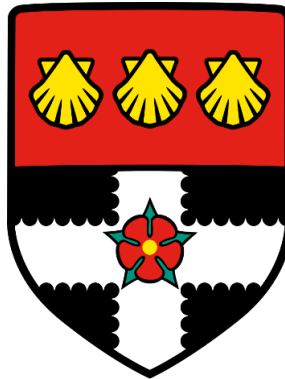# UNIVERSITY OF READING

Impact of meal fat composition on postprandial vascular function & cardiovascular disease risk markers in postmenopausal women

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A report submitted in partial fulfilment of the requirements for the degree of PhD

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August 2017

ABSTRACT

Replacement of saturated fat (SFA) with unsaturated fats is recognised as an effective strategy to lower cardiovascular disease (CVD) risk; however, the optimal type of fat is unclear. The majority of studies examining the effects of dietary fat composition on lipids and vascular function have been conducted in the fasting state, with very little known about the acute effects of meal fat composition on postprandial lipaemia. This is particularly important since individuals spend up to 18 h in the postprandial (fed) state, with non-fasting triacylglycerol levels now recognised as an important CVD risk factor in women. However, conclusions from my literature review revealed that little is known about the effects of meal fat composition on postprandial lipaemia and vascular function in postmenopausal women, an understudied subgroup of the population at increased CVD risk. It was hypothesised that, relative to test meals rich in SFA, those rich in monounsaturated (MUFA) or n-6 polyunsaturated fat (n-6 PUFA) would improve vascular function, postprandial lipaemia and other CVD risk markers, with differential responses dependent on the Glu298Asp polymorphism (eNOS genotype) and *APOLIPOPROTEIN E (APOE)* (epsilon) genotype in postmenopausal women. In a double-blind, randomised, cross-over, postprandial study, 32 postmenopausal women consumed sequential test meals (0 min, 50 g fat; 330 min, 30 g fat) rich in SFA (butter), MUFA fat (olive oil and MUFA-rich spreads) or n-6 PUFA fat (safflower oil and n-6 PUFA-rich spreads) on three separate occasions, each 4-6 weeks apart. Postprandial flow-mediated dilatation (FMD, primary outcome measure), laser Doppler imaging and digital volume pulse responses were not different after consumption of the test fats. For diastolic blood pressure, there was a significantly greater reduction in the incremental area under the curve (IAUC) for the postprandial response after the MUFA than SFA-rich meals ($P=0.009$), with a similar trend found for systolic blood pressure ($P=0.012$). These findings were linked with a greater reduction in the IAUC for postprandial plasma nitrite (a biomarker for nitric oxide, an important vasodilator) after the SFA than MUFA-rich meals ($P=0.010$). An effect of meal fat composition was also evident on the postprandial intracellular adhesion molecule response, with a greater reduction after the n-6 PUFA than SFA and MUFA-rich ($P\leq0.001$) meals. There was little impact of the different dietary fats on postprandial lipid, glucose and insulin responses, although a significant meal fat effect was evident for postprandial IL-1β production by *ex-vivo* whole blood culture lipopolysaccharide (LPS)-stimulated blood samples (a real time measure of inflammation), with higher concentrations observed after the SFA than MUFA and n-6 PUFA-rich meals

($P<0.0001$). When the data were stratified according to genotype, the eNOS Glu298Asp polymorphism was revealed to be a likely determinant of the inter-individual variability in the postprandial % FMD ($P=0.019$) and insulin responses to acute dietary fat intake. As expected *APOE* genotype was associated with baseline fasting plasma lipids and inflammatory markers (C-reactive protein) but our study also revealed a novel impact on arterial stiffness. However, there was limited impact of *APOE* genotype on cardio-metabolic risk markers in response to acute and chronic fat manipulation. In conclusion, this PhD has shown for the first time that meal fat composition has a significant impact on blood pressure, plasma nitrite, markers of endothelial activation, and *ex-vivo* IL-1β cytokine production, but no effect on postprandial lipaemia in postmenopausal women. The eNOS Glu298Asp polymorphism and *APOE* genotype may also be important in relation to vascular function in this population group, and warrant further investigation. Overall, these findings will contribute to the evidence base for dietary fat recommendation and may be important in identifying population subgroups with greater responsiveness to the beneficial effects of targeted dietary fatty acid manipulation through personalised nutrition.

Declaration

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

Kumari M Rathnayake, August 2017

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Papers submitted for publication

Impact of meal fatty acid composition on postprandial lipaemia, vascular function and blood pressure in postmenopausal women: a review

Kumari M Rathnayake, Michelle Weech, Kim G Jackson & Julie A Lovegrove

Submitted to Nutrition Research Reviews (manuscript provisionally accepted June 2017 pending revisions)

Meal fatty acids have differential effects on postprandial blood pressure and biomarkers of endothelial function but not vascular reactivity in postmenopausal women: findings from the randomized, controlled DIVAS-2 study

Kumari M Rathnayake, Michelle Weech, Kim G Jackson & Julie A Lovegrove

Submitted to Journal of Nutrition (July 2017)

Papers prepared for submission

Manuscripts have been prepared and will be submitted once the primary paper describing the DIVAS-2 study has been published

Greater ex-vivo IL-1β production after sequential saturated than unsaturated fat-rich meals in postmenopausal women

Kumari M Rathnayake, Michelle Weech, Julie A Lovegrove & Kim G Jackson

Prepared for submission to Cytokine Journal

Glu298Asp (rs1799983) polymorphism influences postprandial vascular reactivity and insulin response to meals of varying fat composition in postmenopausal women: findings from the randomized, controlled DIVAS-2 study

Kumari M Rathnayake, Michelle Weech, Julie A Lovegrove & Kim G Jackson

Prepared for submission to Journal of Nutrition

Impact of the *APOLIPOPROTEIN E* (epsilon) genotype on cardio-metabolic risk markers and responsiveness to acute and chronic dietary fat manipulation

Kumari M Rathnayake, Michelle Weech, Kim G Jackson & Julie A Lovegrove

Prepared for submission to Journal of Nutrition

Abstracts published in conference proceedings

Meal fat composition has differential effects on biomarkers of postprandial endothelial function in postmenopausal women. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *Nutrition Society Summer Meeting, King's College London (London, UK), July 2017 (oral presentation).*

Meal fat composition has a significant impact on postprandial blood pressure in postmenopausal women: Findings from the DIVAS-2 study. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *The 12th Congress of the International Society for the Study of Fatty Acids & Lipids (ISSFAL), Stellenbosch (South Africa), Sept 2016 (oral presentation). **Awarded young scientist award**.*

Meal fatty acid composition has a differential effect on postprandial blood pressure in postmenopausal women. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *Nutrition Society Summer Meeting, University College (Dublin, Ireland), July 2016 (oral presentation).*

Impact of meal fatty acids on postprandial vascular reactivity: Overview of the DIVAS-2 study. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *Nutrition Society Postgraduate Conference, Trinity Hall (Cambridge, UK) Sept 2015 (oral presentation).*

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ABBREVIATIONS

%TE	Percentage of total energy
ACE	Angiotensin-converting enzyme
Ach	Acetylcholine
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
Apo	Apolipoprotein
ASC	Apoptotic speck protein
AU	Arbitrary units
AUC	Area under the curve
BH4	Tetrahydrobiopterin-4
BMI	Body mass index
CD14	Cluster of differentiation-14
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
CMR	Chylomicron remnant
CRP	C reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DRV	Dietary reference value
DVP	Digital volume pulse
eNOS	Endothelial nitric oxide synthase
FMD	Flow-mediated dilatation
GLM	General linear model
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
HR	Heart rate
IAUC	Incremental area under the curve
IKKβ	IκB kinase β
IL	Interleukin
LDI	Laser Doppler imaging

LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MaxC	Maximum concentration
MD2	Myeloid differentiation protein-2
MinC	Minimum concentration
MUFA	Monounsaturated fat
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor kappa B
NLRP3	Nucleotide-binding domain, leucine-rich, repeat containing family, pyrin domain-containing-3
NO	Nitric oxide
NOx	Total nitrate and nitrite
PA	Palmitic acid
PP	Pulse pressure
PUFA	Polyunsaturated fat
RAS	Renin-angiotensin system
RCT	Randomised controlled trial
RE	Retinyl esters
RI	Reflection index
ROS	Reactive oxygen species
rQUICKI	Revised quantitative insulin sensitivity check index
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
Sf	Svedberg flotation rate
SFA	Saturated fat
SI	Stiffness index
sICAM-1	Soluble intercellular cell adhesion molecule-1
SNP	Sodium nitroprusside
sVCAM-1	Soluble vascular cell adhesion molecule-1
TC	Total cholesterol
TLR	Toll-like receptors

TNFα	Tumour necrosis factor alpha
TRL	TAG-rich lipoproteins
TTMax	Time to reach maximum concentration
TTMin	Time to reach minimum concentration
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cells
WBC	Whole blood culture

1. INTRODUCTION

1.1 Dietary fatty acids and cardiovascular disease

1.1.1 Dietary fatty acids

Fatty acids play diverse roles within the human body. They are important as i) sources of energy, ii) structural components of cell membranes, iii) cell signalling molecules and regulators of gene expression, iv) precursors of bioactive lipid mediators, such as eicosanoids that regulate inflammation, and v) aid the absorption of fat-soluble vitamins. Dietary fats consist of 95% triacylglycerol (TAG), formed when a glycerol backbone bonds to three fatty acids (hydrocarbon chains of varying length with methyl and carboxyl groups at either end). Fatty acids are classified according to their degree of saturation: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The chemical properties, types and dietary sources of the fatty acids classes are summarised in **Table 1.1**.

Table 1.1 Classes of dietary fatty acids: chemical properties, types and dietary sources

	SFA	MUFA	n-6 PUFA	n-3 PUFA
No. of double bonds	0	1	≥ 2 n-6: First on carbon 6 from methyl end n-3: First on carbon 3 from methyl end	
Structure				
Consistency at room temperature	Solid	Liquid or semi-liquid	Liquid	
Common types of dietary fatty acids	Lauric (C12:0) Myristic (C14:0) Palmitic (C16:0) Stearic (C18:0)	Oleic (C18:1 n-9)	Linoleic (C18:2 n-6)	α-Linolenic (C18:3 n-3) Eicosapentaenoic (C20:5 n-3) Docosapentaenoic (C22:5 n-3) Docosahexaenoic (C22:6 n-3)
Dietary sources	Dairy products, Meat fat, Coconut oil, Manufactured goods using the above or palm oil	Olive oil, Rapeseed oil, High oleic, sunflower oil, Hazelnuts	Sunflower oil, Safflower oil, Peanuts	Oily fish, Walnuts, Linseeds, Flaxseed oil

MUFA and PUFA also exist as geometric isomers: *cis* and *trans*. Both are mostly present in food in the *cis* configuration (hydrogen atoms on the same side of the double bond), although they also exist in the *trans* configuration (hydrogen atoms on the opposite sides of the double bond) but in much smaller quantities. They are naturally present in dairy products and meat from ruminant animals, but are also formed during the hydrogenation of vegetable oils, which are used to produce spreads and manufactured goods, e.g. biscuits and cakes. However, the use of hydrogenated vegetable oils, and subsequently dietary intakes of these *trans* fatty acids, has dramatically fallen in recent years due to studies reporting their detrimental effects on the plasma lipid profile (1, 2).

In the UK, population-based recommendations for intakes of dietary fatty acids are given as Dietary Reference Values (DRV) (3). **Table 1.2** presents the corresponding mean daily intakes for UK men and women aged 19-64 y as determined by the National Diet and Nutrition Survey 2008/2009 – 2011/2012 (4). Although adults meet the recommendation for total fat (≤33% of total energy (%TE)), current intakes of SFA exceed the DRV of ≤11%TE. However, SFA intake has dramatically fallen over the past 50 y in the UK after the introduction of the dietary fat recommendations and the lower population consumption of meat and dairy products (4).

Table 1.2. Mean daily intake of fatty acids and dietary reference values for adults in UK

Dietary fat	Mean daily intakes (%TE)[a]		Dietary reference values including alcohol (%TE)[b]
	Men	Women	
Total fat	32.8	33.0	≤ 33
SFA	11.9	12.1	≤ 10
cis-MUFA	12.0	11.9	12[c]
n-6 PUFA	4.8	4.9	*Total PUFA*
n-3 PUFA	0.9	1.0	6.0

[a] Mean daily intakes taken from the National Diet and Nutritional Surveys of adults aged 19-64 y from Years 1-4 of the Rolling Programme between 2008/09-2011/12 (4)

[b] UK dietary reference values as % of total energy (%TE) (3)

[c] No recommendation for MUFA, value based on population averages

1.1.2 Dietary fats and cardio vascular disease

Cardiovascular disease (CVD) is the collective term for diseases of the heart and circulation, including coronary heart disease (CHD), angina, myocardial infarction, congenital heart disease, peripheral vascular disease and stroke. In the UK, CVD is the leading cause of death, accounting for 25% of all deaths in women and 27% in men and costing the National Health Service £4,292 million in 2013/14 (5). The aetiology for CVD is multifactorial, including numerous modifiable risk factors, such as cigarette smoking, inactivity, obesity, type 2 diabetes mellitus, hyperlipidaemia and hypertension (6). Diet, in particular dietary fat composition, has also been recognised to play an important part in the aetiology of CVD. A reduction in population-wide intakes of SFA is a major dietary guideline recommended by the UK's Department of Health for the prevention of CVD, although the optimal unsaturated fat (MUFA or n-6 PUFA) to replace SFA is unclear. Strong convincing evidence has shown the risk of CVD increases with higher intakes of SFA (7, 8). The large Seven Countries epidemiological study reported a positive association between intakes of certain SFA (lauric and myristic acids) and CHD mortality risk (9). Furthermore, the Strong Heart study reported total fat, SFA and MUFA intake as strong predictors of CVD mortality in middle-aged American Indians, independent of other established risk factors, although this was not observed for older adults (10). However, others have reported contrasting findings. A meta-analysis of prospective epidemiological studies claimed there was no significant evidence to conclude dietary SFA is associated with increased risk of CVD (11, 12), whereas a large follow up study in Japan reported a positive association between SFA intake and the incidence of myocardial infarction (13). However, these analyses did not take into account the macronutrient which was replacing SFA in the diet.

Substituting SFA with unsaturated fats is thought to have a beneficial impact on CVD risk. A critical review reported a 17% reduction in CVD risk when replacing SFA with unsaturated fatty acids (PUFA and MUFA), offering a greater benefit than by reducing total fat intake (14). Likewise, a pooled analyses of prospective cohort studies showed strong evidence of dietary MUFA in Mediterranean being associated with a 20% risk reduction in CVD events (15), while the Nurses' Health Study reported lower risk of CVD and stroke in women with greater adherence to the Mediterranean diet, which is characterised by high intakes of MUFA from olive oil (16). However, pooled data from 11 American and European cohorts reported a significant inverse association between high PUFA (including n-3 and n-6 fatty acids; primarily n-6 linoleic acid) diets and both coronary events and deaths but no

impact of MUFA. The authors concluded that replacing SFA with PUFA, rather than MUFA, would lower CVD risk over a wide range of intakes (17). Supporting this finding, the Nurses' Health Study reported an inverse relationship between both n-6 and n-3 PUFA intakes and CVD risk after a 20 y follow up, particularly among younger or overweight women, and the findings of a meta-analysis of randomised controlled trials (RCT), which included dietary intervention studies last for one year or more, indicated that replacing SFA with n-6 PUFA reduces both CHD events and deaths (18, 19). The Cardiovascular Health Study of 2792 older adults (20) reported high circulating linoleic acid, as measured in plasma phospholipids, but not other n-6 PUFA, was inversely associated with CVD mortality after 18 y. Likewise, a meta-analysis of prospective observational studies showed dietary linoleic acid to be inversely associated with CHD risk in a dose-response manner, supporting current recommendations to replace SFA with n-6 PUFA for the primary prevention of CHD (21). Another systematic and meta-analysis of prospective, observational and RCT did not support guidelines encouraging high consumption of PUFA and low consumption of SFA (11), but this analysis has been criticised for failing to take into account the macronutrient substituting SFA, and the presence of trans fatty acids in the PUFA interventions. Furthermore, a meta-analysis of RCT showed n-6 PUFA-specific interventions tend to elevate CHD risk, recommending that the advice to maintain or increase intakes of n-6 PUFA is reconsidered as there was no indication of benefits, yet there was the possibility of adverse effect (22). Overall, these findings are not conclusive and further research is required to determine both the impact of SFA on CVD risk and the optimal unsaturated fat to replace SFA.

1.1.3 Dietary fats and classic CVD risk factors

There is much evidence to suggest that classic risk factors of CVD, such as plasma lipids and blood pressure, are influenced by the composition of dietary fatty acids. High plasma concentrations of TAG, total cholesterol and low-density lipoprotein cholesterol (LDL-C) and low concentrations of high-density lipoprotein cholesterol (HDL-C) are all linked to an increased risk of CVD (23-25). Raised blood pressure is also a risk factor of CVD (26), and, compared with men, women have an increased risk of hypertension as they age, particularly after the menopause (27). Both hyperlipidaemia and hypertension are widely prevalent within the UK population and are a burden to the National Health Service. Therefore, dietary strategies, such as fatty acid manipulation, aimed at improving these risk factors are important. Two meta-analyses of 27 (28) and 60 studies (29) identified positive associations between SFA intake and the ratio of total cholesterol to HDL-C, a risk factor of CVD.

Furthermore, a number of studies have also reported a relationship between high intakes of SFA and raised circulating total cholesterol and LDL-C (28-31). However, not all types of SFA are considered to have the same impact on plasma lipids. Lauric, myristic and palmitic fatty acids are reported to be mostly responsible for the detrimental effects on plasma lipids, whereas stearic acid is considered neutral (28, 29, 32, 33). Replacement of SFA with MUFA or PUFA is associated with a reduction in CVD risk through the reduction of LDL-C, without impacting on HDL-C or TAG (34). Both PUFA and MUFA-rich diets decreased total cholesterol and LDL-C in adults (n=130, age, 46 y ± 14) with moderate CVD risk compared with baseline in a randomised, double blind crossover study, although the MUFA-rich diet increased HDL-C and reduced both TAG and systolic blood pressure (SBP) more than the PUFA-rich diet (35). Moreover, a critical assessment of RCT revealed that, relative to replacement with carbohydrates, replacing SFA with MUFA prevented CVD risk by favourably improving blood lipids, blood pressure and insulin sensitivity. However, compared to PUFA, the MUFA diet was hypotensive but had slightly less or comparable plasma LDL-C and total cholesterol lowering effects (36).

