 who withdrew from the study at the end of the first diet (n=6 due to time or work commitments, n=2 due to loss of interest in the study, n=1 due to newly prescribed medication). The remaining n=109 participants completed both the first (high-SFA) and second dietary intervention period (low-SFA diet), giving an overall drop-out rate of 7.6%. Baseline characteristics of participants are presented in *Table 3*. Participants mean age was 48 (SD 11) y, with a BMI of 25.1 (SD 3.3) kg/m^2.

Participants were of Asian or UK Asian (7.3%), Black or UK Black (2.8%), Chinese (1.8%), Mixed Ethnic (1.8%), or White (86.2%) self-reported ethnic backgrounds. Finally, most participants had moderate or high, self-reported physical activity levels (31.2% and 47.7%, respectively).

Dietary consumption

Nutrient intakes during each dietary intervention period are shown in *Table 4*. Out of the n=109 participants who completed the RISSCI-1 study, nine were excluded from the dietary analyses due to insufficient or incomplete dietary data. There were no significant differences between the dietary energy, macronutrients (total fat, carbohydrates, and proteins), Association of Analytical Chemists (AOAC) dietary fibre or alcohol consumption during the high-SFA and low-SFA diets. Data on average daily nutrient consumption indicated a successful exchange of dietary SFAs for MUFAs and PUFAs during the second dietary intervention period, with dietary SFA consumption decreasing from 19.1 %TE (SD 3.5) during the high-SFA diet to 8.9 %TE (SD 2.1) during the low-SFA diet (p-value <0.001). The observed decrease in SFA intake was compensated for by a rise in MUFA and PUFA consumptions from 11.1 %TE (SD 2.8) and 3.7 %TE (SD 1.3), respectively during the high-SFA diet to 13.4 %TE (SD 2.9), and 11.1 %TE (SD 3.6) during the low-SFA diet (both p-values <0.001). In addition, participants consumed

less TFAs (p-value <0.001), dietary cholesterol (p-value <0.001), and sodium (p-value = 0.04) during the low-SFA diet compared to the high-SFA diet.

Energy balance

There was no statistically significant impact of the dietary interventions on participants' BMI (p-value = 0.7 for the high-SFA diet, and 0.1 for the low-SFA diet, compared to baseline). Estimated marginal means for BMI at baseline, following the high-SFA diet, and following the low-SFA diet were 25.1 kg/m^2 (95%CI 24.4-25.7), 25.1 kg/m^2 (95%CI 24.4-25.7), and 25.0 kg/m^2 (95%CI 24.4-25.7), respectively. The proportions of under-reporters of energy intake at baseline, following the high-SFA diet and following the low-SFA diet were estimated at 28%, 17%, and 27%, respectively, based on the assumption that participants remained in energy balance throughout the study.

Analysis of plasma PL-FAs

Relative concentrations of plasma PL-FAs after each 4-week dietary intervention period are shown in *Table 5*. All plasma PL-FA concentrations were significantly different between the high-SFA and low-SFA diets apart from those of elaidic acid (18:1 n-9 *trans*, p-value=0.37), γ-linolenic acid (18:3 n-6, p-value=0.26), and α-linolenic acid (18:3 n-3, p-value=0.53). Overall, plasma samples after the high-SFA diet had higher abundances of 16 plasma PL-FAs which included palmitic acid (16:0, difference between high-SFA and low-SFA diet (Δ) =1.23 wt%, p-value $<10^{-4}$), total SFAs (Δ=0.84 wt%, p-value $<10^{-4}$), n-3 PUFAs (Δ=0.52% total FA, p-value $<10^{-4}$), dihomo-γ-linolenic acid (20:3 n-6, Δ=0.41 wt%, p-value $<10^{-4}$), and total MUFAs (Δ=0.31 wt%, p-value $<10^{-2}$). In contrast, plasma samples collected after the low-SFA diet were characterised by higher abundances of 10 plasma PL-FAs, which included linoleic acid (18:2 n-6, Δ= -1.87 wt%, p-value $<10^{-4}$), n-6 PUFAs (Δ= -1.69 wt%, p-value $<10^{-4}$), total PUFAs (Δ= -1.15 wt%, p-value $<10^{-4}$), stearic acid (18:0, Δ= -0.53 wt%, p-value $<10^{-4}$), and arachidonic acid (20:4 n-6, Δ= -0.31 wt%, p-value $<10^{-2}$).

In OPLS-DA of the plasma PL-FA abundances during the high-SFA and low-SFA diets, the first component of the model, which explained 13.6% of the total variation, was retained for interpretation (*Figure 2A*). The OPLS-DA, which aimed to discriminate plasma PL-FA profiles specific to each dietary intervention period, revealed moderate fitness (R^2Y=0.66, empirical permutation p-value < 0.01 (0/1000)) and predictive accuracy (Q^2=0.57, empirical permutation p-value < 0.01 (0/1000)). As shown in *Figure 2B*, discriminating plasma PL-FAs during the high-SFA diet included pentadecanoic acid (15:0, p(corr)=0.72), *trans* vaccenic acid (18:1 n-7 *trans*, p(corr)=0.69), palmitic acid (16:0, p(corr)=0.58), myristic acid (14:0, p(corr)=0.46), and n-6 docosapentaenoic acid (22:5 n-6, p(corr)=0.38). In contrast, the low-SFA plasma PL-FA profile showed higher abundances of eicosenoic acid (20:1 n-9, p(corr)= -0.63), arachidic acid (20:0, p(corr)= -0.60), behenic acid (22:0, p(corr)= -0.48), linoleic acid (18:2 n-6, p(corr)= -0.41), and stearic acid (18:0, p(corr)= -0.36).

Analysis of dietary patterns

For the recorded consumption of 40 food categories during the high-SFA and low-SFA diets, the first component of the model (OPLS-DA) was retained for the discrimination of dietary patterns during the two diets and explained 7.5% of the overall variation (*Figure 3A*). The retained model showed adequate fitness (R^2Y=0.82, empirical permutation p-value < 0.01 (0/1000)) and predictive accuracy (Q^2=0.68, empirical permutation p-value < 0.01 (0/1000)). As shown in *Figure 3B*, the high-SFA dietary pattern was characterised by higher intakes of SFA-rich fat (correlation scaled loading p(corr)=0.89), full-fat dairy foods (p(corr)=0.57), and biscuits and cakes (p(corr)=0.27). In contrast, the low-SFA dietary pattern was characterised by higher consumptions of MUFA-rich fat (p(corr)= -0.80), PUFA-rich fat (p(corr)= -0.71), nuts (p(corr)= -0.63), savoury snacks (p(corr)= -0.31), and low-fat dairy (p(corr)= -0.23).

Other food categories, such as cereals and grains, meats, fish, or fruits and vegetables, did not contribute significantly to the dietary pattern discrimination between the low-SFA and high-SFA diets.