Fatty acid composition is also thought to impact on blood pressure. Strong positive associations were identified between SBP and both plasma phospholipid SFA in 4033 healthy middle-aged men (37) and dietary SFA intake in 1497 randomly selected adults (38). In the DIVAS study, replacement of 9.6 %TE dietary SFA with MUFA for 16 wk significantly attenuated the increase in night SBP in 195 men and women at moderately increased risk of CVD, although there was no impact of replacing SFA with n-6 PUFA (30). Another 3-month controlled parallel study also reported significant reductions in both fasting diastolic blood pressure (DBP) and SBP in response to a MUFA-rich diet (23%TE MUFA) compared with a SFA-rich diet (17%TE SFA) (39). A meta-analysis of 9 RCT identified beneficial effects of long-term (≥6 months) high-MUFA dietary interventions (>12%TE MUFA) compared to low-MUFA dietary interventions (≤12%TE MUFA) on blood pressure (40). However, the current data are conflicting and limited for the impact of n-6 PUFA on blood pressure. Even though the majority of dietary PUFA comes from n-6 PUFA, many epidemiological studies do not differentiate between n-3 PUFA and n-6 PUFA. An inverse association between linoleic acid (plasma phospholipid and dietary) and SBP has been reported in over 4000 healthy middle-aged men and adults aged 25-93 y, respectively (37, 41). However, a 4-year follow-up consisting of 60,000 US females from the Nurses' Health study did not demonstrate an association between n-6 PUFA intakes and hypertension (42).

Likewise, the Sydney Diet Heart Intervention Study that replaced dietary SFA with n-6 PUFA in men aged 30-59 y with recent coronary events did not report a change in SBP or DBP after 12-months relative to a control group (no intervention) (43). However, this paper used data from a study performed in the 1980s which may not relate to populations who have not previously suffered a coronary event. Therefore, it is important to investigate the effects of n-6 PUFA on blood pressure further.

1.2 Vascular function

1.2.1 Dietary fats and vascular function

Vascular function is a measure of cardiovascular health. It refers to the regulation of blood flow, arterial pressure, capillary recruitment and filtration, and central venous pressure, all of which are controlled by a multitude of extrinsic and intrinsic mechanisms of the body. The components of impaired vascular function, including hypertension (26), arterial stiffness (44) and impaired endothelial dependent vasodilation (45), are all associated with cardiovascular mortality and are considered to be risk factors of CVD. Evidence is emerging that vascular function is influenced by diet, of which dietary fat is a potentially important modulator. The impact of dietary fat on classic risk factors of CVD has been well-researched, however, their effects on vascular function is less clear, prompting two reviews by Hall and Vafeiadou et al to conclude there is a requirement for suitability powered, robust RCT to investigate this further (46, 47).

1.2.2 The role of endothelium and its role in regulating vascular tone

The endothelium is a monolayer of endothelial cells that line the internal surface of blood vessels. Under normal physiological situations, the endothelium preserves vascular wall homeostasis by maintaining the balance of vasodilators (e.g. nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor) and vasoconstrictors (e.g. endothelin-1, angiotensin-II, thromboxane, prostaglandin H_2, and oxidant radicals), all of which are produced by the endothelium. The vasodilators are anti-thrombotic, anti-inflammatory, anti-atherogenic, anti-coagulant and fibrinolytic, whereas the vasoconstrictors are pro-thrombotic, pro-inflammatory, pro-atherogenic, and pro-coagulant (48). NO is a potent endothelial-derived relaxing factor, which regulates vascular tone and reactivity (49). The NO gas is produced following activation of the enzyme, endothelial nitric oxide synthase (eNOS) (**Figure 1.1**). Inactive eNOS is located in the endothelial cell membrane bound to

caveolin. Changes to the local environment, including pharmacological stimuli, such as acetylcholine (ACh), and physical stimuli, such as shear stress, trigger the release of calcium ions from the endoplasmic reticulum (50). An increase in intracellular calcium ions activates eNOS through its binding with calmodulin and subsequent release from caveolin, where it converts L-arginine into NO and L-citrulline. NO diffuses into the underlying vascular smooth muscle cells (VSMC), where it binds to soluble guanylate cyclase, producing guanosine monophosphate from guanosine triphosphate (51). This in turn activates protein kinase G, reducing the release of calcium ions from the sarcoplasmic reticulum (52). A reduction in myosin light chain kinase activity relaxes the VSMC, allowing blood vessels to dilate and blood flow to increase. This is known as endothelium-dependent vasodilation.
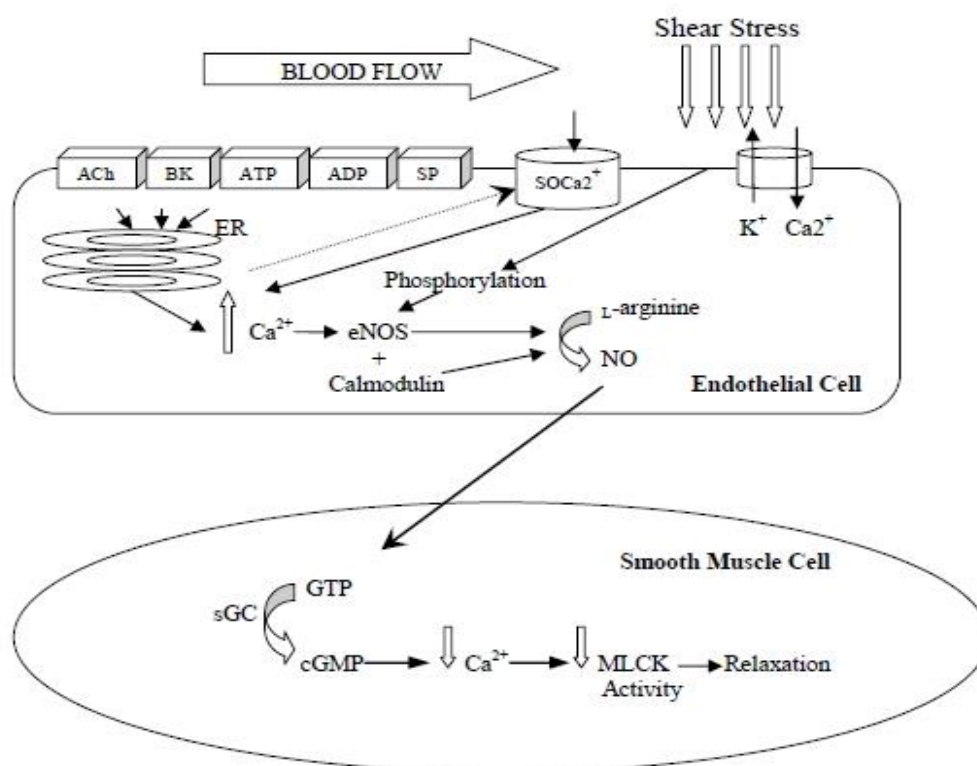
Figure 1.1 Endothelial-dependent vasodilation by endothelial NO production. Image taken from (51). Abbreviations: Ach: acetylcholine; BK: bradykinin; ATP: adenosine triphosphate; ADP: adenosine diphosphate; SP: substance P; $SOCa^{2+}$: store-operated Ca^{2+} channel; ER: endoplasmic reticulum; NO: nitric oxide; sGC: soluble guanylyl cyclase; cGMP: cyclic guanosine-3', 5-monophosphate; MLCK: myosin light chain kinase.

1.2.3 Endothelial dysfunction and the progression of atherosclerosis

CVDs are clinical manifestations of advanced atherosclerosis (Schwenke, 1998), a chronic inflammatory disease characterised by the gradual build-up of fibrous and fatty material inside the innermost layer of the artery walls (intima) causing them to narrow (53-55). The endothelium regulates vascular wall homeostasis, but prolonged activation of these protective mechanisms to adverse stimuli impairs the balance of endothelial regulation, known as 'endothelial dysfunction'. This is characterised by reduced production or availability of endothelial-derived vasodilators, particularly NO, creating an imbalance between vasodilators and vasoconstrictors and impairing endothelium-dependant vasodilation (48). Endothelial dysfunction is a key early step in the development and progression of atherosclerosis as well as the occurrence of atherosclerotic complications (53) and therefore, the vasculature has emerged as an important modifiable target for dietary therapies targeting CVD risk reduction.

Early atherosclerotic lesions (fatty streaks) originate early in life (infants and young children) and may even be influenced by maternal hypercholesterolaemia (56). At the initial stages of atheroma development, excess LDL-C accumulates within the arterial intima. Due to lack of antioxidants in the blood vessel wall, the lipid fraction is oxidised by oxygen radicals in the intima, producing minimally modified LDL, which is further degraded into oxidised LDL (**Figure 1.2**). Exposure to modified LDL promotes the recruitment of circulating monocytes and T lymphocytes. Adhesion molecules (e.g. intercellular-cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)), selectins (e.g. E-selectin and P-selectin) and chemoattractant chemokines (e.g. monocyte chemoattractant protein-1) present on the surface of activated endothelial cells mediate monocyte adhesion, permitting their entry into the intima. A healthy endothelium will generally resist monocyte adhesion, however, pro-inflammatory stimuli, such as a diet rich in SFA, hypercholesterolaemia, obesity, insulin resistance, hypertension, and smoking, promote adhesion. Here, the monocytes differentiate into macrophages (57), and scavenger receptors present on the surface of the macrophages recognise and internalise oxidised LDL. After excessive engulfing of LDL, the macrophages transform into lipid-laden foam cells, promoted by macrophage colony stimulating factor and cytokines, e.g. interferon-γ. Together, the accumulation of LDL and activated cellular components further promote VSMC cell proliferation, uptake of oxidized LDL, production of foam cells and localised inflammation. Over decades, the initial reversible fatty streak develops into a larger and

complex raised lesion and a fibrous cap surrounding the lipid core develops (58). In the final stages of atherosclerosis, the death of foam cells and VSMC creates a 'necrotic core' within the plaque. Symptoms of CVD occur when the plaque is large enough to restrict blood flow to the organs (ischaemic injury) or a weak fibrous cap ruptures, activating platelets to produce a thrombus. Partial or complete blockage of the lumen results in myocardial infarction, stroke or sudden cardiac death.
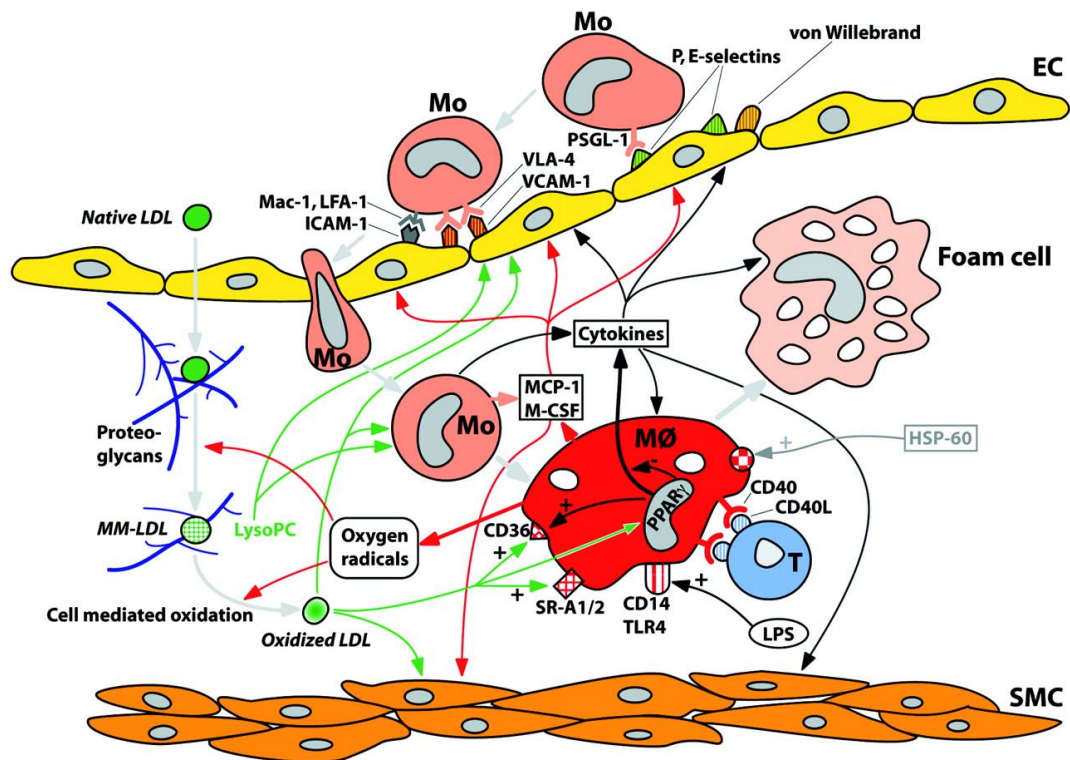
Figure 1.2 Development of atherosclerosis in the intima. Image taken from (57). Abbreviations: EC, endothelial cells; HSP-60, heat shock protein; IFN- γ, interferon-gamma; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony stimulating factor; MO, monocytes; MM-LDL, minimally modified LDL; PAF, platelet activating factor; PPAR-γ, peroxisome proliferator-activated receptor; SMC, smooth muscle cells; TLR4, toll-like receptor 4; VCAM-1, vascular cell adhesion molecule.

1.3 Assessment of vascular function: methods and clinical relevance

Vascular function includes endothelial function and arterial stiffness, which can be assessed non-invasively in the peripheral circulation by a number of techniques as described below.

1.3.1 Flow-mediated dilatation (FMD)

FMD is considered the gold standard non-invasive technique used to assess endothelial function of the conduit arteries in the peripheral circulation. Studies have reported a strong correlation between the FMD in the brachial artery and invasive coronary endothelial function (59). The technique uses high resolution ultrasound to visualise a two-dimensional longitudinal image of the brachial artery, and electrocardiogram monitoring ensures that the artery is recorded at the same phase of the cardiac cycle. During the FMD measurement, a sphygmomanometer pressure cuff is placed on the upper arm or forearm and inflated to a suprasystolic pressure (above 220 mm Hg) causing arterial occlusion for 5 min after which it is immediately deflated. This inflation and deflation increases blood flow in the brachial artery (reactive hyperaemia) (60). The resulting action of shear stress on the arterial wall induces the release of NO from the endothelium via the activation of eNOS as previously described (Figure 1.1). The subsequent dilation of the brachial artery is referred to as FMD, which is quantified as the percentage increase in the diameter of the brachial artery during reactive hyperaemia relative to the pre-occlusion diameter; a larger % FMD response refers to greater endothelial function in the macrovasculature. Impaired % FMD is a predictor of CVD risk. A meta-analysis of prospective studies with a 1 y follow-up showed brachial FMD to be inversely associated with future CVD events in adults (61), supporting the findings of Inaba et al (62). FMD was also identified as an independent predictor of CVD events in a prospective study of 2,264 asymptomatic postmenopausal women (63), a population-based multi-ethnic cohort study (64) and a cardiovascular health study in older adults (45). Furthermore, impaired FMD was reported in patients with CHD and hypertension (65, 66).

1.3.2 Laser Doppler imaging (LDI) with iontophoresis

LDI is a technique used to determine the vasodilatory response of the peripheral microvasculature to vasoactive stimuli, such as administration of vasodilators or reactive hyperaemia. Impaired blood flow in the microcirculation has been proposed to be an indicator of initial endothelial damage in subjects at risk of CVD (67). The LDI technique is based on the reflection of a beam of low-intensity infrared laser light, whose wavelength changes when light hits the moving blood cells in the underlying small blood vessels from which a 2D colour-coded image is produced for the quantification of blood flow at the site of stimuli administration. Blood flow or 'flux' (measured in arbitrary perfusion units) is proportional to the average speed and concentration of blood cells. LDI can be coupled with iontophoresis to deliver vasoactive stimuli to the microvessels using a weak electrical current

to increase blood perfusion of the microvasculature. Two such stimuli are ACh and sodium nitroprusside (SNP). ACh determines endothelial-dependent vasodilation via the production of NO from eNOS, whereas SNP breaks down to release NO and assesses endothelial-independent vasodilation. It was reported that the vasodilatory responses to Ach and SNP were lower in patients with peripheral arterial occlusive disease than in age and sex matched healthy controls (68). Furthermore, a strong positive linear relationship ($r= 0.92$) was found between the LDI response to ACh and FMD suggesting a strong association between endothelial function in the macro- and microvasculature (69).

1.3.3 Digital volume pulse (DVP)

A DVP is a simple non-invasive method used to measure vascular tone (DVP-RI) and arterial stiffness (DVP-SI). A finger cuff attached to the index finger measures infrared light transmission through the pulp of the fingertip (photoplethysmography). The amount of light detected is proportional to the pressure pulse of the brachial artery and produces a wave form (**Figure 1.3A**). The first part of the waveform (systolic component) determines the pressure pulse transmitted between the aorta and fingertip, whereas the second part (diastolic component) determines the pressure pulse that is transmitted from the ventricle via the aorta to the lower body where it is reflected back along the aorta to the finger.
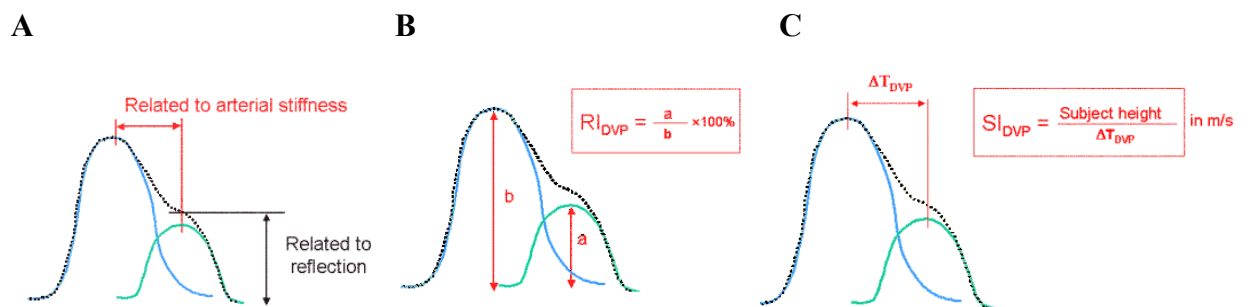
Figure 1.3 The formation of the DVP waveform (A) and its measures of vascular function: (B) Reflection Index (RI) and (C) Stiffness Index (SI).