Associations between dairy consumption and plasma PL-FAs

In accordance with the dietary fat exchange model developed for the RISSCI-1 study (*Table 1*), dietary intakes from the 4-day weighed diet diaries showed that total dairy foods were important contributors of total fat (39.6%, SD 11.5) and SFA consumption (50.1%, SD 12.6) during the high-SFA diet compared to baseline (16.6% SD 11.4 for total fat, and 28.5% SD 17.5 for SFA) (*supplementary table 2*).

Prospective associations

Prospective constraint-based feature selection analyses identified two independent predictors of changes in dairy fat consumption among plasma PL-FAs after the high-SFA diet compared to baseline: pentadecanoic acid (15:0) and *trans* vaccenic acid (18:1 n-7 *trans*). In prospective multiple linear regression models between the end of the high-SFA diet and baseline (n=104 participants), each 1% total FA increment of circulating pentadecanoic acid was associated with an additional 158g of consumed dairy fat (95% CI 81-235, p-value $<10^{-3}$). In a separate linear regression model, a 1% total FA increment of circulating *trans* vaccenic acid was associated with an 84g increase in dairy fat consumption (95%CI 26-142, p-value=0.005). In addition, the linear regression model based on pentadecanoic acid abundance had a slightly better predictive accuracy (predictive R^2=0.27) than the model based on *trans* vaccenic acid (predictive R^2=0.21).

Cross-sectional associations

In cross-sectional analyses of baseline data (n=106), pentadecanoic acid and *trans* vaccenic acid were also identified as two independent predictors of dairy fat consumption. However, linear regression models for both pentadecanoic acid (β=92, 95%CI 42-142, p-value $<10^{-3}$) and *trans* vaccenic acid (β=100, 95%CI 50-150, p-value $<10^{-3}$) showed weaker prediction accuracy, compared to prospective models (predictive R^2=0.10 for pentadecanoic acid, and 0.12 for *trans* vaccenic acid). In further exploratory analyses, baseline abundances of pentadecanoic acid in plasma PL were moderately correlated with the consumptions of total dairy (Pearson correlation r=0.34, p-value $<10^{-3}$), milk (r=0.31, p-value=0.001), and butter (r=0.25, p-value=0.01), but not with other dairy food groups (i.e. dairy desserts, cheese, cream, and yogurts, *supplementary table 3*). While circulating *trans* vaccenic acid was also correlated with consumptions of total dairy (r=0.33, p-value $<10^{-3}$) and milk (r=0.26, p-value=0.007), it was also weakly related to intakes of dairy desserts (r=0.19, p-value=0.048) and cream (r=0.19, p-value=0.049), but not with butter, cheese, or yogurts (*supplementary table 3*).

Discussion

The analyses of 4-day weighed diet diaries and plasma PL-FA profiles confirmed that the participants reached the nutritional targets set in our model, by reducing their consumption of dietary SFAs by 10.2%TE from the high-SFA diet to the low-SFA diet. This decrease in SFAs was compensated by an increase in dietary MUFAs and PUFAs by 2.3%TE and 7.4%TE, respectively, while maintaining other macronutrient intakes. The exchange of dietary SFAs for UFAs was achieved without affecting total energy intake or BMI, which confirmed that participants remained in energy balance throughout the study. In addition, discriminant analyses of dietary patterns constituted a novel method of confirming compliance to the RISSCI-1 dietary guidelines, by showing that participants integrated the recommended and supplied study foods into their diets to exchange dietary SFAs for UFAs, without modifying their overall dietary patterns (e.g. via changes in intakes of meat, fish, cereals and grains, fruits, and vegetables).

The analysis of plasma PL-FAs during the two dietary intervention periods provides further evidence in support of the successful implementation of the RISSCI-1 dietary fat exchange, by revealing a 0.84 wt% decrease in total SFAs, 0.31 wt% decrease in total MUFAs, and 1.15 wt% increase in total PUFAs during the low-SFA compared to the high-SFA diet. The rise in plasma PL PUFAs during the low-SFA diet was driven by n-6 PUFAs (1.70 wt% increase), whereas circulating n-3 PUFAs decreased by 0.53 wt%. These results reflect the type of dietary fat consumed during the two diets, albeit on a much smaller scale. Indeed, even-chain SFAs and UFAs are subject to endogenous synthesis and oxidation in humans, which limits their reliability and utility as biomarkers of fat consumption [33]. For instance, total circulating palmitic acid have been reported to be associated with dietary intakes of carbohydrates and alcohol [34, 35], although in the RISSCI-1 study, intakes of these macronutrients were not significantly different between the diets.

Furthermore, dietary analyses revealed small but significantly higher intakes of dietary TFAs and cholesterol during the high- compared to the low-SFA diet (decreases in 0.6%TE and 72mg during the low-SFA diet, respectively). Since the abundance of elaidic acid (a *trans* FA mostly found in industrially processed food) in plasma PL did not differ between the high- and low-SFA diets, these differences may be explained by the guidelines to consume full-fat dairy foods and butter during the high-SFA diet, which contain naturally occurring ruminant *trans* FAs and cholesterol [36, 37]. However, participants remained well below the dietary reference value for TFAs of 2%TE [7], and small variations in dietary cholesterol (i.e. equivalent to less than that from a single egg yolk [21]) are unlikely to impact on plasma LDL-C. Moreover, current epidemiological evidence suggests that TFAs from dairy may not be associated with deleterious cardiometabolic outcomes as opposed to industrial TFAs [38, 39]. Similarly, higher sodium intakes were observed during the high-SFA diet compared to the low-SFA diet. This may reflect the dietary guidelines for this diet, which recommended daily servings of salted butter and cheese with higher salt content (e.g. Cheddar and Red Leicester) than those recommended during the low-SFA diet (e.g. cottage cheese and spreadable cream cheese). On average, study participants exceeded UK dietary recommendations for sodium of 2.4 g/d (6 g/d salt) at baseline and throughout the RISSCI-1 dietary intervention, but remained below the national average for men aged 19-64y which was estimated at 3.7 g/d (SD 1.7) in 2020 [40].