DVP derives two indices of vascular function (70). The DVP-RI (**Figure 1.3B**) is derived from the height of the diastolic component expressed as a percentage of the systolic component and represents the degree of pressure wave reflection, an indicator of vascular tone in the small arteries. It is a measure of endothelial function in which greater impairment

is reflected by a higher DVP-RI. DVP-SI (expressed as m/s) reflects arterial stiffness and is associated with pulse wave velocity (the gold-standard measure of arterial stiffness) in the large arteries (71, 72). It is calculated from a person's height divided by the time delay between the systolic and diastolic peaks (**Figure 1.3C**); the time delay represents the time it takes for the reflected pressure wave to travel from the lower body back to the fingertip. DVP-SI is positively correlated with classical CVD risk factors, such as age, waist-to-hip ratio, blood pressure, and carotid intima-media thickness (73, 74).

1.4 Inflammation and cytokines: modulation by dietary fatty acids

Inflammation is involved at all stages of the atherosclerotic process, from the initial stages of lesion development to plaque rupture (75), and it has been recognised that both the innate (monocyte-derived macrophages) and the acquired immune system (T-cells) are involved in the atherogenic process. Both monocyte-derived macrophages and T-cells (mainly CD4+ and CD8+) present in atherosclerotic lesions produce a wide range of cytokines that can exert both pro-and anti-inflammatory effects. Dietary fat is a modulator of inflammation., in particular SFA pro-inflammatory and n-3 PUFA anti-inflammatory. Many *in vitro* and *in vivo* studies have shown the detrimental effects of SFA on the inflammatory responses (76, 77) and the proposed mechanism is that SFA stimulate inflammatory cytokine production (such as IL-6 and IL-1 β) by a pathway involving Toll-like receptor (TLR)-4. Briefly, as described by Teng et al. (**Figure 1.4)**, two potential pathways are involved in the SFA-mediated inflammatory mechanisms, including TLR-4 dependant and independent pathways (78-80). TLR-4 activates pro-inflammatory cytokine production by stimulating the expression and translocation of transcription factors including IKKβ and NF-κB to the nucleus leading to increased expression of pro-inflammatory cytokines via the TLR-4 dependant pathway. Furthermore, SFA is also thought to stimulate the production of pro-inflammatory IL-1β via a TLR-4 independent pathway involving the production of reactive oxygen species and stimulation of NF-κB, as shown in the **Figure 1.4.** A recent *in vitro* study has demonstrated that palmitic acid, a long chain saturated fatty acid mostly in the Western diet, stimulates pro-inflammatory cytokine IL- 1β via TLR-4.
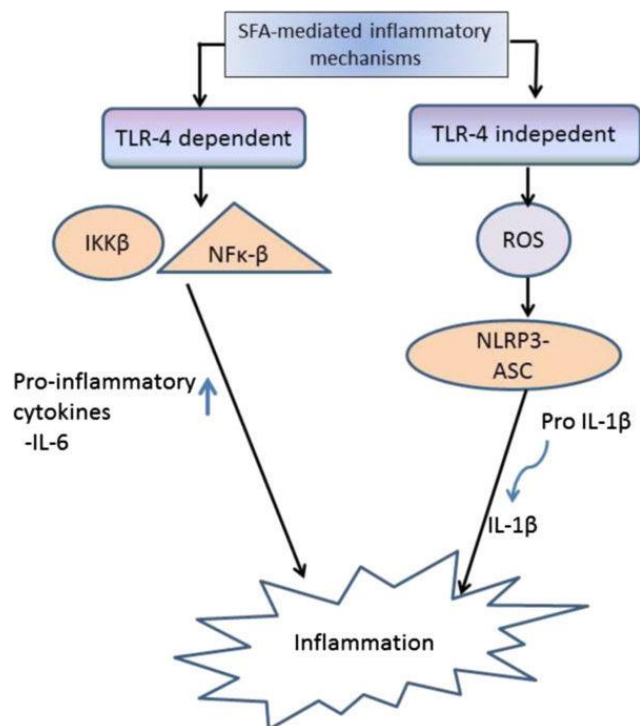
Figure 1.4 SFA mediated inflammatory mechanisms. Image taken from (80). Abbreviations: ASC, apoptotic speck protein; CD14, cluster of differentiation 14; IKKβ, IκB kinase β; IL, interleukin; MD2, myeloid differentiation protein 2; NF-κB, nuclear factor kappa B; NLRP3, nucleotide-binding domain, leucine-rich repeat containing family, pyrin domain-containing 3; ROS, reactive oxygen species; SFA, saturated fatty acids; TLR, Toll-like receptors

1.5 Postprandial lipaemia

Postprandial lipaemia describes the metabolic events that occur following the digestion and absorption of a meal that contains fat (81) and will be discussed further in Chapter 2. It is characterised by a rise in TAG-rich lipoproteins (TRL) after meal ingestion and is a dynamic non-steady-state condition, in which humans spend the majority of the day. The postprandial TAG response is influenced by a number of metabolic pathways including: a) rate of TAG release from the intestine and liver; b) activity of enzymes (lipoprotein lipase and hepatic lipase) that process TRL, and c) rate of clearance of TRL remnants by receptor-mediated processes. Increasing evidence highlights the importance of determining postprandial lipaemia to assess cardiovascular risk. Non-fasting TAG concentrations are associated with increased CVD risk, with hazard ratios of 2-4 in the highest versus the lowest levels of non-fasting TAG (82-84). Furthermore, these large prospective cohort studies have shown that the association with CVD events are two-fold greater for non-fasting than fasting TAG, in women (83, 85, 86).

1.5.1 Postprandial lipaemia and vascular function

CVD is considered to be a postprandial phenomenon, as oxidative stress created during postprandial stage appears to mediate postprandial vascular dysfunction (87). Previous studies have reported postprandial lipaemia associated CVD risk and the hypothesised mechanism for this effect is via lipaemia-induced oxidative stress (88, 89). The increased production of superoxide radicals due to excess mitochondrial lipid oxidation results in less bioavailability of vascular NO, which is the preferred scavenger for superoxide radicals. The reaction between superoxide radical and NO not only creates a loss of NO bioavailability but also results in formation of peroxynitrite (potent oxidant). Furthermore, superoxide radical and peroxynitrite will cause oxidation of tetrahydrobiopterin (BH4), a cofactor necessary for normal production of NO via eNOS, reducing NO bioavailability.

1.5.2 Acute studies investigating dietary fatty acid composition on postprandial lipaemia and vascular function

Traditionally, CVD risk factors have been measured in the fasting state. However, individuals living in Western societies, who consume regular meals and snacks, are thought to spend up to 18 h in the postprandial (fed) state. The postprandial lipaemic response is influenced by various factors, including lifestyle factors and physiological determinants, such as gender and age. The amount and composition of dietary fat in the test meal are also largely influential (90). With regards to fat quantity, it has been observed that single meals containing 30-50 g of fat give rise to dose-dependent increases in postprandial plasma TAG, with small differences in response to meals containing ≥80 g fat (81). Although the effects of substituting SFA with MUFA and n-6 PUFA on fasting lipid levels have been extensively studied in chronic interventions, the acute influence of fatty acid composition on postprandial lipaemia remains unclear. A recent meta-analysis with 12 crossover clinical trials investigated the postprandial TAG response to fat tolerance tests of varying fat composition reported no differences between SFA and unsaturated fats over 4-h whereas after 8-h, a lower TAG response to PUFA (both n-3 and n-6) and a trend to a lower response to MUFA were observed (91). Furthermore, it was suggested that postprandial studies shorter than 8-h may not be sufficient to differentiate TAG responses between fat tolerance tests with different fatty acid compositions.

The majority of RCT conducted in healthy volunteers have reported a clear impairment in postprandial vascular function following a high fat meal (92-97). However, the impact of

fatty acid composition on postprandial vascular function is not clear. Three reviews reported that the evidence for the vascular effects of meal fatty acid composition was limited and inconclusive (46, 47, 81). The authors of one review concluded there is weak evidence that SFA-rich meals modestly reduce postprandial vascular function, whereas the effects of MUFA and n-6 PUFA-rich meals were inconsistent (98). However, since the design of the postprandial test meal studies varied greatly (e.g. source of test fats, fat content and number of test meals, comparator meals, study populations, methods of assessing vascular function, and duration of postprandial measurements), the authors stated that comparisons were difficult. In conclusion, all reviews recommended that well-designed, suitably powered, RCT are performed to establish the postprandial effects of SFA, MUFA and n-6 PUFA-rich meals on postprandial vascular function.

1.6 The role of hormonal status on vascular function and postprandial lipid metabolism

It has been long identified that gender differences impact on the incidence of CVD as well as vascular function. In particular, premenopausal women have been shown to have a significantly reduced risk of cardiovascular events compared with men of a similar age, however, the risk is greater in older women, particularly after the menopause (99). Although ageing is associated with progressive endothelial dysfunction in both genders, it occurs earlier in men than women. Celermajer at al reported a decline in FMD in men aged ≥40 y at a rate of 0.21 % per y, whereas both the age of onset (≥50 y) and rate of decline (0.49 %/y) were greater in women (99). The most prominent gender-related difference in physiological ageing is the menopause in women (the cessation of menstruation), which usually occurs between the ages of 45 and 55 y (mean age 51 y) in the UK (100). The menopause contributes a significant cardiovascular milestone in terms of both physiology and pathology since premenopausal women have a significantly reduced risk of CVD compared with men of a similar age, although the incidence of events increases dramatically after the menopause (99) (**Figure 1.5**). CVD remains the leading cause of death in women in the UK but, despite this increased CVD risk, postmenopausal women remain an understudied group within the population. Hashimoto et al showed a steep decline in FMD in women around the time of the menopause highlighting the protective effect of physiological oestrogen levels on endothelial function (101). They also reported maximal endothelial-dependant vasodilation during the follicular and luteal phases of the menstrual cycle where there are elevated levels of endogenous oestrogen, such as oestradiol, which modulates endothelium-dependent vasodilation by increasing the endothelial production of NO and prostacyclin (101). Several

studies have also reported a significant association between oestrogen replacement in postmenopausal women and improved endothelial-dependant vasodilation in both the coronary and systemic circulations (102, 103). Furthermore, oral oestrogen replacement therapy in postmenopausal women showed an improvement in endothelial function after 9-weeks. The role of menopause on conventional CVD risk factors has been extensively researched. For example, oestrogen has been shown to lower total and LDL-C and increase HDL-C concentrations (104, 105). However, studies that determined the effect of menopause on vascular function, including those that investigate the impact of diet and meal composition, are hampered by a lack of data from well-designed, suitably powered RCT. A review of the literature regarding the impact of meal fat composition on vascular function, postprandial lipaemia and other risk markers of CVD in postmenopausal women is presented in Chapter 2.
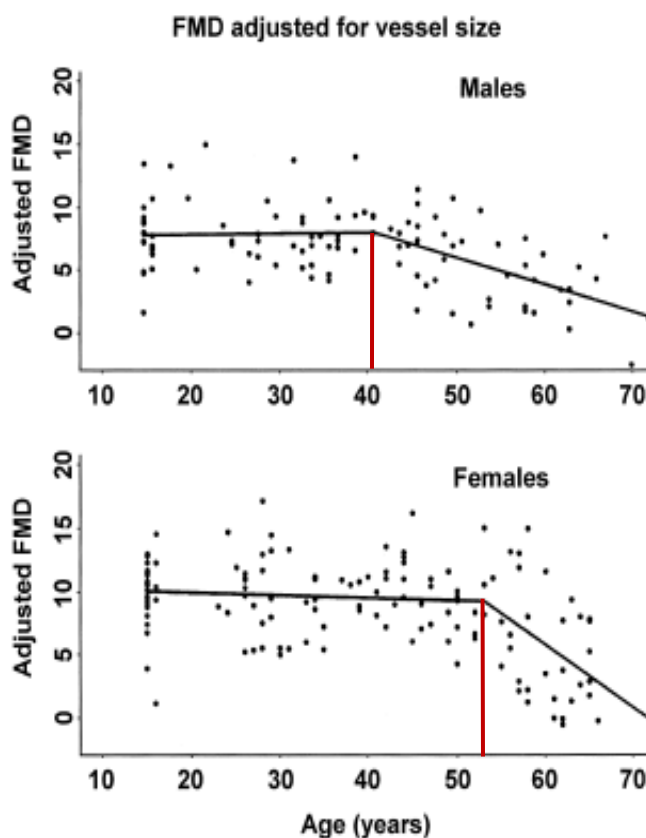
Figure 1.5 Comparison of aging associated progressive endothelial dysfunction in healthy men (n=103) and women (n=135) between the ages of 15 and 70 years. Image taken from (99). The red line denotes the age of onset for declining %FMD. Abbreviations: FMD, flow-mediated dilatation.

1.7 The role of genetic variations on vascular function and postprandial lipid metabolism

As well as modifiable risk factors, non-modifiable risk factors, such as genetic make-up, are thought to impact on CVD risk. Research is emerging to suggest that genetic variations can influence the responsiveness of CVD risk markers to changes in dietary fat intake. Therefore, personalised nutrition based on genotype may be an effective strategy to reduce the burden of CVD (106). However, the complex interactions between genotype and diet require a greater understanding before effective personalised nutritional advice can be administered.

1.7.1 eNOS genotype

As described in section 1.3.1, eNOS plays a vital role in endothelial function as it synthesises the potent vasodilator, NO. A common genetic variant of the eNOS protein is the single nucleotide polymorphism where thymine replaces guanine at position 984 of exon 7 (rs1799983). This changes the amino acid at position 298 of the eNOS protein in which aspartate replaces the wild-type glutamate (Glu298Asp polymorphism). Asp298 carriers (Asp/Asp and Asp/Glu, approximately 52% of the UK population (107)) have a greater incidence of CVD compared to Glu298 homozygotes (Glu/Glu) (107-109). This may result from a reduced capacity of eNOS to synthesise NO (110). Individuals who carry the minor Asp298 allele have been shown to have impaired vascular function (111, 112), plasma lipids (113, 114), blood pressure (115, 116), insulin resistance (114) and inflammation (117), although others dispute these findings (113, 118).

Limited evidence is emerging to suggest there is an impact of the eNOS Glu298Asp polymorphism on the responsiveness of postprandial vascular function to intakes of dietary fatty acids. A high-fat meal rich in SFA has been shown to lower fasting concentrations of nitrates and nitrates and impair microvascular reactivity in young, healthy male Asp298 carriers compared with Glu298 homozygotes (119). Another study by the same group also reported an impaired postprandial endothelial function to three different MUFA rich meals containing varying amounts of phenolic compounds in Asp298 carriers compared to Glu298 homozygotes (120). Gender may also be important. Previous diet-genotype studies reported impaired postprandial endothelial-dependant vasodilation, in particular in females homozygous for the minor Asp298 allele following a SFA-rich meal than SFA-rich meal containing long chain n-3 PUFA-rich (121, 122). However, to date, studies have not been conducted comparing SFA, PUFA and MUFA according to eNOS genotype which is

important considering population dietary recommendations for CVD risk advise the replacement of SFA with unsaturated fatty acids.

1.7.2 *APOE* genotype

The *APOE* genotype is the most widely researched single nucleotide polymorphism in relation to CVD risk. It is well known that *APOE* gene plays a key role in lipoprotein and cholesterol metabolism. The apolipoprotein (apo)E protein is a structural component of TRL (chylomicrons and VLDL), and HDL, and also acts as a high affinity ligand recognised by the LDL and hepatic chylomicron remnant receptors, mediating hepatic uptake of intermediate density lipoprotein and chylomicron remnants (123-125). The *APOE* gene has several polymorphic variants, however, the two most commonly examined single nucleotide polymorphisms are rs429358 and rs7412 derived from three common alleles in the population, ε4, ε3, and ε2. These combine to form 6 different genotypes, *E2/E2*, *E2/E3*, *E2/E4*, *E3/E3* (wild-type), *E3/E4*, and *E4/E4*. There is much evidence to suggest that the *APOE4* allele is linked with increased total cholesterol, LDL-C, CVD risk and mortality (126-130).

The *APOE* genotype has been shown to interact with total fat content and fatty acid composition both acutely and chronically. Given that the key role of apoE is the clearance of TRL, it has been shown that the *APOE* genotype may affect the postprandial lipid response (81, 90, 131). *APOE4* carriers have shown a greater responsiveness to changes in the amount and type of dietary fat compared with wild-type *E3/E3* individuals (127, 130, 132-134). In contrast, the SATgenε study showed dietary fat manipulation (high SFA diet, low SFA diet, and high SFA plus n-3 PUFA diet) to be a greater modulator of the postprandial lipid response compared with the *APOE* genotype (135). The impact of the *APOE* genotype on CVD risk biomarkers has been mainly researched with respect to SFA and long chain n-3 PUFA-rich diets/meals, with limited data on the effects of MUFA and n-6 PUFA-rich diets/meals (136-138). Therefore, there is a gap in the data reported to date regarding the influence of interactions between *APOE* genotype and diets/meals of varying fatty acid composition on vascular function and other CVD risk markers.

Chapter 2: Impact of meal fatty acid composition on postprandial lipaemia, vascular function and blood pressure in postmenopausal women: a review

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Contribution towards the manuscript

All authors agreed the search terms for the systematic search. I was responsible for conducting the literature searches on PubMed, sorting the abstracts for relevance and obtaining the complete articles. I was also responsible for interpreting the data and preparing the manuscript, which was critically appraised by all authors.

Impact of meal fatty acid composition on postprandial lipaemia, vascular function and blood pressure in postmenopausal women: a review

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Disclaimer: There are no conflicts of interest.

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Running title: Meal fat, lipaemia and vascular function

Abbreviations in the text
ACE: angiotensin-converting enzyme; Apo: apolipoprotein; AUC: area under the curve; BMI: body mass index; CETP: cholesteryl ester transfer protein; CM: chylomicron; CMR: chylomicrons remnants; CVD: cardiovascular disease; FMD: flow-mediated dilatation; HDL: high density lipoprotein; HDL-C: High density lipoprotein cholesterol; LDL: low density lipoprotein; LDL-C: low density lipoprotein cholesterol; LPL: lipoprotein lipase; MUFA: monounsaturated fat; NEFA: non-esterified fatty acids; NO: nitric oxide; PUFA: polyunsaturated fat; RAS: renin-angiotensin system; RCT: randomised clinical trial; RE: Retinyl esters; ROS; reactive oxygen species; Sf: Svedberg flotation rate; SFA: saturated fat; TAG: triacylglycerol; TC: total cholesterol; TRL: triacylglycerol-rich lipoprotein; VLDL: very low density lipoprotein

2.1 ABSTRACT

Cardiovascular diseases (CVD) are the leading cause of death in women globally, with aging associated with progressive endothelial dysfunction and increased CVD risk. Natural menopause is characterised by raised non-fasting triacylglycerol concentrations and impairment of vascular function but little is known about the mechanisms underlying the increase in CVD risk as women transition through the menopause. Dietary fat is an important modifiable risk factor in relation to both postprandial lipaemia and vascular reactivity. Meals rich in saturated and monounsaturated fatty acids are often associated with greater postprandial lipaemic responses compared with those containing n-6 polyunsaturated fatty acids, but studies comparing the effects of these fatty acids on vascular function during the postprandial phase are limited, particularly in postmenopausal women. A systematic search of the literature identified 778 publications, from which 12 relevant articles describing 11 independent studies examined the effects of high fat meals on postprandial lipaemia whereas one study also determined the impact on vascular reactivity. Although there is moderately consistent evidence to suggest detrimental effects of high fat meals on postprandial lipaemia in postmenopausal than premenopausal women, there is insufficient evidence to establish the impact of meals of differing fat composition. Furthermore, there is no robust evidence to conclude the impact of meal fatty acids on vascular function. In conclusion, there is an urgent requirement for suitably powered robust randomised controlled trials to investigate the impact of meal fat composition on postprandial lipaemia, vascular function and blood pressure in postmenopausal women, an understudied population at increased cardiometabolic risk.

2.2 INTRODUCTION

Cardiovascular diseases (CVD) include coronary heart disease (myocardial infarction and angina), stroke and peripheral vascular disease (139) are a key contributor to the burden of disease globally (140). Over the past 50 years, the prevalence of CVD has fallen in Western populations, however, CVD are currently the major cause of death in women in the UK, accounting for 32% of all deaths (141). Furthermore, the prevalence of CVD is dramatically increasing in other areas, including Eastern Europe, Asia and the Indian subcontinent (142).

The aetiology for CVD is multifactorial and includes several modifiable risk factors, such as cigarette smoking, a sedentary lifestyle, obesity, elevated blood pressure, dyslipidaemia, type 2 diabetes mellitus, and non-modifiable factors, such as advancing ageing, sex, family history of heart disease and ethnicity. Among the non-modifiable risk factors, ageing is associated with progressive endothelial dysfunction in both sexes, although it appears to occur earlier in men than women (99). The most prominent sex related difference in physiological ageing is the menopause (cessation of menstruation) in women, which usually occurs between the ages of 45 and 55 y, with 51 y being the average age of menopause in the UK (100). This natural part of aging in women contributes a significant cardiovascular milestone in terms of both physiology and pathology since oestrogen deficiency is known to impair cardiovascular function and metabolism, and the menopause is a recognised risk factor for CVD (143). It has been shown that premenopausal women have a significantly reduced risk of cardiovascular events compared with men of a similar age, although the incidence of such events increases dramatically after the menopause (144). It has further been shown by Schouw *et al.* (145) that for each year of delay in the age of onset of the natural menopause, CVD risk falls by 2%. This was further supported by data from the Nurses' Health Study in which a 50% reduction in the incidence of cardiac events after a 10 y follow-up was observed in women who underwent oestrogen replacement therapy (146).

Postprandial lipaemia and the Menopause

Postprandial lipaemia refers to the metabolic events that occur following the digestion and absorption of a meal that contains fat (81). With frequent meal ingestion, humans spend the majority of their time, approximately 17 h during each 24 h period, in this dynamic non-steady-state condition. Kolovou *et al.* (147) defined postprandial lipaemia as a complex syndrome characterised by non-fasting hypertriacylglycerolaemia that is associated with an

increased risk of cardiovascular events (148). Following a fat containing meal, there is a transient rise in circulating triacylglycerol (TAG) rich lipoproteins (TRL), such as chylomicrons (CM) and very low density lipoprotein (VLDL), which undergo two different metabolic pathways called exogenous and endogenous pathways, respectively. In brief, upon digestion of dietary fat, short-chain (fatty acids with fewer than 6 carbons) and medium-chain fatty acids (fatty acids with 6-12 carbons) are bound to albumin and transported to the liver directly via the hepatic portal vein. In contrast, long-chain fatty acids are re-esterified into TAG within the intestinal enterocytes and are transported in newly formed CM particles, which contains cholesteryl ester and TAG within their core with a surface of unesterified cholesterol, phospholipids and apolipoproteins (apo), in particular apo-B-48. After entering the circulation, the TAG component in the CMs is hydrolysed into free fatty acids (also referred to as non-esterified fatty acids (NEFA)) by lipoprotein lipase (LPL) present on the surface of endothelial cells in adipose and skeletal muscle tissues. The hydrolysed particles are known as CM remnants, which are rich in cholesteryl ester and are cleared by the liver by receptor-mediated uptake. Delayed hepatic clearance of CM remnants has been related to increased CVD risk in both animal and human studies(149, 150).

The VLDL particles facilitate the transport of endogenous TAG from the liver to the peripheral tissues. In the postprandial state, secretion of VLDL is regulated by insulin sensitive mechanisms in which insulin stimulates endothelial LPL to hydrolyse CM, releasing NEFA that are used by the liver to produce TAG for VLDL synthesis. VLDL follows a similar route of metabolism in the circulation as CM particles but VLDL is hydrolysed at a slower rate as it is smaller in size than CM, which are the preferential substrate for LPL. VLDL undergoes TAG depletion producing smaller VLDL known as intermediate density lipoprotein or VLDL remnants, and ultimately a proportion of these particles will be metabolised to low density lipoprotein (LDL). LDL particles are cleared from the bloodstream via the hepatic LDL receptor using apoB-100 as a ligand. During the postprandial period, there is an accumulation of TRL in the circulation due to competition between intestinal and hepatic TRL for the same lipolytic and receptor mediated uptake (151).

Since atherosclerosis is now also considered to be a postprandial phenomenon, three large prospective cohort studies have demonstrated the link with cardiovascular events to be two-fold greater for non-fasting than fasting TAG, especially in women (83, 85, 86). It has further

been shown in the Women's Health study that the strongest association with non-fasting TAG was reported to be 2-4 h after the last meal(85). Although raised LDL cholesterol (LDL-C) is an established risk factor for CVD, large prospective studies have shown that non-fasting TAG is considered to be a better predictor of CVD risk in women than fasting LDL-C (84, 152, 153). The relationship between postprandial lipaemia and CVD is a topic of current interest, yet the impact of menopausal status on the variability of the postprandial lipaemic response is less researched. It has been shown that, in general, premenopausal women have lower postprandial lipaemic responses than men (154-157), which is in contrast to the higher reported responses observed in postmenopausal women compared with men of a similar age (158). Postprandial hyperlipidaemia is strongly linked with atherosclerosis (159). A delayed clearance of TRLs in the circulation enhances the accumulation of TRLs particles carrying acceptor sites for the cholesteryl ester transfer protein (CETP) which is a plasma protein that facilitates the reciprocal transport of cholesteryl esters and TAG between lipoproteins. CETP transfers TAG from TRLs such as CM and VLDL and exchanges it with cholesteryl esters from high density lipoprotein (HDL) and LDL. Remodelling of the lipid content of the LDL and HDL particles make them suitable substrates for LPL and hepatic lipase, leading to the formation of smaller denser LDL (LDL_3) and HDL (HDL_3) particles. HDL_3 is rapidly removed from the circulation, thereby decreasing circulating HDL cholesterol (HDL-C) concentrations, which is one proposed mechanism for the inverse association between exaggerated postprandial lipaemia and CVD risk (160). Another possible mechanism is that LDL_3 has a lower binding affinity to the LDL-receptor, reducing their rate of clearance from the circulation and enabling them to infiltrate the arterial wall (81).

A reduction in endogenous oestrogen and progesterone is the major biochemical change that occurs in women after the menopause. These hormones not only play a major role in sexual physiology, but are also involved in various physiological processes associated with the vasculature and lipid metabolism. There are several mechanisms to explain the effects of oestradiol (the predominant type of oestrogen) on lipid metabolism. One possible mechanism, that was identified in *in vitro* animal studies, was an increase in the number of high affinity LDL receptors on liver cell membranes that enhances LDL uptake by the liver (161). In addition, oestradiol has a marked impact on the clearance of remnant lipoprotein particles (162). Exaggerated postprandial lipaemia is observed after the menopause (163) but the administration of even short term (two to six weeks) oestradiol therapy reduces

menopause-related postprandial TAG in postmenopausal women (164, 165). These findings indicate that 17 beta-estradiol may accelerate the postprandial clearance of TRL.

Vascular function and blood pressure: Menopause

In a healthy blood vessel, the endothelium (a single layer of endothelial cells that forms the innermost layer of blood vessels) plays a pivotal role in vascular wall homeostasis by immediately responding to blood-borne and locally produced stimuli to regulate blood flow, blood pressure and vascular tone. It does so by maintaining a precise balance between the release of endothelium-derived vasodilators, such as nitric oxide (NO), and vasoconstrictors, such as endothelin-I, which actively regulates vascular permeability to plasma constituents, platelets and leukocyte adhesion molecules (166) as well as aggregation and thrombosis (167). However, when the production or bioavailability of NO is reduced, the resulting imbalance of these vasoactive substances disrupts vascular homeostasis. This 'endothelial dysfunction' is characterised by vasoconstriction, increased expression of adhesion molecules and pro-inflammatory cytokines, platelet activation and increased oxidative stress (48), and has emerged as a critical early modifiable event in the development of coronary atherosclerosis (168) and CVD risk in postmenopausal women (63). There is supporting evidence of impaired endothelial function after the menopause, which has been associated with a lack of endogenous oestrogen (99, 169).

There are a number of non-invasive methods which are used to assess endothelial function (170). Flow-mediated dilatation (FMD) is the gold standard technique that uses ultrasound to assess endothelium-dependent vasodilation in the conduit arteries in the peripheral circulation and is used as a surrogate measure of NO production (171). It is therefore recognised as a screening tool to assess future cardiovascular risk (61, 62, 172). Rossi *et al.* (63) reported that postmenopausal women in the lowest tertile of % FMD response (reflective of impaired vascular reactivity) had the greatest relative risk of cardiovascular events . Furthermore, it has been shown that endothelial function is impaired across the stages of the menopause transition in healthy women with the highest % FMD response reported in premenopausal women, with a progressive decline in perimenopausal and postmenopausal women (173). This suggests the perimenopausal stage (the transition towards the menopause where oestrogen production starts to fall) is a crucial turning point in women where changes in CVD risk commence. It is still unclear how changes in ovarian

hormones during the potentially critical perimenopausal period contribute to the age-associated decline in endothelial function.

Current evidence suggests that oestrogen has several cardio-protective effects, including lowering of total and LDL-C, elevation of HDL-C (105) and reduction of fibrinogen and factor VII (174) (**Figure 2.1**). However it is estimated that lipid modifications only contribute 25% of the protective effect of oestrogens (175). Majmudar *et al.* (176) revealed that the menopausal status is associated with reduced NO activity, which is restored with oestrogen replacement therapy. It is recognised that changes in vascular NO activity may be an important mechanism facilitating the detrimental effect of the menopause on CVD risk and mortality. Another study that acutely administered oestrogen (17β-oestradiol) to postmenopausal women demonstrated protective effects on forearm microvascular responses to both endothelium-dependent (acetylcholine) and -independent vasodilation (sodium nitroprusside) via improvements in NO activity (102). Impaired blood flow in the microcirculation has been proposed to be an indicator of initial endothelial damage in subjects at risk of CVD (67). Furthermore, it has been repeatedly shown that 17β-oestradiol stimulates the production of vasodilatory prostaglandins, such as prostacyclin (PGI_2) (177, 178). These vascular effects are believed to be partly responsible for the long-term benefit of oestrogen therapy on cardiovascular events in postmenopausal women.

Hypertension (high blood pressure) is one of the main age-related disorders in postmenopausal women (179, 180) which has been identified as a leading risk factor for CVD, myocardial infarction and stroke (181) in women (27). The renin-angiotensin system (RAS) is a hormonal cascade, which plays a key role in the regulation of fluid and electrolyte balance, and arterial blood pressure. Upon activation of the RAS cascade, angiotensin II is produced in the liver by angiotensin-converting enzyme (ACE) following conversion of angiotensin I to angiotensin II (182). Angiotensin II is a potent vasoconstrictor which degrades bradykinin (a vasodilator) causing arterioles to constrict, resulting in increased blood pressure (183). It is well documented in the literature that oestrogen acts on RAS at different points of the cascade including the inhibition of ACE activity. Loss of oestrogen-dependent cardiovascular protection induces endothelial dysfunction, and may be involved in the activation of the RAS cascade. Evidence from both clinical and animal studies have shown an inverse association between oestrogen and the activation of RAS (184-187). *In vitro* and *in vivo* animal studies have also demonstrated the potential effects of oestrogen on

the endothelial-dependent vasodilator response to acetylcholine due to oestrogen induced sensitisation measured by vessel responsiveness to acetylcholine (188-190). It has been shown that an augmented level of angiotensin II due to oestrogen induced downregulation of angiotensin receptor I expression (185), which is a major component of RAS system, has several harmful effects on the vascular wall including vasoconstriction, vascular smooth muscle cell proliferation, reactive oxygen species (ROS) generation, and endothelial cell apoptosis (191-193). Oestrogen deficiency has also been reported to lead to an upregulation of ACE activity causing an accumulation of angiotensin II (194).

Impact of meal fat composition on postprandial lipaemia

Diet is one of the most important modifiable risk factors in relation to CVD (195). As a strategy to reduce the incidence of CVD, public health policy makers recommend that intakes of dietary saturated fatty acids (SFA) are reduced to <10% total energy in the UK. Substituting SFA with unsaturated fatty acids may provide additional benefits with regards to CVD risk factors, including reductions in the postprandial lipaemic response and improvements in endothelial function. A systematic review proposed that lowering dietary SFA intake by modifying dietary fat composition rather than reduction in total fat intake, may reduce cardiovascular events by 14% (196). Since individuals spend a large proportion of the day in the fed (postprandial) state, modifications to the fatty acid composition of our meals that are repeated on a daily basis may impact on postprandial lipaemia and vascular health, which over time could affect our risk of CVD.

The chronic effects of substitution of SFA with polyunsaturated fat (PUFA) on fasting lipid levels have been extensively studied (18), however, the acute affects are less well known. One systematic review and meta-analysis of crossover RCT has compared the effects of oral fat tolerance tests with differing fatty acid compositions on postprandial lipaemia in men and women (91). Relative to a single SFA-rich meal challenge, a PUFA-rich meal significantly reduced the postprandial lipaemic response over 8 h, whereas a trend for a reduced response was identified following a monounsaturated (MUFA) rich meal challenge. However, differences were not evident at 4 h suggesting that a longer follow-up time after the test meal (i.e. 8 h) is required to observe the acute effects of meal fatty acid composition on postprandial lipaemia. Of the 18 studies included in the review by Monfort-Pires *et al.* (91) none of the studies included postmenopausal women which reflects the paucity of data in this population subgroup.

Menopause oestrogen decline

Lipid metabolism

Vascular function

Blood pressure

- Dyslipidaemia in fasting state (↑ LDL-C and ↓ HDL-C)
- Increased fasting and non-fasting TAG
- Insulin resistance

- Reduced NO-mediated vasodilation via inhibition of following processes;
  - eNOS gene expression
  - eNOS phosphorylation which helps to increase enzyme activity and correlate with increased eNOS activity
  - expression of calmodulin, which is necessary for calcium activation of eNOS
- Decreased vasodilator PGI2 production by elevating levels of the two key synthetic enzymes, COX-1 and PGI2 synthase
- Increased vascular smooth muscle cell proliferation
- Increased arterial stiffness
- Increased adhesion of leukocytes
- Promote expression of adhesion molecules by endothelial cells
- Increased vascular inflammatory response via improved induction of vascular inflammatory mediators, such as iNOS and COX-2
- Increased production of ROS

Activation of renin-angiotensin system via

- Increased angiotensin II production due to increased AT 1 receptor density
- Increased ACE activity

Figure 2.1: Consequences of the decline in oestrogen during the menopause on the lipid profile, endothelial function and blood pressure.
Adapted from Davis *et al.*(197) Abbreviations: ACE; angiotensin converting enzyme, AT-1; angiotensin I receptor, COX; cyclooxygenase, eNOS; endothelial nitric oxide synthase, HDL; high density lipoprotein, iNOS; inducible NO synthase, LDL; low density lipoprotein, NO; nitric oxide, PGI; prostaglandin, RAS; renin-angiotensin system; ROS; reactive oxygen species, TAG; Triacylglycerol

Impact of meal fat composition on vascular function

West and colleagues (198) reported that consumption of a single high-fat meal (50-105 g of fat) can impair FMD by 45% to 80% with observations of impaired FMD within 2 to 5 h after a high-fat meal (94, 97, 199, 200). Prolonged postprandial lipaemia is known to induce endothelial dysfunction by promoting the formation of free radicals by accelerating the rate of β-oxidation of free fatty acids (e.g. superoxide radicals). Increased production of ROS or free radicals reduce the amount of bioactive NO by chemical inactivation to form toxic peroxynitrite (201). In addition, it has been shown that persisting oxidative stress will render endothelial nitric oxide synthase dysfunctional, markedly reducing NO production (202). Indeed, high concentrations of TRLs during postprandial lipaemia enhance inflammation by inducing the secretion of pro-inflammatory cytokines (203) and expression of soluble cell adhesion molecules (204).

Reviews by Hall (47) and Vafeiadou *et al.* (46) stated that the acute effects of dietary fats on vascular function is less researched. The authors concluded that high-fat meals have a detrimental effect on postprandial vascular function and there is limited and inconclusive evidence for the comparative effects of test meals rich in MUFA or n-6 PUFA with SFA. Of note, the data derived from these reviews were mainly from studies where the effects of a single high-fat meal on postprandial vascular function in different subject groups were determined; however, none of the studies identified in these reviews included postmenopausal women only. Since the published data is limited and inconsistent, the effect of test meals rich in SFA, n-6 PUFA and MUFA on postprandial lipaemia, vascular reactivity, blood pressure and biomarkers of vascular function and inflammation needs addressing in individuals at greater risk of CVD. Therefore, this review aimed to address this knowledge gap by systematically reviewing and critically evaluating the existing evidence from postprandial test meal studies including meals rich in these fatty acids in postmenopausal women. It is very timely to focus on postmenopausal women since they represent an understudied group within the population at increased CVD risk.