The plasma PL-FA profile related with the high-SFA diet was characterised by higher proportions of pentadecanoic acid (C15:0) and vaccenic acid (C18:1 n-7 *trans*). These two fatty acids have been previously used as biomarkers of dairy fat consumption, as odd-chain SFAs and ruminant TFAs are synthesised in the rumen of cows before being integrated into the fat fraction of dairy foods [36, 41]. As plasma PL-FAs are thought to reflect short to medium-term dietary FA consumption [12, 16, 17], the importance of these two FAs in the high-SFA diet plasma PL-FA profile may be explained by a higher consumption of full-fat dairy products, which contributed to 39.6% of dietary total fat and 50.1% of dietary SFAs during the high-SFA diet. The strong association between dairy fat consumption and pentadecanoic acid or vaccenic acid in plasma PL from the RISSCI-1 study participants was further confirmed in prospective and cross-sectional multiple linear regression models, which identified these FAs as two independent predictors of dairy fat consumption among the 25 other FAs measured in plasma PL. In cross-sectional analyses, these two FAs were correlated with the baseline consumption of total dairy foods and milk, while pentadecanoic acid was additionally correlated with butter consumption. These findings from plasma PL-FAs are consistent with those from previous RCTs, which reported moderate but consistent associations between total dairy consumption and circulating levels of pentadecanoic acid in serum or plasma total lipids [42–44]. However, these findings from the RISSCI-1 study provide novel evidence for the utility of vaccenic acid as a biomarker for dairy fat consumption, a ruminant TFA that has been previously under studied in intervention studies. Finally, in this study the predictive accuracy of circulating pentadecanoic or vaccenic acids as biomarkers of dairy fat consumption, reflected by the predictive R^2 value, was significantly improved when using prospective multiple regression models (i.e. changes between baseline and high-SFA diet) compared to cross-sectional models. This might provide an important area of future research for the use of these FAs in

observational epidemiology studies, which often rely on a single measurement of dairy-specific FAs (e.g. pentadecanoic, heptadecanoic, or vaccenic acids) to investigate associations with mortality or incidence of cardiometabolic diseases [45–47].

In contrast, the low-SFA diet was associated with higher abundances of long-chain MUFAs and n-6 PUFAs such as eicosenoic and linoleic acids in plasma PL, which may reflect the increased dietary consumption of MUFAs and PUFAs from sunflower oil and vegetable spread during the low-SFA diet [16, 17]. Moreover, the low-SFA plasma PL-FA profile was also characterised by higher concentrations of long-chain SFAs (i.e. ranging from 18 to 22 carbons). These results might be partly explained by the endogenous synthesis of long-chain SFAs in humans together with the fat composition of sunflower oil, vegetable spreads, and nut-based snacks recommended during the low-SFA diet, which contain very small amounts of long-chain SFAs [48, 49]. In line with this idea, a prospective study of changes in plasma PL-FA concentrations over 13 y among participants of the EPIC-Norfolk study reported that each additional 100 g/d of nut and seeds intake was associated with a 2.33% increase in plasma PL long-chain SFAs (20 to 24 carbons, 95%CI: 0.15-4.55) [50].

A major strength of the RISSCI-1 dietary intervention was its success in replacing dietary SFAs with UFAs from commonly available commercial foods in healthy, free-living men living in the UK. The reduction of dietary SFAs achieved in the dietary intervention exceeded public health guidelines by reducing dietary SFA consumption to below 10 %TE [8]. The dietary intervention was also reported to be well received by the participants, on the basis of self-reports and low attrition rate. This may be explained, in part, by the wide range of commercially available food products recommended and supplied during each dietary intervention period, which facilitated compliance, and minimised disruption to the participants' habitual dietary habits.

Limitations of the dietary intervention included the use of self-reported dietary records, which may have influenced the eating behaviour of participants, and introduced bias towards healthier dietary patterns and under-reporting of energy intakes [51, 52]. Such self-reporting bias may partly account for the moderate proportion of under-reporting of energy intakes among participants at baseline (28%) and during the low-SFA diet (27%), which were similar to that observed in previous dietary intervention studies in free-living participants [11–13]. Interestingly, under-reporting of dietary energy was much less prevalent during the high-SFA diet (17%), which might, in part, be explained by increased awareness of the importance of accurate dietary records after being enrolled in the study. However, this might have been attenuated throughout the course of the 8-week intervention, as reflected in the higher degree of under-reporting observed at the end of the study, which may reflect participants' fatigue. Nutrient consumptions were calculated using food composition databases, which could have introduced measurement errors through missing values and lack of diversity in food items. PUFAs (n-3 and n-6) were the main nutrients affected by this limitation, and their consumptions were estimated more accurately by using the NDNS nutrient databank [19] to complement missing data from the CoFID database [21]. Finally, participants were healthy men, many with optimal BMI (between 18.5 and 24.9 kg/m^2, n=56, 52.8%), high self-reported physical activity levels (n=52, 47.7%), and white ethnic background (n=94, 86.2%), which may limit the generalisability of the study findings to a wider population. However, self-reported ethnicity from the RISSCI-1 closely match data from the 2011 Census in England and Wales [53].

In conclusion, the RISSCI-1 dietary fat exchange model was successful in exchanging dietary SFAs for UFAs in healthy UK men, in accordance with current UK public health guidelines for adults. The replacement of dietary SFAs with UFAs, was based on commercially available foods and relied mostly on dairy foods, snacks, and cooking oil, did not interfere with the overall dietary patterns of participants. In particular, our findings support the utility of dairy food and snacks as a potential public health strategy to reduce dietary SFAs, helping to lower population CVD risk. Confirmation of the feasibility and efficacy of this food-based dietary exchange model will require its use in larger populations and intervention studies of longer duration.

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List of figures

Table 1. Identified sources of dietary exchangeable fat in the RISSCI-1 food exchange model [a]

	Total Energy	**Total Fat**		**SFAs**		**MUFAs**		**PUFAs**	
	MJ/d	**g/d**	**%TE**	**g/d**	**%TE**	**g/d**	**%TE**	**g/d**	**%TE**
Total baseline intake (including alcohol) [b]	8.80	77.7	32.8	28.4	11.9	28.5	12.0	13.4	5.7
Sources of exchangeable fat									
Added oils [c]	0.38	8.7	3.7	0.8	0.3	3.2	1.4	1.5	0.6
Added fats (butter and spreads)	0.29	7.8	3.3	2.8	1.2	2.9	1.2	1.4	0.6
Milk	0.44	4.3	1.8	2.7	1.2	1.1	0.5	0.1	<0.1
Cheese	0.26	5.0	2.1	3.0	1.3	1.3	0.6	0.2	<0.1
Sweet and savoury snacks[d]	0.86	9.9	4.2	3.8	1.6	3.4	1.5	1.6	0.7
Total exchangeable fat intake	2.15	35.8	15.3	13.1	5.6	12.0	5.1	4.8	2.1
Non-exchangeable fat intake	6.65	41.9	17.9	15.3	6.5	16.5	7.1	8.6	3.7

Abbreviations: %TE, % total energy; **MJ/d**, megajoules/day; **SFAs**, saturated fatty acids; **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids.

[a] Adapted from Weech *et al.* [12]

[b] Calculation based on the National Diet and Nutrition Survey (y 1 to 4) in men aged 19-64y [19].

[c] Calculation based on the Dietary Intervention and Vascular function (DIVAS) randomised controlled trial [12]

[d] Included biscuits, buns, cakes, pastries, fruit pies, savoury snacks, and chocolate.

Table 2. Recommended daily servings of intervention food items for the achievement of the RISSCI-1 dietary fat exchange.

Intervention food item	High-SFA diet		Low-SFA diet	
	Description	*Recommended amount (g/d)*	*Description*	*Recommended amount (g/d)*
Fat spreads	Salted butter [a]	14	Vegetable fat spread [a,b]	17
Cooking fats	Salted butter [a]	6	Sunflower oil [a]	11
Cheese or yogurt	Cheese with ≥ 25% fat, or full-fat yogurt	25 (cheese) or 100 (yogurt)	Cheese with < 25% fat, or virtually fat free yogurt	25 (cheese) or 100 (yogurt)
Milk	Full fat or semi-skimmed	200	< 1% fat	200
Snacks	Chocolates, biscuits, and crackers [a]	50	Crisps and nuts [a]	50

Abbreviations: **SFA**, saturated fatty acid; **MUFA**, monounsaturated fatty acid; **PUFA**, polyunsaturated fatty acid.

[a] Food items provided by researchers. Items provided for the high-SFA diet included: Wyke Farms "Salted Butter", Whitworths "Banana Chips", McVitie's "Gold Bar", Mrs Crimble's "Big Choc Macaroon", McVitie's "Trio Toffee Biscuit bar", Sainsbury's "Belgian Chocolate Chunk Shortbread", Tunnock's "Caramel Wafer", Sainsbury's "Cheddar Cheese Crispies", Arden's "Cream Cheese and Spring Onion Melts", and Jacob's "Savours Sweet Chilli Thins Crackers". Items provided for the low-SFA diet included: Flora "Buttery Spread", KTC "100% Sunflower Seed Oil", Tesco "Crispy Seedy Nutty Bites", Sainsbury's "Unsalted Mixed Nuts and Raisins", Tesco "Sweet Chilli Coated Peanuts", Sesame Snaps ®, Tesco "Bombay Mix", Nik Naks "Nice & Spicy Corn Snacks", Tesco "Ready Salted Crisps", Walkers "Max Paprika Crisps", and Pringles "Original Crisps".

[b] 79% vegetable fat spread with 5% sunflower oil and 24% rapeseed oil.

Table 3. Baseline characteristics of adult men from the RISSCI-1 study (n=109).

	Mean	**SD**
Age, *y*	48.4	10.8
Self-reported ethnicity, n (*%*)		
Asian or UK Asian	8 (7.3)	
Black or UK Black	3 (2.8)	
Chinese	2 (1.8)	
Mixed Ethnic Background (not specified)	2 (1.8)	
White	94 (86.2)	
BMI, *kg/m²*	25.1	3.3
Physical activity level, n (*%*) [a]		
Low	6 (5.5)	
Moderate	34 (31.2)	
High	52 (47.7)	
Missing	17 (15.6)	
Total energy		
kcal/d	2320	635
MJ/d	9.7	2.7
Total fat, *%TE*	36.2	7.8
SFAs, *%TE*	12.7	3.8
MUFAs, *%TE*	13.3	3.5
n-3* PUFAs, *%TE	0.8	0.4
n-6* PUFAs, *%TE	4.6	1.8
Total PUFAs, *%TE*	5.8	2.1
TFAs	0.5	0.3
Cholesterol, *mg/d*	235	116
Protein, *%TE*	16.3	3.3
Carbohydrates, *%TE*	44.3	9.4
Free sugars, *%TE*	7.6	4.8
Dietary fibre (AOAC), *g/d*	25.8	9.5
Alcohol, *%TE* [b]	4.0	(1.4-7.7)
Sodium, *g/d*	2.62	0.99

Abbreviations: **AOAC**, Association of Analytical Chemists; **BMI**, body mass index; **d**, day; **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **SD**, standard deviation; **SFAs**, saturated fatty acids; **TFAs**, trans fatty acids; **%TE**, % total energy

[a] Categories derived from the International Physical Activity Questionnaire (IPAQ) [27].

[b] Values presented as *median (interquartile range)* and based on n=45 participants who consumed alcohol (n=55 non-consumers).

Table 4. Recorded and target daily nutrient intakes following each dietary intervention period (high-SFA and low-SFA diets) in adult men from the RISSCI-1 study (n=100).

	High-SFA Diet			**Low-SFA Diet**			
	Target	**Mean**	**SD**	**Target**	**Mean**	**SD**	**p-value** [a]
Total energy							
kcal/d		2354	546		2282	558	0.13
MJ/d		9.9	2.3		9.6	2.3	0.14
Total fat, *%TE*	34.0	38.4	6.5	34.0	38.2	6.6	0.79
SFAs, *%TE*	18.0	19.1	3.5	10.0	8.9	2.1	< 0.001
MUFAs, *%TE*	12.0	11.1	2.8	14.0	13.4	2.9	< 0.001
n-3* PUFAs, *%TE		0.6	0.4		1.2	0.5	< 0.001
n-6* PUFAs, *%TE		2.5	1.0		9.5	3.5	< 0.001
Total PUFAs, *%TE*	4.0	3.7	1.3	10.0	11.1	3.6	< 0.001
TFAs, *%TE*		0.8	0.3		0.2	0.2	< 0.001
Cholesterol, *mg/d*		273	112		201	166	< 0.001
Protein, *%TE*		16.0	3.0		16.3	3.1	0.28
Carbohydrates, *%TE*		42.6	7.9		42.9	8.0	0.61
Free sugars, *%TE*		5.0	3.9		4.7	3.2	0.35
Dietary fibre (AOAC), *g/d*		24.4	10.3		25.9	11.9	0.06
Alcohol, *%TE* [b]		4.5	(2.2-6.2)		3.6	(2.0-5.6)	0.83 [c]
Sodium, *g/d*		2.67	0.88		2.45	0.91	0.04

Abbreviations: **AOAC**, Association of Analytical Chemists; **d**, day; **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **SD**, standard deviation; **SFAs**, saturated fatty acids; TFAs, trans fatty acids; **%TE**, % total energy

Means and SD based on n = 100 participants, unless specified otherwise.

[a] From paired *t*-tests.

[b] Values presented as *median* (interquartile range) and based on n=45 participants who consumed alcohol (n=55 non consumers).

[c] From paired T-test on log-transformed values between the high-SFA and low-SFA diets.

Table 5. Fasting abundances of plasma phospholipid fatty acids (PL-FAs) following the low-SFA and high-SFA diets in adult men from the RISSCI-1 study (n=108).