2.3 SUBJECTS AND METHODS

A systematic approach was used to identify all relevant published literature according to the method used by Vafeiadou *et al.* (46). The PubMed (http://www.ncbi. nlm.nih.gov/pubmed/) database was used to perform the literature search, which included all studies published in English until October 2016. A protocol that included search terms to conduct the literature

search was prepared by two authors (KMR and MW) and then agreed by all authors. Three categories of search terms were identified: i) study group search term (postmenopausal or post-menopausal or post menopause or menopause or menopausal); ii) exposure search terms (which included descriptors of SFA, MUFA and n-6 PUFA, and relevant food sources, e.g. butter, safflower oil and olive oil); iii) outcomes (which included descriptors of vascular function, blood pressure, biomarkers of vascular function and inflammation, and plasma lipids) (**Appendix I**). The Medical Subject Heading Browser (http://www.nlm.nih.gov/mesh/MBrowser.html) was used to identify relevant exposures and outcomes. Additional studies (n=2) were identified through hand searching of original articles found using the PubMed search. The titles and abstracts of all papers were assessed for relevance at the initial stage by one author (KMR) and any uncertainties were discussed with other members of the review team until a consensus was reached. This review was restricted to epidemiological studies (cross-sectional, case-control and cohort) and RCT in postmenopausal women with respect to SFA, MUFA and/or n-6 PUFA composition. Only published peer-reviewed literature was considered (i.e. 'grey' literature, such as dissertations, conference proceedings, reports, letters to editors and other non-peer-reviewed research were excluded). Although Hall (47) and Vafeiadou *et al*. (46) previously reviewed the chronic and acute studies on vascular function, they did not specifically address the acute effects in postmenopausal women. In this present review, we only considered acute studies as our objectives were to determine the impact of meal fatty acids on non- fasting TAG responses, vascular function and blood pressure as important CVD risk factor in postmenopausal women. A summary of the literature search and reasons for exclusion of the studies is shown in **Figure 2.2.**

2.4 RESULTS

This systematic search identified 778 publications in total. Of these, there were 12 relevant articles describing 11 independent studies in postmenopausal women that examined the acute effects of SFA and/or MUFA and/or n-6 PUFA on postprandial lipaemia (205-216), with one study also determining the effect on vascular function (212) (**Table 2.1**). No studies were identified that reported the acute impact of meal fatty acids on postprandial blood pressure, or biomarkers of vascular function and inflammation in postmenopausal women. Only one RCT, which is considered as the gold standard in terms of scientific evidence, was identified and this single-blind randomised crossover study compared the effects of meal fat composition on postprandial lipaemia using a sequential meal protocol (207, 210). A

multiple meal study design is considered much stronger than a single meal protocol because it more closely mimics the eating pattern of free-living individuals, particularly in westernised societies, and provokes a sustained lipaemic response. Five publications described cross-sectional epidemiological studies, which were single arm studies that did not have comparator meals (205, 212, 213, 215, 216). Among these postprandial studies with blood samples collected between 6 to 10 hours after the test meal, four studies (205-207, 210, 213) used a sequential two meal protocol, whereas the other studies (208, 209, 211, 212, 214-216) incorporated a single meal approach. In addition, five case-control studies were identified (206, 208, 209, 211, 214). Four compared the postprandial lipaemic response among pre-and postmenopausal women (206, 208, 211, 214), whereas one compared the response between normolipaemic, hypercholesterolaemic and mixed hyperlipidaemic postmenopausal women (209).

Data on these human studies will be presented in two sections that address the effects of total fat or fatty acid composition on i) postprandial lipaemia and ii) postprandial vascular function in postmenopausal women.
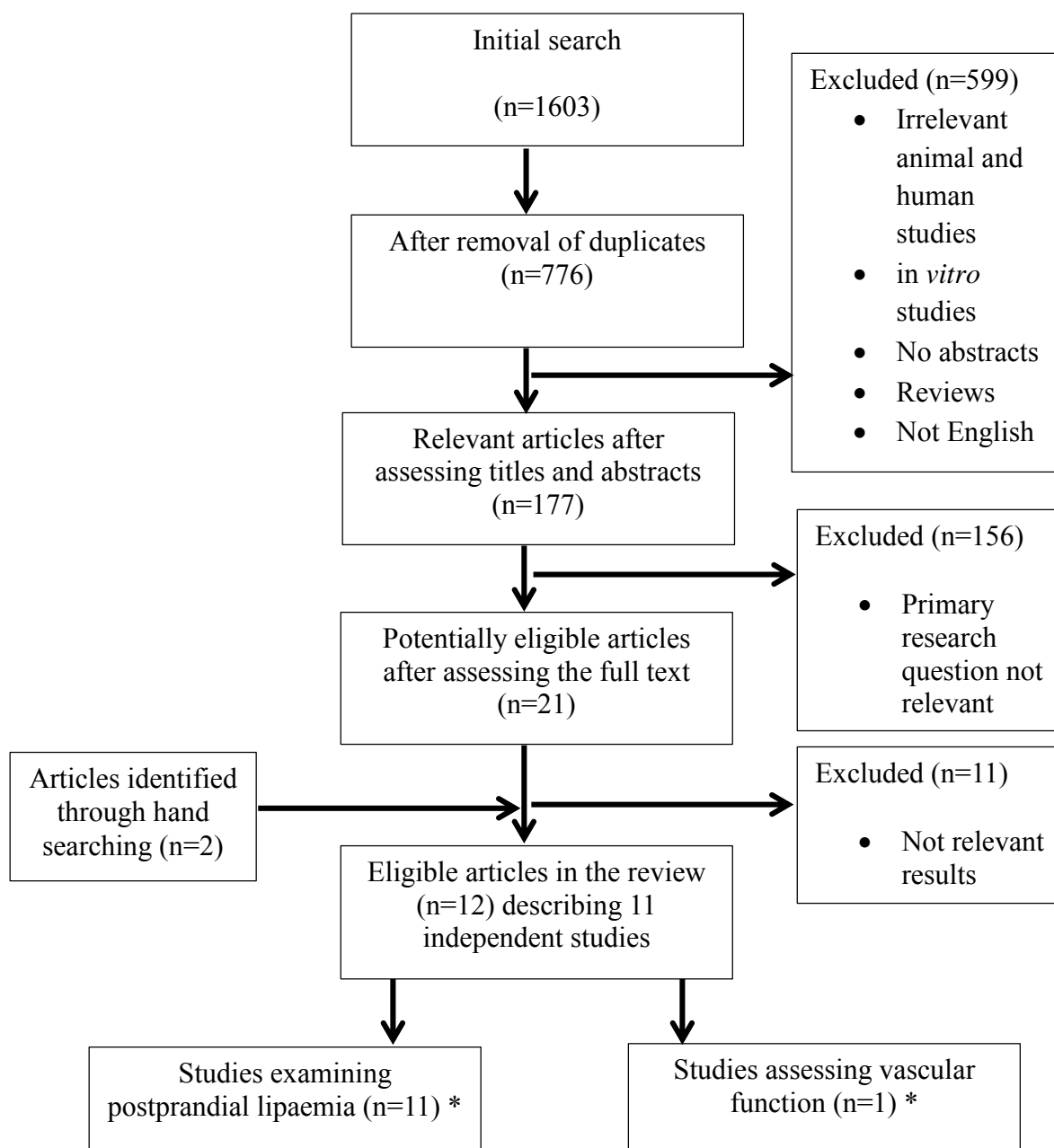
Figure 2.2: Flow of information through the different phases of the review

*Of the studies included in the review, one publication reported both postprandial lipaemia and vascular function.

2.5 DISCUSSION

Acute intervention studies: Postprandial lipaemia

There is consistent evidence from the test meal studies that high-fat meals have a detrimental effect on postprandial lipaemia in postmenopausal women (205, 212, 213, 215, 216) (**Table 2.1**). However, comparison of these findings are challenging due to differences in the nature of the fats and oils used in the test meal, the amount of fat, and postprandial follow up times, as well as the use of both single and sequential test meal protocols. In addition, all four case control studies (206, 208, 211, 214), which compared the postprandial lipaemic responses in postmenopausal with premenopausal women, reported higher TAG concentrations following high fat meals in the former group. Among all 12 articles (11 independent studies) reported in **Table 2.1**, only one study compared the postprandial lipaemic responses to high-fat meals containing SFA, MUFA, n-6 PUFA and a mixture of n-6 PUFA and n-3 PUFA (207, 210). In this study, volunteers ingested a high fat breakfast (40 g total fat) containing the assigned test fat followed by a low fat, high carbohydrate lunch (5.4 g total fat) given 5 h later. The authors observed significantly higher levels of plasma NEFA and lower insulin sensitivity following the SFA meal compared with the other test oils. During the postprandial state it has been shown that up to 50% of the liberated NEFA is dietary-derived CM-TAG due to the action of LPL upon TAG to release NEFA (217). Although this study did not determine the specific fatty acid composition of the circulating NEFA after consumption of the meals, similar studies have reported postprandial changes in the plasma NEFA profile to represent the fatty acid composition of the test meals (218). This study further examined the postprandial TAG and apo B-48 (the apolipoprotein specifically associated with CM) responses in three distinct TRL subfractions and reported significant differences in the apo B-48 time course profiles between the four different test oils. In particular, the MUFA meal resulted in the formation of a greater number of both large (Svedberg flotation rate (S_f)>400 fraction) and moderately (S_f 60-400 fraction) sized apo B-48 particles compared with the other three study meals. All studies identified in this review include postprandial blood sampling between 6 and 12 h after the first meal.

Postprandial lipaemia: Menopause

Four studies were identified which compared postprandial responses among pre- and postmenopausal women (206, 208, 211, 214). Beek et al. (214) investigated whether a natural menopause was associated with reduced protection from exaggerated postprandial lipaemia in normolipidaemic postmenopausal women (50 y, 24.6 kg/m^2, n=23), compared

with age and BMI matched premenopausal women (49 y, 24.1 kg/m^2, n=21). Following a single oral vitamin A fat-loading test, higher concentrations of postprandial plasma TAG and retinyl palmitate (an indirect marker of CM) were observed in postmenopausal women compared with premenopausal women of similar age, BMI, daily energy and fat intake, *APOE* genotype, LPL activity, and HDL-C concentration, even after adjusting for the confounding effect of fasting TAG. Masding *et al.* (208) also reported a significantly higher postprandial TAG response expressed as area under the curve (AUC) calculated over 6 h after the fat load (45 g) in postmenopausal compared with premenopausal women. This was further supported by Schoppen *et al.* (211) who reported similar findings after a single high-fat meal (75.3 g fat), although this study observed a significantly later time to reach the peak concentration and higher maximal TAG concentration in postmenopausal than premenopausal women.

Pirro et al. (209) investigated the changes in postprandial TAG concentrations after a standardised oral fat load (65 g of fat) at baseline, 4, 6 and 8 h in postmenopausal women with hypercholesterolaemia and mixed hyperlipidaemia and compared them with a control group of normolipidaemic women. A significantly greater postprandial TAG response was found in the mixed hypelipidaemic women than in the hypercholesterolaemic and normolipaemic women. Analysis from The Dietary Studies: Reading Unilever Postprandial Trials (DISRUPT) (206) database comparing the postprandial responses to sequential high-fat meals in 37 premenopausal and 61 postmenopausal women reported significantly greater incremental AUC and maximum TAG concentrations over 8 h in postmenopausal women. Post-hoc analysis of the menopausal groups according to age also revealed a greater increase in non-fasting TAG than fasting LDL-C during the late premenopausal period suggesting that age and the menopause have a differential impact on these two lipid CVD risk biomarkers. All fours studies (206, 208, 211, 214) reported consistent results that postmenopausal women have an exaggerated postprandial lipaemia compared with premenopausal women.

Vascular function: Menopause and acute effects of meal fatty acids composition

Only one study has also examined the acute impact of total fat and/or SFA and/or MUFA and/or n-6 PUFA on vascular reactivity. A significant decrease in %FMD at 2 h (2.3 ± 2.6 %) compared with baseline (7.7 ± 2.8 %, $P < 0.05$) was observed in healthy postmenopausal women after an oral fat load of 65 g (212) (**Table 1**). A comparator meal of a different fatty acid composition was not included in this study.

2.6 SUMMARY

A systematic approach was used to review the literature on the impact of meal fat composition (SFA, MUFA and n-6 PUFA) on postprandial lipaemia, blood pressure, vascular function and biomarkers of vascular function and inflammation in women, and the importance of declining vascular function and increasing postprandial lipaemia following transition through the menopause. There was moderately consistent evidence that reported greater postprandial TAG responses following a high fat meal in postmenopausal than premenopausal women. However, there is at present, an extremely limited number of RCT that have investigated the impact of meal fatty acid composition on measures of postprandial lipaemia and vascular function in postmenopausal women. Furthermore, differences in study designs (such as the absence of a comparator test meal, and differences in meal fat composition, study duration and outcome measures) prevent any firm conclusions being drawn from this literature review.

2.7 CONCLUSIONS

In conclusion, there is an urgent requirement for suitably powered RCT to investigate the effects of meal fat composition on postprandial lipaemia and vascular function in postmenopausal women. With the increased prevalence of non-communicable diseases in women, especially after the menopause, future studies should consider both healthy postmenopausal women and those at increased cardiometabolic risk using well-standardised measures of vascular function. Since non-fasting TAG is an important CVD risk factor for women, it is essential to use robust test meal protocols that are more reflective of habitual eating patterns to gain a greater understanding of the postprandial handling of different dietary fats.

2.8 ACKNOWLEDGEMENTS

Table 2.1 Acute test meal studies investigating the effects of meal fat content and composition on postprandial lipaemia and vascular function in postmenopausal women

Reference	Subject group, age (mean) and n	Study design	Meal type	Amount of fat (% meal fat if available)	Fatty acid composition	Time of postprandial data	Postprandial measurements	Significant outcomes compared to baseline, unless otherwise stated
Postprandial lipaemia								
Westerveld *et al.* (1996)[216]	59 y n 16 normolipidaemic	Cross sectional	Single	50 g (40%)	PUFA: SFA 0.06	8 h	TAG, HDL and plasma HDL-Apo A-1	↓HDL at 3 to 8 h (p<0.05), ↓HDL-Apo A-1 at 3 and 6 h (p<0.05) ↑TAG at 8 h (p<0.05)
van Beek *et al.* (1999)[214]	47-52 y (50 y) n 23 PoM women and 47-52 y (49 y) n 21 PrM women	Case control	Single	50 g (40%)	PUFA: SFA 0.06	12 h	TAG and Vitamin A/retinyl palmitate	↑TAG AUC at 0-8h (p=0.024) ↑TAG ΔAUC (p=0.020) in PoM compared to PrM at 0-8h ↑Vitamin A AUC (p=0.001) in PoM compared to PrM at 0-8h
Pirro *et al.* (2001)[209]	57 y n 17 normolipaemic, 54 y n 17 hypercholesterolaemia and 55 y n 16 mixed hyperlipaemia	Case control	Single	65 g (83%)	PUFA: SFA 0.06	8 h	TC, TAG, HDL, HDL$_2$ HDL$_3$, LDL, LDL particle size, and Lp(a)	↑ TAG at 4,6 and 8h, ↓HDL at 6h and ↓Lp(a) at 4 and 6 h in normolipaemic PoM (p<0.05) ↑TAG at 4, 6 and 8h, ↓HDL at 4 and 6h, ↓HDL$_2$ at 4h and ↓Lp(a) at 4h in hypercholesterolaemia PoM (p<0.05) ↑TAG at 4h, 6h, and 8h, ↓LDL size at 4 and 6h, ↓HDL at 4, 6 and 8h, ↓HDL2 at 6h and ↓Lp(a) at 4h and 6h in mixed hyperlipaemia PoM (p<0.05)

Postprandial lipaemia

Robertson *et al.* (2002)[210] Jackson *et al.* (2002)[207]	50-63 y (56y) n 10	Single-blind randomised crossover	Sequential	Breakfast (41 g) * Lunch (6 g)	**High SFA (g/100 g):** 10g n-6 PUFA, 0g n-3 PUFA, 40g MUFA and 50g SFA **High MUFA (g/100 g):** 11g n-6 PUFA, 0g n-3 PUFA, 72g MUFA and 17g SFA **High n-6 PUFA (g/100 g)**: 74g n-6PUFA, 0g n-3 PUFA, 15g MUFA and 11g SFA **High n-3/n-6 PUFA (g/100 g):** 39g n-6 PUFA , 22g n-3 PUFA, 22g MUFA and 19g SFA	8 h	Plasma glucose, NEFA and plasma insulin	High insulin response: SFA > n-6 PUFA > n-3 PUFA > MUFA (p<0.006) Glucose: Not significant effect ↑NEFA at 5h following high SFA breakfast and 30 min after low-fat high-CHO meal ↓insulin sensitivity: SFA < n-6 PUFA < n-3 PUFA < MUFA
							TAG, apo B-48, and RE in three TAG-rich lipoprotein subfractions	apo B-48 IAUC in the S_f60-400 fraction greater than in the S_f> 400 fraction for the palm oil, safflower oil and olive oil meals (p<0.04) ↑apo B-48 IAUC in the Sf> 400 fraction in olive oil than palm, safflower and fish/safflower oils (p<0.02)
Masding *et al.* (2003)[208]	34-56 y (42y) n 8, PrM and 46-68 y (58 y) n 8 PoM	Case and control	Single	45 g	No information	6 h	TAG, NEFA, Glucose, and ^{13}C-palmitic acid	↑TAG AUC in PoW than PrM (P<0.05) ↑^{13}C-palmitic acid in PoM than PrM (P<0.01)
Silva *et al.* (2005)[213]	52-76 y (62y) n 17	Cross sectional	Sequential	Breakfast 30g (46%) Lunch 44g (52%)	Breakfast (27% E SFA, 12% E MUFA, 5% E PUFA and2% E Trans) Lunch (27 % E SFA, 18% E MUFA, 5% E PUFA and 2% E Trans)	10 h	TAG, RE and apo B-48	↑TAG at 210 min after breakfast and 60 min after lunch ↑RE at 210 min after breakfast and 60 min after lunch ↑ Apo B-48 at 150 min after breakfast and 60 min after lunch
Alssema *et al.* (2008)[205]	60.1 y n 76	Cross sectional	Sequential	**Fat rich meal:** Both breakfast and lunch; 50g fat + 56g CHO + 28g protein	No information	8 h	TAG, HDL and CETP	↑ TAG at 8h (p<0.05), ↓HDL at 8h (p<0.05) in fat rich meal ↑ TAG at 8h (p<0.05), ↓HDL at 8h and ↑ CETP in CHO rich meal (p<0.05)

				CHO rich meal: Both breakfast and lunch; 4g fat 162g CHO +22g protein				
Schoppen *et al.* (2010)[211]	18-36 y (20.9 y) n 20 PrW and 51-59 y (55.7 y) n 18 PoW	Case and control	Single	Breakfast 75.3 g (62.3%)	11.8 % E SFA, 39.7 % E MUFA and 6.6% E PUFA	7 h	TAG and TC	↑TAG and TC in PoM than PrM (p < 0.0001) Peak TAG at 240 min in PoM and 120 min PrM (p < 0.0001)
Jackson *et al.*(2010)[206]	PrM: 42 y n 37 PoM: 60 y n 61	Case and control	Sequential	Breakfast:51 g Lunch: 31 g	29g SFA at breakfast and 14g SFA at lunch	8 h	TAG	↑ correlation coefficients for AUC (p<0.0004), IUAC (p< 0.007) and maximum TAG concentration (p<0.0004) in PoM than PrM
Wassef *et al.* (2012)[215]	58 y (45-74 y) n 19 obese PoM	Cross sectional	Single	^{13}C-labeled breakfast 80g fat (68%) + 0.017g ^{13}C-triolein/g	25% E SFA, 26% E MUFA 10% E PUFA and 6% E other sources	6 h	TAG, glucose, NEFA and Insulin	↑TAG after meal ↓ NEFA between 1 to 2h ↑ Glucose at 1h ↑ Insulin AUC at 1h
Postprandial lipaemia and vascular function								
Siepi *et al.* (2002)(212)	57 y n 10	Cross sectional	Single	65 g	PUFA:SFA 0.06	6 h	TAG and GSH	↑TAG at 4h and 6h (p < 0.05) ↓GSH at 2h (p < 0.05)
							Brachial FMD	↓FMD at 2h (p < 0.05)

*values given per 100g of test oil of which 41g was included in the breakfast.

Abbreviations: AUC; area under the curve, CETP; cholesteryl ester transfer protein, CHO; carbohydrate, E; energy, FMD; flow-mediated dilatation, GSH; glutathione, HDL; high density lipoprotein, IAUC; incremental area under the curve, LDL; low density lipoprotein, Lp (a); lipoprotein (a), MUFA; monounsaturated fat, NEFA; non-esterified fatty acid, NS; not significant, PoM; postmenopausal women, PrM; premenopausal women, PUFA; polyunsaturated fat, RE; Retinyl esters, SFA; saturated fat, TAG; triacylglycerol, TC; total cholesterol.

2.9 RESEARCH AIMS AND HYPOTHESIS

2.9.1 Research aims

The aims of this research are three-fold:

1. To investigate the impact of the acute consumption of test meals rich in SFA, n-6 PUFA or cis-MUFA on postprandial vascular function, postprandial lipaemia and associated CVD risk biomarkers in postmenopausal women. A human study will be conducted using a randomised, controlled, double-blind, crossover design incorporating a sequential test meal protocol. The primary outcome measure of this research will be macrovascular function measured by FMD. Secondary outcome measures will be; i) microvascular function, arterial stiffness and vascular tone; ii) blood pressure and heart rate; iii) serum lipids; iv) markers of insulin resistance; v) plasma nitric oxide and; vi) plasma markers of endothelial activation.

2. To investigate whether meal fat composition influences postprandial inflammation assessed using whole blood culture lipopolysaccharide-stimulated cytokine production in postmenopausal women.

3. To investigate whether genetic variants of proteins involved in endothelial-dependent vasodilation (eNOS Glu298Asp polymorphism) and postprandial lipaemia (*APOE*)) influence the responsiveness of vascular function and CVD risk markers to changes in meal (eNOS) and diet (*APOE*) fatty acid composition.

2.9.2 Research hypothesis

Relative to SFA, sequential meals rich in n-6 PUFA and cis-MUFA will improve the postprandial responses of vascular function and CVD risk markers in postmenopausal women. Furthermore, the impact of meal fatty acid composition on these outcome measures will be influenced by genetic variations in eNOS and *APOE*.

Chapter 3: Meal fatty acids have no impact on vascular function but differential effects on postprandial blood pressure and biomarkers of endothelial function in postmenopausal women: findings from the randomized, controlled DIVAS-2 study

Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove

Contribution towards the manuscript

I was responsible for creating the ethics application for the DIVAS-2 study, and M. Weech completed the clinical trial registration.

Volunteer recruitment was primarily the responsibility of M. Weech (with assistance from myself, J. Luff and S. Hargreaves), whilst I was responsible for conducting the initial screening visits including taking fasting blood samples from the volunteers through venepuncture, the subsequent analysis of blood samples on the clinical autoanalyzer, and determining eligibility. M. Weech prepared the volunteer handbook, which I explained to the volunteers during the familiarisation visits along with demonstrating the vascular techniques. M. Weech determined both the random sequences for meal order, which I assigned to eligible volunteers, and the composition of the study meals, which were prepared by S. Wu and Y. Luo on the study visits. During the study visits, my role was to take the anthropometric, LDI, DVP and blood pressure measurements and collect regular blood samples from the cannula, which was inserted by a research nurse (K. Jenkins and R. Mihaylova) and M. Weech performed FMD. Blood samples were centrifuged and prepared for freezing primarily by S. Wu.

With the exception of i) blood pressure and anthropometric measurements (data entry by S. Wu and Y. Luo) and ii) both LDI and dietary assessment analysis (M. Weech), I analysed all other outcome measures for the DIVAS-2 study as described in Chapter 3, and determined both the CVD risk scores using QRISK® and indices of insulin resistance/sensitivity. I also performed all of the statistical analysis (except for LDI and dietary intake (M. Weech)).

Finally, I was responsible for writing the manuscript, which was modified with feedback from the other authors, and journal submission.

Meal fatty acids have no impact on vascular function but differential effects on postprandial blood pressure and biomarkers of endothelial function in postmenopausal women: findings from the randomized, controlled DIVAS-2 study[1,2,3,4]

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Running title: Meal fats and postprandial endothelial function

Abbreviations: ApoB: apolipoprotein B; CVD: cardiovascular disease; DBP: diastolic blood pressure; DIVAS: Dietary Intervention and VAScular function; DVP: digital volume pulse; FMD: flow-mediated dilatation; HDL-C: HDL-cholesterol; IAUC; incremental AUC; LDI: laser Doppler imaging; LDL-C: LDL-cholesterol; MaxC: maximum concentration; MinC: minimum concentration; NEFA: non-esterified fatty acids; NO: nitric oxide; sICAM-1; soluble intracellular cell adhesion molecule; sVCAM-1; soluble vascular cell adhesion molecules; TAG: triacylglycerol; TC: total cholesterol; TE: total energy; TTmax: time to reach maximum concentration; TTmin: time to reach minimum concentration

Registered at www.clinicaltrials.gov (NCT02144454).

3.1 ABSTRACT

Background: Elevated postprandial triacylglycerol concentrations, impaired vascular function and hypertension are important independent cardiovascular disease (CVD) risk factors in women. However, little is known of the effects of meal fat composition on postprandial lipemia and vascular function in postmenopausal women.

Objective: To investigate the impact of sequential test meals rich in saturated fat (SFA), monounsaturated fat (MUFA) or n-6 polyunsaturated fat (PUFA) on postprandial vascular reactivity and associated CVD risk biomarkers in postmenopausal women.

Design: A double-blind, randomized, cross-over, postprandial study was conducted with 32 postmenopausal women. Following an overnight fast, participants consumed sequential meals containing the test fats at breakfast (0 min; 50 g total fat) and lunch (330 min; 30 g total fat) on three separate occasions, separated by 4-6 weeks. Blood samples were collected before breakfast and at regular intervals after the meals for 480 min, with specific time points selected for the vascular function and blood pressure measurements.

Results: Postprandial flow-mediated dilatation (primary outcome measure), laser Doppler imaging and digital volume pulse responses were not different after consumption of the test fats. The incremental AUC (IAUC) for diastolic blood pressure was significantly lower after the MUFA than SFA-rich (P=0.009) meals, with a similar trend for systolic blood pressure (P=0.012). This finding corresponded with a greater IAUC for the plasma nitrite response after the MUFA than SFA-rich meals (P=0.010). The soluble intracellular cell adhesion molecule-1 time course profile, AUC and IAUC were lower after the n-6 PUFA relative to the SFA and MUFA-rich meals (P≤0.001). There were no differences in lipids, glucose and markers of insulin sensitivity between the test fats.

Conclusions: Our study revealed a differential impact of meal fat composition on blood pressure, plasma nitrite and markers of endothelial activation, but no effect on postprandial lipemia in postmenopausal women. These findings warrant further investigation.

Keywords: cell adhesion molecules, diastolic blood pressure, monounsaturated fat, n-6 polyunsaturated fat, nitrate and nitrite, postprandial lipemia, saturated fat, vascular function

3.2 INTRODUCTION

The decline in estrogen at menopause is associated with adverse effects on lipid metabolism, vascular function and blood pressure (197), significantly increasing CVD risk in postmenopausal women. As a key public health strategy to reduce the incidence of CVD, the leading cause of death in women globally (219, 220), the FAO recommends that intakes of dietary SFA are reduced to ≤10% of total energy (TE) (221). Replacement of SFA with unsaturated fats is recognized as an effective strategy to lower risk; however the optimal type of replacement fat is unclear (196, 222). In the Dietary Intervention and VAScular function (DIVAS) study, replacement of 9.5-9.6% TE of dietary SFA with either MUFA or n-6 PUFA for 16-wk showed favourable effects on the fasting lipid profile, with differential beneficial effects of the unsaturated fats on markers of endothelial activation and blood pressure (223). The majority of studies examining the effects of dietary fat composition on lipids and vascular function have been conducted in the fasting state, with very little known about the acute effects of meal fat composition on postprandial lipemia. This is particularly important since individuals spend up to 18 h every day in the postprandial (fed) state, with non-fasting triacylglycerol (TAG) levels now recognized as a valid independent risk factor for CVD, particularly in women (83-85). However, the majority of studies have only looked at the effects of a single high-fat meal on the postprandial response with very little known about the impact of meal fat composition. The only acute study to address this has shown a SFA-rich breakfast meal to reduce postprandial insulin sensitivity with a carryover effect observed after eating a subsequent low fat meal, compared with n-6 PUFA, n-3 PUFA and MUFA-rich breakfast meals (224). Dietary fat induced insulin resistance (225) which can initiate metabolic changes that predispose individuals to CVD.

Acute endothelial dysfunction associated with postprandial lipemia has been reported by several investigators (97, 226). Flow mediated dilatation (FMD) is well recognized as a gold standard measure of endothelium-dependent vasodilation and is used as a surrogate measure of endothelial nitric oxide (NO) production (62, 227). Although it has been shown that a single high-fat meal (50 g) can reduce FMD 2-4 h postprandially (97, 228), there is limited data on the impact of different meals fatty acids, particularly in postmenopausal women who represent an understudied population at increased cardiometabolic risk. Thus the second DIVAS study (DIVAS-2) aimed to investigate the acute effects of sequential meals rich in SFA, MUFA or n-6 PUFA on measures of vascular function, postprandial lipemia and other CVD risk biomarkers in postmenopausal women.

3.3 SUBJECTS AND METHODS

Subjects

This study was conducted at the Hugh Sinclair Unit of Human Nutrition, University of Reading (UK), between June 2014 and September 2015. Thirty-six postmenopausal women were recruited from the University of Reading and surrounding area by sending letters/emails of invite to individuals registered on the Hugh Sinclair Unit volunteer database and the study was advertised using posters (**Appendix III**), social media and local community group newsletters. Interested volunteers were provided with a participant information sheet (**Appendix IV**) and completed a medical and lifestyle questionnaire to assess study eligibility. Participants who met the initial recruitment criteria were invited to attend a short screening visit. After written informed consent was provided, measurements were performed (height, BMI and blood pressure) and a small fasting blood sample (9 ml) was taken. Inclusion criteria included: female; postmenopausal (not menstruated for ≥ 1 y; self-reported); aged ≤ 65 y; non-smokers; not consuming more than the recommended intake of alcohol per week (<14 units/week; self-reported), BMI between 18-35 kg/m^2; blood pressure <160/100 mmHg; fasting glucose concentration <7 mmol/L (not diagnosed with diabetes or any other endocrine disorders); total cholesterol (TC) concentration <8 mmol/L; TAG concentration 0.75-4.1 mmol/L; normal liver and kidney function; and not anemic (hemoglobin ≥ 115 g/L). Further exclusion criteria included: early menopause resulting from medical conditions; myocardial infarction/stroke within the past 12 months; diagnosis of CVD, respiratory, renal, gastrointestinal, cancer or hepatic disease; medication for hyperlipidemia, hypertension, inflammation or hypercoagulation; hormone replacement therapy; vegan; planning or undertaking a weight reducing regime; taking nutritional supplements; participation in a clinical trial within the last 3 months and >3 x 30 min aerobic exercise sessions per week.

Study design

This study was given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (project reference number 14/16; **Appendix V**), registered at www.clinicaltrials.gov (NCT02144454), and the study protocol was conducted in accordance with the Declaration of Helsinki. The DIVAS-2 study was an acute randomized, double-blind, cross-over study. The participants were randomly allocated to the three different treatment arms with the use of a random assignment program (http://www.randomizer.org/form.htm) by one study researcher (KMR). Each of the three

postprandial visits, lasting approximately 10 h, took place on different days and each visit was separated by approximately 4-6 weeks. The primary endpoint was macrovascular reactivity measured by FMD. Secondary outcome measures included clinic blood pressure, peripheral microvascular function (measured using laser Doppler imaging (LDI)), vascular tone and arterial stiffness (both determined by digital volume pulse (DVP)), plasma lipids, and circulating markers of insulin resistance, inflammation and endothelial activation.

Postprandial test meal composition

For this sequential meal protocol, both breakfast (50 g total fat) and lunch (30 g total fat) were provided in the form of a warm chocolate drink containing the specific test fat/oil accompanied by toasted thick white bread with the test fat and strawberry jam. For the SFA-rich meals, butter (Wyke Farm) was used as the fat source, whereas specially prepared spreads (80% total fat) and oils (Unilever R & D) were used for the MUFA-rich (refined olive oil and olive oil/rapeseed oil blended spread) and n-6 PUFA-rich (safflower oil and spread) meals. The nutrient and fatty acid composition of the sequential meals (breakfast and lunch) containing the different test fats are shown in **Table 3.1**. Neither the researchers responsible for performing and analyzing the measurements (KMR and MW) nor the participants were aware of the meal composition at each visit.

Study visits

Volunteers attended five visits: a screening visit, a familiarization visit and three postprandial study visits. At the screening visit, a number of measurements were taken to determine eligibility. Height was measured using a wall-mounted stadiometer to the nearest 0.5 cm. While wearing light clothing, weight and BMI were determined using the Tanita BC-418 scale (Tanita Europe) with the following settings: standard body type and -1 kg for clothing. Blood pressure was measured in triplicate using an OMRON M6 automatic digital blood pressure monitor (OMRON). A 12 h fasted serum blood sample (9 mL) was collected to assess fasting TC, TAG, glucose and markers of kidney and liver function using an autoanalyzer (ILAB600; Werfen (UK) Ltd.). Participants were assessed for anemia by a full blood count which was analyzed by the Pathology Department at the Royal Berkshire Hospital (Reading, UK). Prior to the first study visit, all participants attended a 'familiarization visit' to introduce and demonstrate the vascular reactivity techniques used in the study protocol to minimize the impact of stress on these measures. Participants were also provided with a study handbook and trained researchers gave detailed instructions for completing a 4-day weighed diet diary (one weekend day and three weekdays) to assess habitual dietary intake. Foods eaten outside of the home were estimated using household measures or portion-size images (229), and quantified using food portion tables (230). Habitual nutrient intakes were determined using the nutrient analysis software Dietplan 7 (Forestfield Software, Horsham, UK), which incorporated the National Diet and Nutrition Survey nutrient databank that provided n-3 PUFA and n-6 PUFA compositions for all foods (231).

For 24 h prior to each postprandial study visit, participants were asked to abstain from alcohol and aerobic exercise regimens. Participants were provided with a low-fat meal (<10 g total fat), which was consumed before 19:30 on the evening before the study day, followed by a 12 h overnight fast. Only low-nitrate mineral water (Buxton) was allowed during the fasting period and throughout the postprandial study day. The study visits began at 07:30. Participants attended the clinical unit of the Hugh Sinclair Unit of Human Nutrition where baseline anthropometric measures were taken. Weight, BMI and % body fat were assessed using the same protocol as screening. With participants standing upright and at the end of expiration, waist circumference was measured at the minimum circumference between the iliac crest and lowest rib margin to the nearest 0.5 cm by a trained researcher. After 10 min of rest, an indwelling cannula was inserted into the antecubital vein of the forearm and two

fasting blood samples were collected (-30 min and 0 min) from which the mean baseline values were calculated. Baseline measurements of LDI, DVP, FMD and blood pressure were performed in that order. Participants were then asked to consume the breakfast meal within 15 min and blood samples were collected at regular intervals (every 30 min until 180 min and then every 60 min until 300 min) until lunch was presented at 330 min. This meal was also consumed within 15 min and blood samples were then collected every 30 min up to 420 min, with the final sample taken at 480 min. FMD was performed at baseline, 180, 300 and 420 min; blood pressure, LDI and DVP were measured at baseline, 240 and 450 min.

Assessment of vascular function and blood pressure

Participants rested for 30 min in a supine position in a quiet, temperature controlled environment (22 ± 1 °C) prior to measurements of vascular function being performed. A single trained researcher measured endothelial-dependent vasodilation of the brachial artery (FMD, primary outcome) and conducted LDI and DVP, as previously described (232). Briefly, FMD was performed with the use of an ALT ultrasound HDI-5000 broadband ultrasound system (Philips Health Care) according to standard guidelines (233). Electrocardiogram-gated image acquisition was accomplished at 0.25 frames/s for 650 s using image-grabbing software (Medical Imaging Applications LLC). The obtained image files were analyzed by a single researcher, who was blinded to the test fat allocation, by using wall-tracking software (Brachial Analyzer; Medical Imaging Applications LLC). The %FMD response was computed as the maximum change in post-occlusion brachial artery diameter expressed as a percentage of the pre-occlusion artery diameter. For each image, %FMD was determined in triplicate, from which the mean %FMD response was calculated.