Fatty acid abundances *(wt%)*	**High-SFA Diet**		**Low-SFA Diet**		
	Mean	**SD**	**Mean**	**SD**	**P-value** [a]
Total SFAs	46.0	0.9	45.1	1.1	$< 10^{-4}$
14:0	0.55	0.12	0.46	0.11	$< 10^{-4}$
15:0	0.28	0.05	0.21	0.04	$< 10^{-4}$
16:0	30.3	1.2	29.0	1.3	$< 10^{-4}$
17:0	0.44	0.06	0.42	0.06	$< 10^{-4}$
18:0	14.3	1.0	14.9	1.0	$< 10^{-4}$
20:0	0.09	0.01	0.11	0.02	$< 10^{-4}$
22:0	0.03	0.01	0.03	0.01	$< 10^{-4}$
Total MUFAs	12.6	1.3	12.3	1.3	$< 10^{-2}$
16:1 n-7 *cis*	0.52	0.21	0.42	0.18	$< 10^{-4}$
18:1 n-9 *cis*	10.2	1.2	9.9	1.2	$< 10^{-2}$
18:1 n-7 *cis*	1.43	0.20	1.49	0.22	$< 10^{-4}$
20:1 n-9	0.18	0.04	0.23	0.05	$< 10^{-4}$
16:1 n-7 *trans*	0.01	0.00	0.01	0.00	$< 10^{-4}$
18:1 n-9 *trans*	0.15	0.04	0.15	0.04	0.37
18:1 n-7 *trans*	0.18	0.06	0.11	0.04	$< 10^{-4}$
Total PUFAs	41.4	1.6	42.5	1.6	$< 10^{-4}$
20:3 n-9	0.15	0.04	0.13	0.04	$< 10^{-3}$
Total PUFAs n-6	35.5	2.1	37.1	2.0	$< 10^{-4}$
18:2 n-6 *cis*	21.4	2.5	23.2	2.4	$< 10^{-4}$
18:3 n-6	0.09	0.05	0.09	0.05	0.26
20:2 n-6	0.33	0.05	0.34	0.06	0.01
20:3 n-6	3.38	0.83	2.97	0.74	$< 10^{-4}$
20:4 n-6	9.70	1.71	9.99	1.86	$< 10^{-2}$
22:4 n-6	0.35	0.08	0.32	0.09	$< 10^{-4}$
22:5 n-6	0.20	0.06	0.16	0.06	$< 10^{-4}$
18:2 n-6 *trans*	0.06	0.01	0.06	0.01	$< 10^{-2}$
Total PUFAs n-3	5.76	1.49	5.23	1.19	$< 10^{-4}$
18:3 n-3	0.22	0.07	0.22	0.08	0.53
20:5 n-3	1.25	0.69	0.99	0.53	$< 10^{-4}$
22:5 n-3	1.08	0.20	0.95	0.20	$< 10^{-4}$
22:6 n-3	3.21	0.90	3.07	0.79	$< 10^{-2}$

Abbreviation: **MUFAs**, monounsaturated fatty acids; **PUFAs**, polyunsaturated fatty acids; **SD**, standard deviation; **SFAs**, saturated fatty acids; **wt%**, weight percentage of total fatty acids.

[a] from paired *t*-tests.

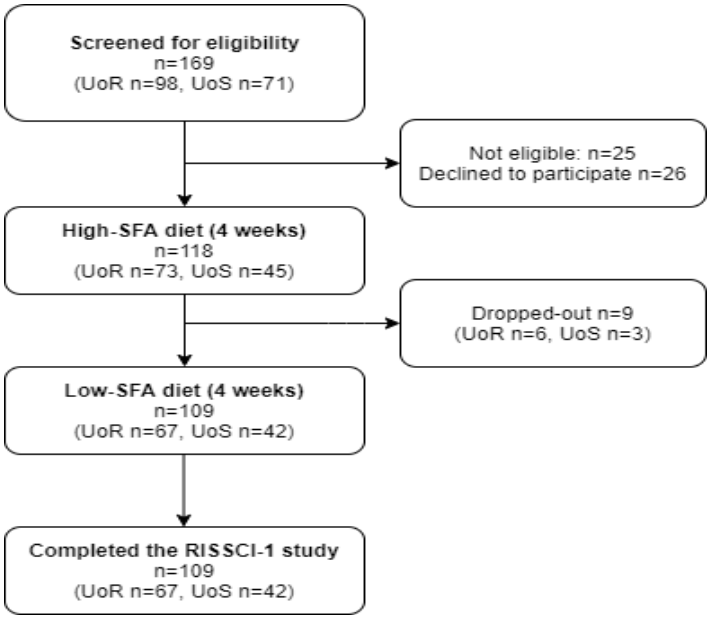

Figure 1. Flow-chart of participants from the RISSCI-1 study.

Abbreviations: UoR, University of Reading; UoS, University of Surrey.

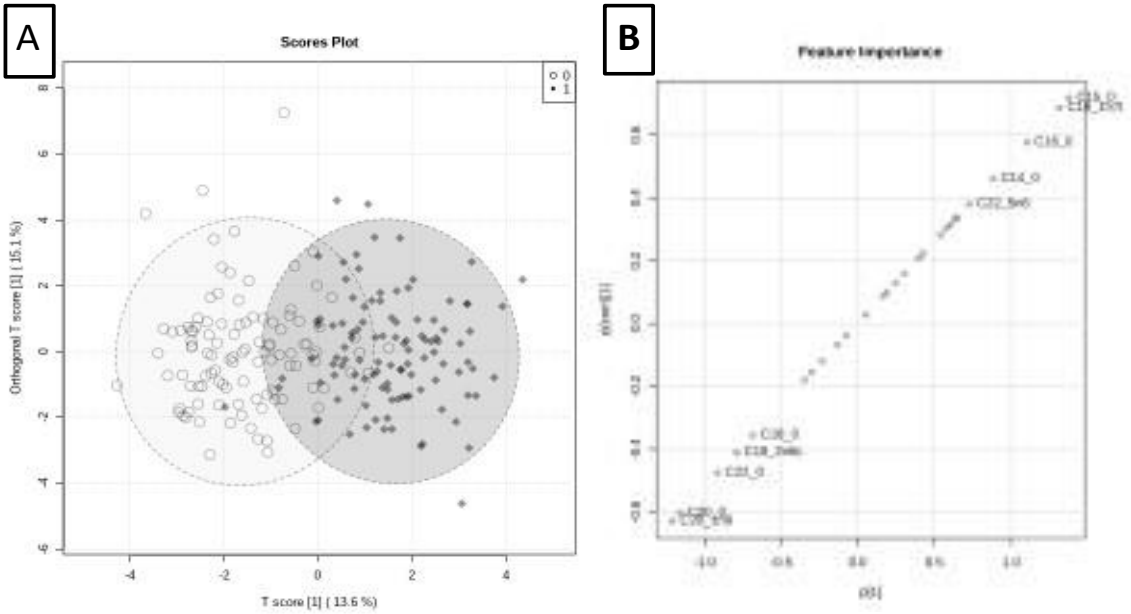

Figure 2. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) based on plasma phospholipid fatty acids (FA) in adult men from the RISSC-I study between the high-SFA and the low-SFA diets (n=108). **A:** Scores plot showing a moderate discrimination between two FA profiles during the high-SFA and low-SFA diets. **B:** Feature loadings scaled as correlation coefficients (p(corr)[1]) towards the OPLS-DA predictive component (p[1]), showing the individual FA contributing to each discriminated FA profile.