LDI was performed with the LDI2-IR laser Doppler imager (Moor Instruments Ltd., Axminster, UK) (232). Microvascular responses to 1% acetylcholine (endothelial-dependent vasodilation) and 1% sodium nitroprusside (endothelial-independent vasodilation), which were subject to transdermal delivery by iontophoresis (MIC2, Moor Instruments Ltd.), were expressed as the AUC for flux (measured in arbitrary perfusion units) vs. time for the 20 scan protocol. In the peripheral arteries, DVP (Pulse Trace PCA2; Micro Medical Ltd.) recorded the systolic and diastolic wave forms of the pulse using an infrared transmitting finger cuff placed on the left index finger, and determined the stiffness index (DVP-SI; m/s) and reflection index (DVP-RI; %) as measures of arterial stiffness and vascular tone, respectively (232).

Clinic systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate measurements were taken using an OMRON blood pressure monitor at least 30 min prior to the commencement of FMD, and three consecutive measurements were obtained to determine the mean. Pulse pressure was calculated as the difference between the mean SBP and mean DBP.

Sample analyses

Blood samples were collected into lithium heparin and K3EDTA coated blood tubes (VACUETTE; Greiner Bio-One) and kept on ice for less than 30 min until centrifugation at 1700 x g for 15 min at $4°C$ to obtain plasma and stored at -80°C until analysis. Blood was also collected into serum separator blood tubes (VACUETTE; Greiner Bio-One) and left at room temperature for 30 min until centrifugation as above at $20°C$ to isolate serum and stored at -20°C. Serum was used to determine lipids (TC, HDL cholesterol (HDL-C), TAG, apolipoprotein B (apoB)), glucose, non-esterified fatty acids (NEFA) and C-reactive protein with the use of an ILAB600 autoanalyzer (reagents: Werfen (UK) Ltd.; NEFA reagent: Alpha Laboratories; apoB reagent: Randox Laboratories Ltd). Fasting LDL cholesterol (LDL-C) was estimated using the Friedewald formula (234). Plasma nitrite and nitrate levels were analyzed using the HPLC based approach, Eicom NOx Analyzer ENO-30 (Eicom; San Diego; USA) as described elsewhere (235). ELISA kits were used to determine concentrations of circulating serum insulin (Dako Ltd.; Denmark), soluble intercellular adhesion molecule-1(sICAM-1), soluble vascular cell adhesion molecule (sVCAM-1), E-selectin and P-selectin (R & D Systems Europe Ltd.; UK & Europe). Mean intra-assay and inter-assay CVs were <5% for the automated assays and <10% for the ELISAs. For the nitrate and nitrite analysis, quality controls with low and high levels were run per 12 samples to check for CV% compliance (<20%).

Using baseline measures, the QRISK®2-2016 online calculator was used to estimate the participant's risk of developing CVD within the next 10 y (http://www.qrisk.org/index.php). Fasting insulin resistance and insulin sensitivity were determined by HOMA-IR and the revised quantitative insulin sensitivity check index, respectively, using standard equations (236).

Statistical analyses

This study required 28 participants for sufficient power to detect a significant change of 1.5% (SD 2.0%) in FMD (primary outcome measure), with a power of 80% at the 5% significance level. To allow for a 22% dropout rate, 36 volunteers were recruited onto the study and randomized. All statistical analyses were performed with the use of IBM SPSS Statistics version 24. All data were checked for normality and log transformed where necessary. For all outcome measures, two-way repeated measures ANOVA were used to analyze the effects of the test fats on the postprandial time course responses using within-subject factors of 'test fat' and 'time', where $P \leq 0.05$ was considered significant. Summary measures for the postprandial responses following the sequential meals were expressed as area under the time response curve (AUC) computed using the trapezoidal rule (237), maximum concentration (maxC) and time to reach maximum concentration (TTMax). The incremental AUC (IAUC) was calculated as AUC minus the fasting concentration. For NEFA, additional summary measures were calculated including the minimum concentration (minC), time to reach minC (TTMin) and % NEFA suppression. Due to the shape of the NEFA curve, AUC and IAUC were calculated from 120-480 min. One-way repeated measures ANOVA were used to analyze the effects of test fat on these summary measures and fasting data. When a significant test fat effect was observed, a paired sample t-test was performed, with the application of Bonferroni's correction (where $P \leq 0.017$ was considered significant). Non-parametric one-way repeated measures ANOVA were applied for data that could not be normalized by transformation. Data are presented in the text, tables, and figures as mean ± SEM.

3.4 RESULTS

Study participation

Of the 36 participants randomly allocated to the intervention meals, 32 (89%) successfully completed all three study visits (see **Figure 3.1** for flowchart). These participants had a mean ± SEM age of 58 ± 1 y, BMI of 25.9 ± 0.7 kg/m^2 and estimated 10 y CVD risk of 4.7 ± 0.4 %. Subject characteristics and baseline levels of all outcome measures were not significantly different between study visits, and the average values for the three visits are shown in **Table 3.2**. The mean habitual dietary intake of the postmenopausal women recorded prior to visit 1 are also presented in this table.

Postprandial vascular function response

For the primary outcome measure, there was no statistically significant difference in the postprandial %FMD response after consumption of the SFA, MUFA or n-6 PUFA-rich sequential test meals (**Table 3.3**). However, there was a tendency for an effect of the test fat on the AUC for the % FMD response ($P = 0.086$), but the differences observed following the MUFA and SFA-rich meals did not reach significance after Bonferroni correction ($P = 0.031$). Furthermore, measures of microvascular reactivity (LDI), vascular tone (DVP-RI) and arterial stiffness (DVP-SI) did not differ after consumption of the different test fats.

Postprandial blood pressure response

There was a significant impact of test fat on the IAUC for the postprandial DBP response ($P = 0.007$), with a greater reduction observed after consumption of the MUFA relative to the SFA-rich meals ($P = 0.009$) (**Table 3.3**). The IAUC for the SBP response showed borderline significance between the test fats ($P = 0.053$), with a tendency for a greater reduction after consumption of the MUFA versus SFA-rich meals ($P = 0.012$). Furthermore, there was a significant effect of test fat ($P = 0.035$) and time ($P < 0.001$) for the incremental DBP time course profile, with a greater reduction after the MUFA than SFA-rich meals ($P = 0.013$; **Figure 3.2A**). A similar tendency was found for the incremental SBP time course response (test fat effect $P = 0.035$ and time effect $P < 0.001$), but the differences between the MUFA and SFA-rich meals were not significant after Bonferroni correction ($P = 0.025$; **Figure 3.2B**). At the end of the postprandial period (450 min), the reduction in DBP after the MUFA-rich meals remained significantly lower (approximately 3 mmHg) compared with those rich in SFA ($P = 0.016$; Figure 3.2A). The AUC for the heart rate response was also found to be influenced by the meal fat composition ($P = 0.022$) but the differences between meals did not reach significance after Bonferroni correction ($P \leq 0.017$) (**Table 3.3**).

Postprandial nitrite and nitrate response

There was a significant effect of the test fat on the IAUC for the postprandial plasma nitrite response ($P = 0.010$), with a greater reduction observed after consumption of SFA than MUFA-rich meals ($P = 0.007$) (**Table 3.3**). The IAUC for the nitrate and NOx (nitrite and nitrate combined) responses did not quite reach significance ($P = 0.054$) (**Table 3.3**) after the meals of varying fat composition.

Postprandial response for markers of endothelial activation

There was a significant test fat*time interaction for the postprandial sICAM-1 time course response ($P < 0.001$; **Figure 3.3**), with lower concentrations found after the n-6 PUFA than SFA and MUFA-rich meals ($P = 0.001$). The AUC and IAUC were also lower after the n-6 PUFA than SFA and MUFA-rich meals ($P \leq 0.002$) (**Table 3.3**). In contrast, meal fat composition had no effect on the postprandial plasma sVCAM-1, E-selectin or P-selectin responses (**Table 3.3**).

Postprandial lipid, glucose and insulin response

No significant effects of meal fatty acid composition were evident for the postprandial TAG, apo B, NEFA, glucose and insulin responses (**Table 3.4**).

3.5 DISCUSSION

To our knowledge, the DIVAS-2 study is the first suitably powered study in postmenopausal women to investigate the impact of sequential meals rich in SFA, MUFA and n-6 PUFA on postprandial macro- and microvascular reactivity (novel CVD risk markers (61, 238, 239)), blood pressure and postprandial CVD risk biomarkers. Our study showed differential beneficial effects of meals rich in unsaturated fatty acids on clinic blood pressure, plasma nitrite and markers of endothelial activation, with little impact of meal fat composition on real time measures of vascular function, postprandial lipemia and markers of insulin resistance.

The lack of effect of meals of varying fat composition on postprandial FMD (our primary outcome measure) and arterial stiffness in our postmenopausal women is similar to previous findings in healthy men and women (240-245). Low bioavailability of NO, the most potent vasodilator produced by the vascular endothelium, has been demonstrated to be closely associated with endothelial damage which may affect the regulation of vascular tone and function (246). Moreover, NO inhibits platelet aggregation, smooth muscle cell proliferation and adhesion of monocytes and endothelial cells (247). An effective method for estimating endogenous NO availability is to measure its more stable oxidation products nitrite and nitrate in plasma or other biological fluids. We observed a greater decrease in plasma nitrite levels post-consumption of the SFA relative to MUFA-rich meals, with little impact of the test fats on nitrate and NOx responses. Similar findings were observed in the LIPGENE study which reported a beneficial effect of a MUFA-rich meal (refined olive oil; 12% SFA,

43% MUFA, 10% PUFA) on plasma nitrites compared with a SFA-rich meal (vegetable sources of SFA; 38% SFA, 21% MUFA, 6% PUFA) in patients classified with metabolic syndrome (248). Although differences in the biomarkers of NO production were evident between the test fats in the current study, comparable changes in the real-time measures of vascular reactivity were not quite significant, suggesting possible indirect effects of meal fatty acids on vascular function. These may include differences by which SFA, n-6 PUFA and MUFA influence fat-induced oxidative stress, the magnitude of the lipemic response and also duration of exposure of the endothelium to circulating TAG-rich lipoproteins (chylomicrons and very low density lipoprotein) during the postprandial phase (249, 250).

In the current study, each test fat reduced blood pressure over 450 min, with a greater decrease in DBP and to a lesser extent SBP observed after consumption of the MUFA than SFA-rich meals. There are very limited and inconclusive data in the literature regarding the relative effects of acute consumption of meals varying in fat composition on postprandial blood pressure in postmenopausal women. However, findings from previous chronic interventions have shown significant effects of replacing SFA with unsaturated fat on blood pressure (39, 223). In our chronic DIVAS study, replacement of 9.5% TE of dietary SFA with MUFA for 16 wk significantly attenuated the increase in night SBP in 195 men and women with a moderately increased risk of CVD (223). Another 3-month controlled parallel study in which participants followed a high fat diet (37%TE) rich in either SFA (17%TE from SFA) or MUFA (23%TE from MUFA) also reported significant reductions in both fasting DBP and SBP in response to the MUFA-rich diet (39). The results from these chronic studies and our more recent acute study, provide evidence to support the replacement of dietary SFA with MUFA as a potential strategy for blood pressure lowering, although more studies are needed to confirm these findings.

Endothelial dysfunction is associated with an increased expression of adhesion molecules due, in part, to increased endothelial cell activation. This triggers leukocyte homing, adhesion and migration into the sub-endothelial space, all of which are associated with the initiation, progression and destabilization of atherosclerosis (251). During the process of atherosclerotic plaque formation, soluble adhesion molecules, such as sICAM-1 and sVCAM-1, and cell surface adhesion molecules, such as E-selectin and P-selectin, are activated (252). In the DIVAS-2 study, we observed a lower postprandial sICAM-1 response after the n-6 PUFA than SFA and MUFA-rich meals, with little effect evident on other

adhesion molecules. Our finding is similar to that of a previous study in overweight men which showed a reduction in sICAM-1 after consumption of a single mixed meal rich in n-6 PUFA (40 g margarine and 10 g safflower oil) compared with SFA (50 g butter fat) (253). In contrast to our study, others have reported significant effects of meal fat composition on sVCAM-1 with a reduction observed following a n-6 PUFA meal in overweight men (45), whereas an increase was found in both postprandial sICAM-1 and sVCAM-1 after a SFA-rich meal in healthy adults (254). Endothelial cell studies also support a differential effect of fatty acids on cell adhesion molecules, where fatty acids with the same chain length, but increasing double bonds accompanying the transition from MUFA to n-6 PUFA, had a greater inhibitory effect of cytokine-induced expression of adhesion molecules, although the specific mechanisms of action are not clear (255).

One systematic review and meta-analysis has compared the effects of single oral fat tolerance tests with differing fatty acid compositions on postprandial TAG responses in men and women (91). Relative to a SFA-rich meal challenge, a PUFA-rich meal significantly reduced the postprandial lipemic response over 8 h, whereas there was only a trend for a reduced response following a MUFA-rich meal. In our study in postmenopausal women, meal fatty acids did not impact on postprandial lipid, glucose or insulin responses following sequential meals. However, Robertson *et al* (224) reported significantly higher levels of plasma NEFA and lower insulin sensitivity following a SFA-rich meal compared with other test oils. The sequential postprandial protocols used in these studies may provide an explanation for the differences observed on postprandial lipemia. In the Robertson study, volunteers ingested a high fat breakfast containing 40 g of the assigned test fat followed by a low fat, high carbohydrate lunch (5.4 g total fat) given 5 h later. Furthermore, in the Robertson study, the SFA meal contained vegetable sources of SFA (palm oil and cocoa butter) whereas the DIVAS-2 study used butter. Therefore, the fat content of the sequential meals and type of SFA may impact on the postprandial outcome measures, and warrants further investigation.

A strength of the study is the use of a two meal sequential postprandial protocol, which more closely mimics a habitual dietary intake pattern compared with a single test meal challenge (218, 256). However, there are some potential limitations of our study. As only healthy postmenopausal women were included, the findings may not reflect responses in men, premenopausal women or postmenopausal women with increased CVD risk. Furthermore,

The SFA-rich meal naturally contained higher quantities of cholesterol and trans fatty acids compared with the two unsaturated fat rich meals. These differences could have contributed to the responses observed, although the amounts consumed in the SFA-rich meals were below that which has been associated with adverse effects on CVD risk factors.

In conclusion, the findings of this novel study, which are timely and important, suggest that MUFA-rich meals had favourable effects on postprandial DBP, as well as improving plasma nitrite concentrations compared with SFA-rich meals. Furthermore, n-6 PUFA rich meals reduced postprandial sICAM-1 concentrations, a marker of endothelial activation relative to the SFA and MUFA-rich meals. Compared with SFA our chronic and acute DIVAS studies consistently show that unsaturated fatty acids to have beneficial effects on blood pressure and biomarkers of endothelial activation. Together these findings will contribute to the evidence base for future dietary fat recommendations for CVD risk reduction. Further studies are necessary to examine the mechanisms underlying the effects of these fatty acids on postprandial endothelial function.

FIGURE LEGENDS

Figure 3.1: Flow of participants through the different stages of the DIVAS-2 study

Figure 3.2: Mean ± SEM for the incremental A) diastolic blood pressure (ΔDBP, mmHg) and B) systolic blood pressure (ΔSBP, mmHg) responses following sequential meals enriched in SFA (■), MUFA (●) and n-6 PUFA (□) in the postmenopausal women (n=32). There was a significant effect of the test fat ($P \leq 0.049$) and time ($P = 0.0001$) for the DBP and SBP responses by repeated measures ANOVA.

Figure 3.3: Mean ± SEM (n=27) for the change in postprandial sICAM-1 responses following sequential meals (0 min and 330 min) enriched in SFA (■), MUFA (●) and n-6 PUFA (□). Two-way repeated measures ANOVA revealed a significant effect of the test fat, time and a test fat*time interaction (all $P < 0.001$).

Table 3.1 Macronutrient composition of the sequential test meals consumed on the three study visits

	Breakfast			Lunch		
	SFA	MUFA	n-6 PUFA	SFA	MUFA	n-6 PUFA
Energy (MJ)	3.8	3.8	3.8	3.0	3.0	3.0
Fat (g)	53.7	53.1	53.1	31.8	31.1	31.1
SFA	32.9	9.4	7.6	19.1	6.1	5.4
MUFA	13.3	35.2	6.7	7.7	19.4	4.1
n-6 PUFA	1.8	5.1	36.2	1.3	3.4	20.0
n-3 PUFA	0.6	0.9	0.1	0.3	0.6	0.1
Trans	1.95	0.13	0.12	1.12	0.12	0.12
Cholesterol (mg)	150	12	12	90	12	12
Carbohydrate (g)	98.4	98.0	98.0	98.2	98.0	98.0
Protein (g)	19.6	19.2	19.2	19.5	19.2	19.2

Table 3.2 Subject characteristics and mean baseline measures of the study participants[1]

Characteristic	Mean	SEM	Range
Age, y	58	1	48-65
Weight, kg	70.1	2.1	47.6-91.9
BMI, kg/m^2	25.9	0.7	17.6-33.9
Waist circumference, cm	90.2	1.6	70.0-108.3
Body fat, %	36.8	1.2	21.1-47.3
Blood pressure, mm Hg			
Systolic	136	3	108-177
Diastolic	78	1	64-94
Pulse pressure	58	2	41-85
Heart rate, beats/min	59	1	50-72
Fasting biochemical profile			
Total cholesterol, mmol/L	5.74	0.12	4.30-7.09
HDL cholesterol, mmol/L	1.62	0.05	1.15-2.17
Total cholesterol : HDL cholesterol ratio	3.57	0.14	2.55-5.24
LDL cholesterol, mmol/L	3.50	0.11	2.33-4.94
Triacylglycerol, mmol/L	1.30	0.07	0.76-2.42
C-reactive protein, mg/L	1.29	0.27	0.14-8.07
Glucose, mmol/L	5.17	0.09	4.36-6.57
Insulin, pmol/L	38.0	4.2	8.9-109.7
NEFA, µmol/L	606	27	414-1055
HOMA-IR	1.48	0.19	0.33-5.33
Rquicki	0.40	0.00	0.33-0.52
Habitual macronutrient intake			
Energy, MJ	7.3	0.3	3.2-11.6
Total fat, %TE	35.4	1.3	21.3-64.9
SFA, %TE	13.1	0.6	7.6-26.9
MUFA, %TE	12.6	0.5	7.1-23.3
n-6 PUFA, %TE	5.2	0.3	2.4-9.7
n-3 PUFA, %TE	0.9	0.1	0.4-1.5
Trans fat, %TE	0.9	0.1	0.1-1.6
Protein, %TE	15.9	0.5	11.5-22.8
Carbohydrate, %TE	45.3	1.3	21.0-65.4
Total sugars, %TE	19.7	1.1	8.0-40.0
Dietary cholesterol, mg	228	18	45-466
Dietary fibre (AOAC), g	22.1	1.1	10.9-35.3

[1]Data represent the average of the three baseline visits (n=32), with the exception of the habitual macronutrient intake that was determined from a single 4-day weighed diet diary recorded prior to visit 1 (n=31). Abbreviations: %TE: percentage of total energy; AOAC: Association of Official Analytical Chemists; CVD: cardiovascular disease; NEFA: non-esterified fatty acids; rQUICKI: revised quantitative insulin sensitivity check index.