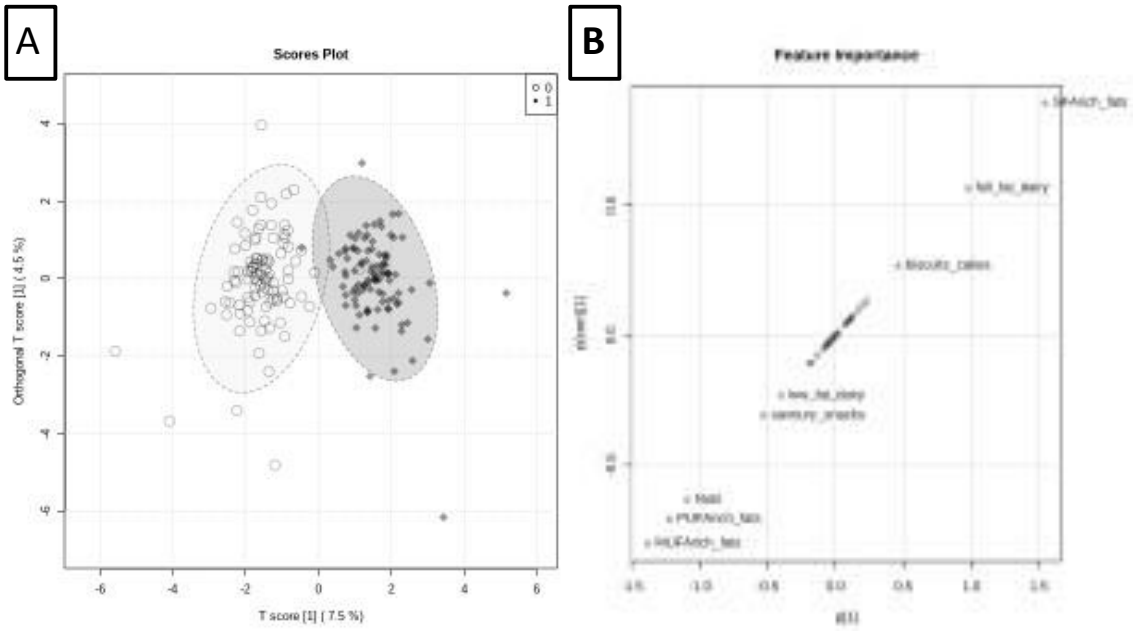

Figure 3. Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) based on dietary intakes in adult men from the RISSC-I-1 study between the high-SFA and the low-SFA diets (n=100). **A:** Scores plot showing the discrimination between two dietary patterns during the high-SFA and low-SFA diets. **B:** Feature loadings scaled as correlation coefficients (p(corr)[1]) towards the OPLS-DA predictive component (p[1]), showing the food groups contributing to each discriminated dietary pattern.

Supplementary table 1. Definition of food categories used to assess dietary patterns in the RISSCI-1 study.

Food category	Example items	Calculation details
Fruits	Banana, apple, berries, etc.	Canned, stewed, and dried fruit as equivalent weight of whole fruit, including fruit within composite dishes
Whole vegetables	Cucumber, tomatoes, spinach, etc.	All cooked or raw vegetables, including tomato puree as equivalent weight of whole vegetable.
Pulses	Lentils, beans, chickpeas, etc.	Equivalent cooked weight
Vegetarian processed foods and ready meals	Potato dishes, pizza, salads, egg dishes,	Weight as consumed
Soups	any vegetable soup, including meat or fish soups	Weight as consumed
Cooking sauces	Tomato sauce, creamy sauces, pesto, etc.	Weight as consumed
Sauces and stock	Gravy, chicken stock, etc.	Weight as consumed
Nut butters	Peanut butter, Tahini paste, etc.	Weight as consumed
Nuts	Walnuts, hazelnuts, etc.	Weight as consumed
Seeds	Sesame seeds, etc.	Weight as consumed
Red and processed meats, offals	Beef, lamb, cured meats, sausages, etc.	Equivalent cooked weight, excluding waste (e.g. bones)
Poultry	Turkey, chicken, etc.	Equivalent cooked weight, excluding wastage (e.g. bones)
Meat alternatives	Quorn, tofu, etc.	Weight as consumed
Red and processed meat dishes	Meat pies, meat curry dishes, etc.	Weight as consumed
White fish	Cod, plaice, etc.	Equivalent cooked weight
Oily fish	Salmon, mackerel, etc.	Equivalent cooked weight
Shellfish	Mussels, clams, crab, etc.	Excluding wastage (e.g. shells)
Fish dishes	Fish pies, breaded fish, etc.	Oily and white fish, and shellfish included
Full-fat dairy foods	Whole milk, medium and full-fat cheese, full-fat yogurts, dairy desserts.	Weight as consumed
Reduced-fat dairy foods	Semi-skimmed and skimmed milk, low-fat and fat free yogurts, low-fat cheese	Weight as consumed
Dairy alternatives	Plant-based milks, plant-based yogurts, etc.	Included fortified and non-fortified dairy alternatives
Eggs	All types of eggs	Weight as consumed
Refined grains	Pasta, rice, etc.	Equivalent cooked weight
Refined grain foods	Bread, flour, crackers, etc.	Weight as consumed

Whole grains	Pasta, rice, etc.	Equivalent cooked weight
Whole grain foods	Bread, flour, crackers, etc.	Weight as consumed
Oats	Porridge and rolled oats	Equivalent dry weight
Condiments	Vinegar, mustard, salad dressing, herbs, spices, etc.	Weight as consumed
MUFA-rich fat	Olive oil, vegetable fat spread	Weight as consumed
PUFA-rich fat	Sunflower oil, vegetable fat spread	Weight as consumed
SFA-rich fat	Butter, animal fat, coconut fat	Weight as consumed
Biscuits and cakes	Sweet bakery products, biscuits, etc.	Weight as consumed
Savoury snacks	Crisps, crackers, corn/maize based snacks, etc.	Weight as consumed
Sugary products	Marmalades, jams, syrups, sugar, etc.	Weight as consumed
Sugar alternatives	Stevia, aspartame, etc.	Weight as consumed
Coffee	All coffee drinks	Weight as consumed
Tea	Green, black, herbal tea drinks	Weight as consumed
Sweetened drinks	Sodas, tonics, squashes, etc.	Equivalent ready to drink weight
Sugar free drinks	Sodas, tonics, squashes, etc.	Equivalent ready to drink weight
Alcoholic drinks	Beers, liqueurs, spirits, cocktails, etc.	Weight as consumed

Supplementary table 2. Contribution of total dairy foods to nutrient intakes (%) in the RISSCI-1 study participants. [a]

Nutrients	Baseline [b]		High-SFA diet [c]		Low-SFA diet [d]	
	Mean, %	SD	Mean, %	SD	Mean, %	SD
Energy	10.6	6.4	20.8	6.3	6.3	3.0
Protein	14.0	8.5	20.9	7.8	15.3	6.9
Carbohydrates	5.4	4.2	5.9	3.5	6.6	3.6
Sugars	2.6	5.3	0.2	1.0	0.5	0.8
AOAC Fibre	0.6	1.7	0.1	0.7	0.4	1.1
Total fat	16.6	11.4	39.6	11.5	3.3	4.0
SFAs	28.5	17.5	50.1	12.6	8.1	8.4
MUFAs	12.5	9.6	35.8	12.5	2.5	3.3
PUFAs	4.2	4.9	13.7	7.4	0.4	0.9
n-3 PUFAs	5.7	7.9	19.7	13.8	0.4	1.5
n-6 PUFAs	2.9	3.7	10.6	7.3	0.2	0.7
TFAs	45.2	26.0	74.3	18.9	22.5	25.7
Cholesterol	21.7	16.5	44.0	19.4	13.0	16.4
Sodium	10.6	7.8	21.9	9.2	9.8	5.6
Iodine	50.5	22.4	66.1	18.5	61.7	20.9
Calcium	37.5	17.1	53.4	14.0	42.5	15.1

Abbreviations: AOAC, American Association of Analytical Chemists; **MUFAs**, monounsaturated fatty acids; **PUFAs,** polyunsaturated fatty acids; **SD** standard deviation; **SFAs**, saturated fatty acids; **TFAs**, trans fatty acids.

[a] total dairy foods included milk, cheese, yogurt, dairy cream, butter, and dairy from milky drinks (e.g. milkshakes and cappuccino).

[b] based on n=106 participants.

[c] based on n=104 participants.

[d] based on n=100 participants.

Supplementary table 3. Cross-sectional Pearson correlations between the consumption of dairy foods and relative concentration of C15:0 or C18:1t11 in plasma phospholipids at baseline in the RISSCI-1 study participants (n=104).

PL-FA	Dairy foods	Correlation Coefficient	p-value [a]
15:0	Total dairy [b]	0.34	**$<10^{-3}$**
	Dairy desserts	0.08	0.42
	Milk	0.31	**0.001**
	Butter	0.25	**0.01**
	Cheese	0.07	0.48
	Milky drinks	-0.03	0.75
	Cream	0.13	0.19
	Yogurts	0.15	0.12
18:1 n-7 *trans*	Total dairy	0.33	**$<10^{-3}$**
	Dairy desserts	0.19	**0.048**
	Milk	0.26	**0.007**
	Butter	0.19	0.06
	Cheese	0.08	0.42
	Milky drinks	0.04	0.66
	Cream	0.19	**0.049**
	Yogurts	0.08	0.42

Abbreviations: **PL-FA**, phospholipid fatty acid; **15:0**, pentadecanoic acid; **18:1 n-7 *trans***, *trans* vaccenic acid.

[a] p-values obtained from statistical tests for association between paired samples, using Pearson's product-moment correlation coefficient

[b] total dairy foods included milk, cheese, yogurt, dairy cream, butter, and dairy from milky drinks (e.g. milkshakes and cappuccino).

Appendix II: Abstracts published in conference proceedings

Impact of replacing dietary saturated with unsaturated fats on the expression of genes related to cholesterol metabolism in peripheral blood mononuclear cells: Findings from the RISSCI-1 study. E. Ozen[1], A. Koutsos[1], R. Antoni[2], G. Wong[1], L. Sellem[1], B. Fielding[2], M.D. Robertson[2], B.A. Griffin[2], J.A. Lovegrove[1] and K.G. Jackson[1] *Nutrition Society summer meeting, online, 6-8 July 2021 (poster presentation)*

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Human studies suggest that replacing dietary saturated fatty acids (SFAs) with unsaturated fatty acids (UFAs) has a beneficial effect on fasting low-density lipoprotein cholesterol (LDL-C)(1). Regulation of the LDL receptor (LDL-R) expression in response to dietary fat intake has been proposed in animal and in vitro studies, but findings are limited in humans(2). Since gene expression in peripheral blood mononuclear cells (PBMCs) is being increasingly recognised as a surrogate marker of hepatic cholesterol regulation, we measured the expression of the LDL-R, and three other genes related to cholesterol metabolism in circulating PBMCs after high and low SFA diets.

The RISSCI-1 (Reading Imperial Surrey Saturated fat Cholesterol Intervention) study was a non-randomised, single-blind, controlled dietary intervention study in which 109 healthy men aged 30 to 65 y followed two iso-energetic diets (35% total energy (TE) total fat); Diet-1 (high SFA (18% TE) and lower UFA (15% TE)) followed by Diet-2 (low SFA (10% TE), high UFA (24% TE) diet) for 4 weeks each. Blood lipids were measured in fasting blood samples collected at baseline and at the end of each dietary intervention period. PBMCs were isolated from fasting blood collected into a BD Vacutainer cell preparation tube and total RNA was extracted and transcribed to cDNA. TaqMan gene expression assays were performed to determine the expression of two housekeeping genes and four target genes (LDL-R, sterol regulatory element binding transcription factor 1 (SREBF1), Nuclear receptor subfamily 1 group H member 3 (NR1H3) and ATP-binding cassette sub-family G member 1 (ABCG1)) in a subset of RISSCI-1 participants. Expression of each target gene was normalised to the housekeeping genes and the fold change in mRNA expression relative to the baseline visit for each diet was calculated by using the ΔΔCt method(3). Paired t tests were used to assess the effect of two diets on the changes in blood lipids and PBMC gene expression. Changes in Log2 gene expression were correlated with changes in total cholesterol (TC) and LDL-C after the diets using Pearson's correlations.

Fasting serum TC and LDL-C levels were 12.2% and 15.4% lower, respectively after Diet-2 compared to Diet-1 ($p<0.001$). The expression of all the genes tested increased after Diet-2 versus Diet-1, but only the mRNA expression of the LDL-R (n=58), NR1H3 and ABCG1 (n=57) were significantly upregulated ($p=0.04$, $p=0.01$, $p<0.01$ respectively). Changes in LDL-R, NR1H3 and ABCG1 gene expression were not associated with the changes in serum TC and LDL-C.

In summary, we found an upregulation in the LDL-R and two other genes, along with reductions in TC and LDL-C after the UFA compared with SFA diet. These findings are in line with previous studies and suggest a role for dietary fat composition on LDL-R regulation.

1. Siri-Tarino PW, Sun Q & Hu FB (2010) *Curr Atheroscler Rep* 12, 384-390
2. Fernandez ML & West KL (2005) *J.Nutr* 135, 2075-2078
3. Livak KJ & Schmittgen TD (2001) *Methods* 25, 402-408

Association between *APOE* genotype with body composition and cardiovascular disease risk markers is modulated by BMI in healthy adults: Findings from the BODYCON study. E. Ozen, R. Mihaylova, N. J. Lord, J. A. Lovegrove and K. G. Jackson. *Nutrition Society Winter meeting, online, 7-8 December 2021 (poster presentation)*

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The relationship between *APOLIPOPROTEIN (APO)E* genotype and cardiovascular disease (CVD) risk is extensively studied due to its effect on the plasma lipid profile[1]. However, studies investigating the associations between *APOE* genotype with CVD risk markers have generated inconsistent results, with a small number of human studies suggesting that BMI might play an important role in this relationship[2, 3].Therefore, we assessed the association between *APOE* genotype with body composition and CVD risk markers, with further examination of the role of BMI on this association.

BODYCON (impact of physiological and lifestyle factors on body composition) was a cross-sectional observational study in which 360 healthy men and women aged 18-70 y with a BMI of 18.5-39.9 kg/m^2 underwent a measure of body composition by dual energy x-ray absorptiometry, assessment of physical activity level using a tri-axial accelerometer and habitual dietary intake using a 4-day weighed food diary. Circulating lipid CVD risk markers were measured in a fasting blood sample and participants were genotyped retrospectively for *APOE* (rs429358 and rs7412). A general linear model was used to determine the impact of genotype on body composition measures and CVD risk markers, and interaction between *APOE* and BMI on these outcome measures.

In the study cohort, n=46 participants were *APOE2/E3*, n=228 the wild type *APOE3/E3* group and n=81 *E4* carriers (*APOE3/E4* and *APOE4/E4*). The *APOE2/E3* group had on average 9%-18% lower fasting total, low-density lipoprotein and non-high density lipoprotein cholesterol concentrations compared to the *APOE4* carrier and *APOE3/E3* groups ($p \leq 0.01$). Significant *APOE* x BMI interactions were observed for body weight and android fat mass ($p \leq 0.01$). When the group were stratified into normal-weight and overweight/obese BMI subgroups, lean body mass was 6.4% lower in the *APOE3/E3* group (mean±SE,45.2±0.5 kg) compared to the *APOE4* carriers (48.1±0.9 kg) in the normal BMI subgroup ($p \leq 0.02$), while in the overweight/obese BMI subgroup, the android:gynoid fat ratio was 7.6% lower in the *APOE4* carriers (1.10±0.03) compared to the *APOE3/E3* group (1.19±0.02)($p=0.04$).Differences in fasting lipid concentrations between the *APOE2/E3* and other genotype groups was only found within the normal weight ($p \leq 0.04$) but not overweight/obese BMI subgroup. Moreover, the *APOE2/E3* participants within the normal-weight BMI subgroup had a lower dietary fibre and trans-fat intake compared to the *APOE4* carriers and lower carbohydrate intake compared to the *APOE3/E3* group while there were no differences between genotypes in the overweight/obese BMI subgroup. Physical activity levels were similar between genotype groups within each BMI subgroup.

Our findings confirm previous studies suggesting that the impact of *APOE* genotype on lipid CVD risk markers is modulated by BMI but indicate that diet may also play a role in this relationship. Further research is needed to draw a firm conclusion on the underlying mechanisms.

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Background: Diets higher in fibre have been associated with beneficial effects on cardiometabolic disease (CMD) risk markers including obesity, blood pressure and cholesterol levels. However, the relationship between dietary fibre intake and body composition is unclear. Therefore, the objective of the study was to further assess the association between fibre intake, body composition and CMD risk markers.

Materials and Methods: A single-centred cross-sectional study was conducted in 277 healthy adults (n=107 men and n=170 women) with a mean age of 41 (SD 16) y and body mass index (BMI) of 23.9 (SD 3.8) kg/m^2. Total body composition was measured by dual energy x-ray absorptiometry and dietary intake was assessed with a 4-day weighed food diary.CMD risk markers included fasting lipids and glucose quantified using an ILAB 600 clinical chemistry analyser and clinic blood pressure measured using an Omron blood pressure monitor.

Results: Average AOAC fibre intake in the cohort was 23.0 (SD 9) g/day, with higher intakes found in men (25.0 (SD 10.3) g/day) than women (21.9 (SD 7.8) g/day; P=0.015*)*. AOAC fibre intakes were significantly weakly correlated with weight (r_s =0.142), percentage body fat (r_s=0.193), bone mineral density (r_s=0.156) and fat free mass (r_s= 0.257; $P\leq$0.009), and inversely correlated with fasting total cholesterol (r_s =-0.124), low-density lipoprotein (LDL)-cholesterol ($r_{s=}$ 0.144) and total to high-density lipoprotein-cholesterol ratio (r_s =-0.129; $P\leq$0.042). After stratifying data according to quartiles of AOAC fibre intake and adjusting for covariates (including age, sex, BMI, weight, energy expended per day through physical activity and total energy intake per day) total and LDL-cholesterol concentrations were significantly lower in quartiles (Q)3 (21.0-29.5 g/d) and Q4 (30-63.5 g/d) than Q1 (3.0-18.8 g/d) and Q2 (19.3-20.9 g/d). Systolic blood pressure was also lower in Q4 than Q1 and Q2 *(*P<0.05*)*. Anthropometric and body composition measures were not found to be different across quartiles of increasing AOAC fibre intake.

Discussion: Findings from this cross-sectional study have revealed daily fibre consumption greater than 21 g to be associated with lower fasting total and LDL cholesterol, and intakes ≥30 g also associated with lower systolic blood pressure. With only 9% of UK adults meeting the current recommended intake, raising public awareness of the importance of dietary fibre is an important strategy for CMD prevention.

Appendix III: Explanation of DXA adiposity measures

Measures of adiposity	
Body fat %	The percent of the body that is composed of fat
Android fat, %	Higher percentage refers to having most of the body fat around the mid-section
Gynoid fat, %	Higher percentage refers to having most of the body fat stored around the hips
A/G fat ratio	Describes where the fat is stored
Fat mass, kg	Total fat mass in the body
Lean mass, kg	Total lean mass in the body
Android fat mass, kg	Fat mass in the mid-section
Android lean mass, kg	Lean mass in the mid-section
Abdominal VAT	Adipose tissue lining internal organs