Table 3.3 Summary measures for the fasting and postprandial vascular outcomes, blood pressure and circulating markers of endothelial activation after sequential meals of varying fat composition

	Test fats			
	SFA	**MUFA**	**n-6 PUFA**	**P value**
Vascular function				
FMD (n=31)				
% FMD response				
Fasting, %	4.69 ± 0.44	4.99 ± 0.60	4.74 ± 0.44	0.994
AUC, % x min	2025 ± 116	2313 ± 165	2117 ± 138	0.086
IAUC, % x min	55 ± 147	216 ± 183	127 ± 135	0.542
Pre-occlusion artery diameter				
Fasting, mm	3.32 ± 0.09	3.31 ± 0.10	3.34 ± 0.10	0.872
AUC, mm x min	1411 ± 39	1401 ± 39	1415 ± 39	0.660
IAUC, mm x min	10.4 ± 12.5	15.8 ± 13.8	12.6 ± 14.7	0.657
Maximum change in artery diameter				
Fasting, mm	0.15 ± 0.01	0.16 ± 0.02	0.15 ± 0.01	0.955
AUC, mm x min	66.7 ± 3.5	74.4 ± 4.6	69.1 ± 3.9	0.172
IAUC, mm x min	2.90 ± 5.12	8.51 ± 5.28	4.08 ± 4.08	0.419
LDI (n=25)[1]				
LDI-Ach, AU				
Fasting, AU	1633 ± 117	1786 ± 151	1805 ± 167	0.386
AUC, AU x min x 10^3	736 ± 41	779 ± 65	778 ± 48	0.468
IAUC, AU x min x 10^3	1.2 ± 29.4	-24.4 ± 49.1	-34.4 ± 46.1	0.595
LDI-SNP, AU, 10^3				
Fasting, AU	1651 ± 132	1832 ± 125	1655 ± 175	0.177
AUC, AU x min x 10^3	745 ± 45	813 ± 63	721 ± 55	0.293
IAUC, AU x min x 10^3	2.3 ± 46.2	-1.1 ± 45.5	-24.1 ± 50.7	0.527

DVP (n=32)

Reflection index

Fasting, %	61.9 ± 1.5	60.7 ± 1.7	62.8 ± 1.8	0.641
AUC, % x min x 10^3	25.9 ± 0.7	25.2 ± 0.6	26.2 ± 0.7	0.374
IAUC, % x min x 10^3	-1.9 ± 0.6	-0.2 ± 0.6	-2.0 ± 0.5	0.687

Stiffness index

Fasting, m/s	7.0 ± 0.3	7.3 ± 0.3	7.1 ± 0.2	0.497
AUC, m/s x min x 10^3	3218 ± 96	3153 ± 75	3276 ± 106	0.596
IAUC, m/s x min x 10^3	89.3 ± 79.1	-121.4 ± 87.3	90.7 ± 91.2	0.666

Blood pressure (n=32)

SBP

Fasting, mmHg	134 ± 3	137 ± 3	136 ± 3	0.207
AUC, mmHg x min x 10^3	57.1 ± 1.2	56.9 ± 1.1	57.5 ± 1.2	0.297
IAUC, mmHg x min x 10^3	-3.4 ± 0.6	-4.8 ± 0.6	-3.8 ± 0.5	0.053

DBP

Fasting, mmHg	76.6 ± 1.4	78.3 ± 1.3	77.7 ± 1.5	0.139
AUC, mmHg x min x 10^3	33.0 ± 0.6	32.9 ± 0.6	33.3 ± 0.7	0.699
IAUC, mmHg x min x 10^3	-1.5 ± 0.3[a]	-2.3 ± 0.3[b]	-1.7 ± 0.3[ab]	**0.007**

Pulse pressure

Fasting, mmHg	57.5 ± 2.1	58.9 ± 1.8	58.6 ± 2.1	0.494
AUC, mmHg x min x 10^3	24.0 ± 0.7	24.0 ± 0.6	24.2 ± 0.7	0.711
IAUC, mmHg x min x 10^3	-19.1 ± 0.4	-25.5 ± 0.4	21.4 ± 0.4	0.755

Heart rate

Fasting, mmHg	58.6 ± 0.8	60.7 ± 1.5	58.8 ± 1.0	0.158
AUC, beats/min x min x 10^3	29.2 ± 0.4	30.0 ± 0.6	29.3 ± 0.5	**0.022**[2]
IAUC, beats/min x min x 10^3	28.1 ± 0.3	27.2 ± 0.3	28.3 ± 0.3	0.380

Circulating markers of endothelial activation (n=27)

Nitrite

Fasting, µmol/L	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.243
AUC, µmol/L x min	52.8 ± 2.5	53.0 ± 2.8	53.7 ± 2.6	0.661
IAUC, µmol/L x min	-1.19 ± 0.6[a]	0.45 ± 0.7[b]	0.72 ± 1.4[ab]	**0.010**
Nitrate				
Fasting, µmol/L	19.1 ± 1.5	18.8 ± 1.8	16.9 ± 1.9	0.127
AUC, µmol/L x min	6094 ± 411	6057 ± 487	5659 ± 527	0.381
IAUC, µmol/L x min	-1915 ± 248	-1835 ± 314	-1460 ± 301	0.054
NOx				
Fasting, µmol/L	19.2 ± 1.5	18.9 ± 1.8	17.1 ± 1.9	0.127
AUC, µmol/L x min	6147 ± 411	6110 ± 487	5712 ± 527	0.384
IAUC, µmol/L x min	-1916 ± 248	-1835 ± 315	-1459 ± 301	0.054
sVCAM-1				
Fasting, ng/mL	633 ± 18	629 ± 20	625 ± 16	0.907
AUC, µg/mL x min x 10^3	266 ± 7	268 ± 8	259 ± 6	0.147
IAUC, µg/mL x min x 10^3	0.5 ± 4.1	3.9 ± 3.9	-3.6 ± 2.4	0.717
sICAM-1				
Fasting, ng/mL	208 ± 7	204 ± 7	206 ± 5	0.690
AUC, µg/mL x min x 10^3	84.8 ± 2.5[a]	86.4 ± 2.9[a]	68.7 ± 4.1[b]	**<0.001**
IAUC, µg/mL x min x 10^3	-2.4 ± 1.1[a]	0.7 ± 1.2[a]	-18.0 ± 3.3[b]	**<0.001**
E-selectin				
Fasting, ng/mL	29.0 ± 1.7	28.0 ± 1.7	27.8 ± 1.9	0.226
AUC, µg/mL x min x 10^3	11.3 ± 0.8	11.1 ± 0.7	10.8 ± 0.6	0.551
IAUC, µg/mL x min x 10^3	-0.15 ± 0.3	0.05 ± 0.1	-0.06 ± 0.1	0.895
P-selectin				
Fasting, ng/mL	32.8 ± 1.4	31.6 ± 1.6	31.5 ± 1.6	0.170
AUC, µg/mL x min, 10^3	13.3 ± 0.6	13.5 ± 0.8	13.3 ± 0.7	0.925
IAUC, µg/mL x min, 10^3	-0.5 ± 0.2	0.2 ± 0.2	0.1 ± 0.2	0.084

Values are mean ± SEM, for FMD and circulating markers of endothelial activation, the time interval for the AUC and IAUC represents 420 min whereas for DBP, SBP, DVP measures (stiffness index and reflection index) and LDI this is 450 min. Data was analyzed using one-way repeated-measures ANOVA (non-

parametric for data that could not be normalized); if the effect of test fat was significant, post-hoc analysis (paired sample t-test) was performed with Bonferroni correction ($P \leq 0.017$). Different superscript letters within a row indicate significant differences between test fats.

[1] LDI-Ach and LDI-SNP were expressed as AUC for the 20-scan protocol. IAUC was also determined for the 20-scan protocol but differences between test fats for subsequent AUC and IAUC were not significant (data not shown).

[2] Post-hoc tests for heart rate were not significant with Bonferroni correction.

Abbreviations: Ach: acetylcholine; AU: arbitrary units; DBP: diastolic blood pressure; DVP: digital volume pulse; FMD: flow-mediated dilatation; IAUC: incremental AUC, LDI: laser Doppler imaging; NOx: total nitrite and nitrate, SBP: systolic blood pressure; sICAM-1: soluble intercellular cell adhesion molecule-1, SNP: sodium nitroprusside, sVCAM-1: soluble vascular cell adhesion molecule-1.

Table 3.4 Fasting and postprandial summary measures for the serum TAG, lipid, glucose and insulin responses after the sequential meals rich in SFA, MUFA and n-6 PUFA

	Test fats			
	SFA meal	**MUFA meal**	**n-6 PUFA meal**	**P value**
TAG response				
Fasting, mmol/L	1.35 ± 0.08	1.32 ± 0.07	1.42 ± 0.11	0.735
MaxC, mmol/L	2.87 ± 0.21	3.14 ± 0.20	3.19 ± 0.26	0.140
TTMax, min	333 ± 15	333 ± 19	326 ± 13	0.907
AUC, mmol/L x min	981 ± 68	1020 ± 63	1058 ± 92	0.546
IAUC, mmol/L x min	333 ± 38	385 ± 41	377 ± 53	0.135
NEFA response				
Fasting, μmol/L	593 ± 32	623 ± 36	590 ± 32	0.609
MinC, μmol/L	122 ± 8	111 ± 8	124 ± 10	0.330
TTMin, min	295 ± 34	260 ± 30	254 ± 29	0.734
Suppression, %	56 ± 5	62 ± 5	62 ± 5	0.819
MaxC, μmol/L	752 ± 37	710 ± 37	698 ± 36	0.223
TTMax, min	231 ± 31	278 ± 26	264 ± 28	0.301
$AUC_{120-480}$, mmol/L x min	136 ± 7	129 ± 8	128 ± 6	0.404
$IAUC_{120-480}$, mmol/L x min	45.1 ± 10.2	50.4 ± 12.3	49.8 ± 10.6	0.089
Apo B response				
Fasting, μg/mL	999 ± 29	998 ± 40	995 ± 38	0.850
MaxC, μg/mL	1064 ± 35	1060 ± 40	1062 ± 40	0.908
TTMax, min	218 ± 29	176 ± 26	148 ± 23	0.177
AUC, mg/mL x min	479 ± 14	481 ± 18	478 ± 18	0.893
IAUC, mg/mL x min	-291 ± 3913	1290 ± 3585	262 ± 3812	0.891
Glucose response				
Fasting, mmol/L	5.19 ± 0.11	5.16 ± 0.10	5.15 ± 0.09	0.929
MaxC, mmol/L	8.88 ± 0.31	9.12 ± 0.38	9.13 ± 0.30	0.641

TTMax, min	328 ± 25	321 ± 29	352 ± 24	0.502
AUC, mmol/L x min	2953 ± 63	2986 ± 91	2980 ± 80	0.933
IAUC, mmol/L x min	463 ± 53	508 ± 64	508 ± 64	0.304
Insulin response				
Fasting, pmol/L	42.5 ± 8.1	38.2 ± 4.0	35.7 ± 5.0	0.491
MaxC, pmol/L	457 ± 40	488 ± 40	434 ± 32	0.290
TTMax, min	228 ± 33	245 ± 33	205 ± 32	0.736
AUC, μmol/L x min	102.1 ± 7.3	102.5 ± 8.4	9.8 ± 6.6	0.777
IAUC, μmol/L x min	81.7 ± 7.1	84.2 ± 7.0	81.3 ± 5.1	0.607

Values are mean ± SEM, n=26. Data were analyzed using one-way repeated-measures ANOVA (non-parametric for data that could not be normalized by transformation); if the effect of test fat was significant, post-hoc analysis (paired sample t-test) was performed with Bonferroni correction ($P \leq 0.017$). Abbreviations: apo; apolipoprotein, IAUC; incremental AUC, maxC; maximum concentration, minC; minimum concentration, NEFA; non-esterified fatty acids, TAG; triacylglycerol, TTMax; time to reach maxC, TTMin; time to reach minC.
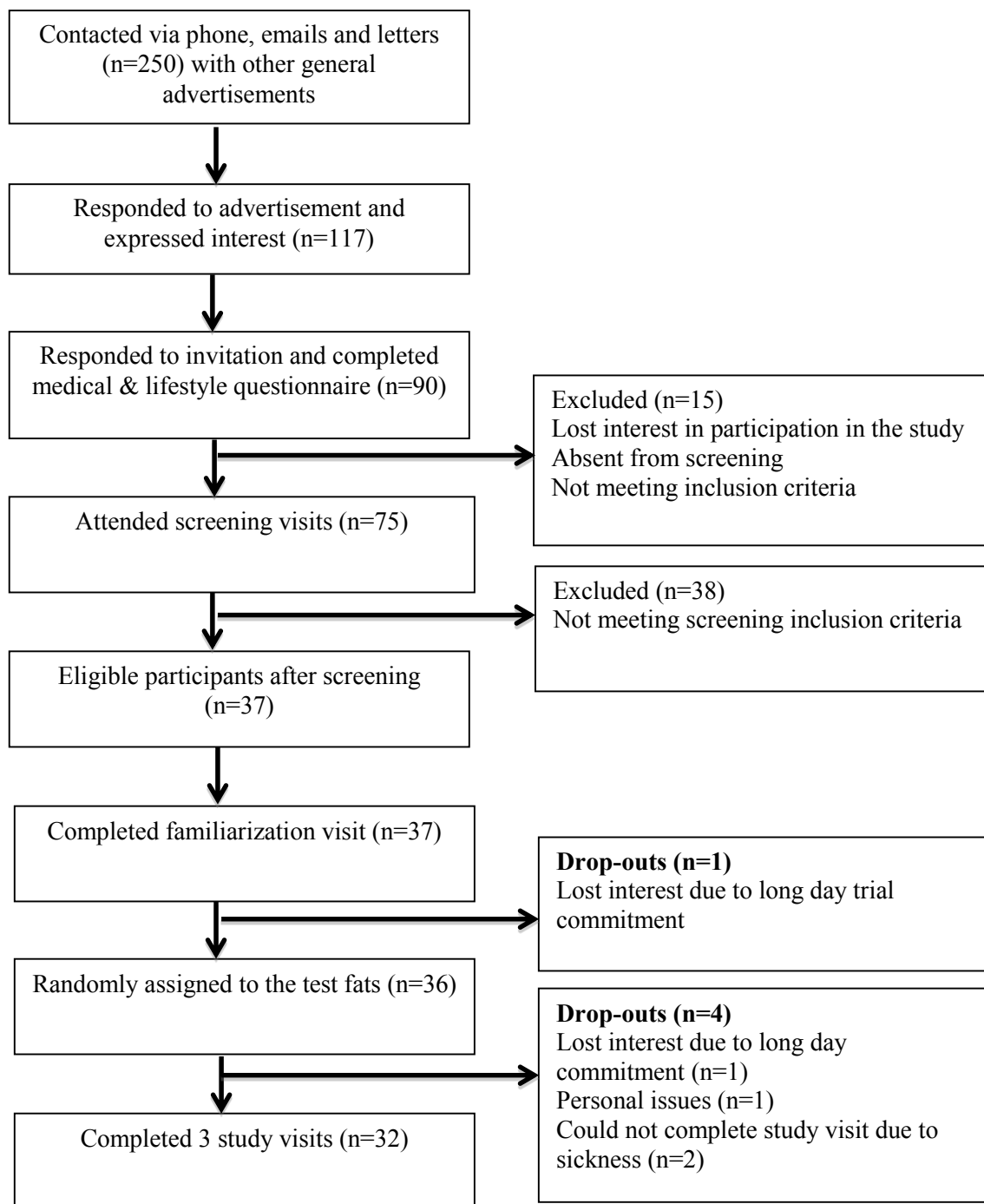
FIGURE 3.1

FIGURE 3.2
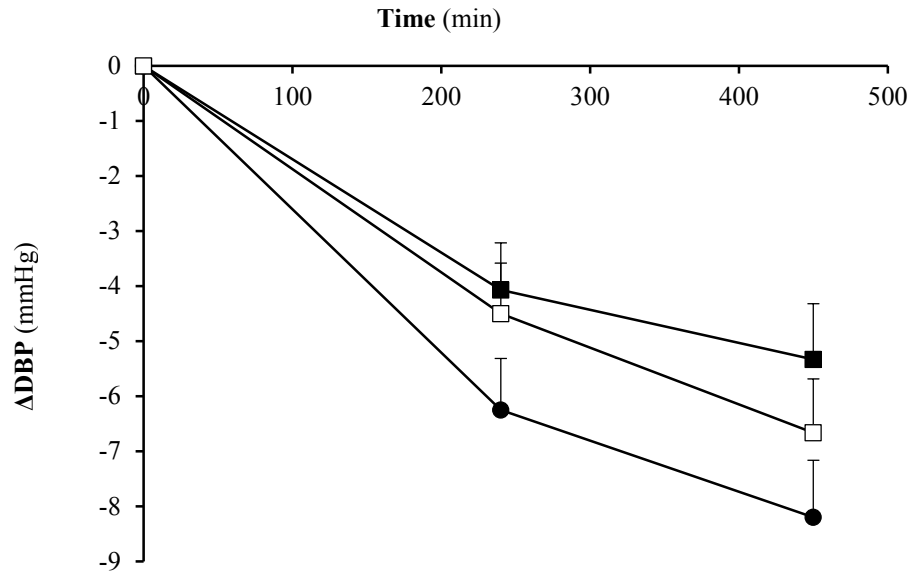
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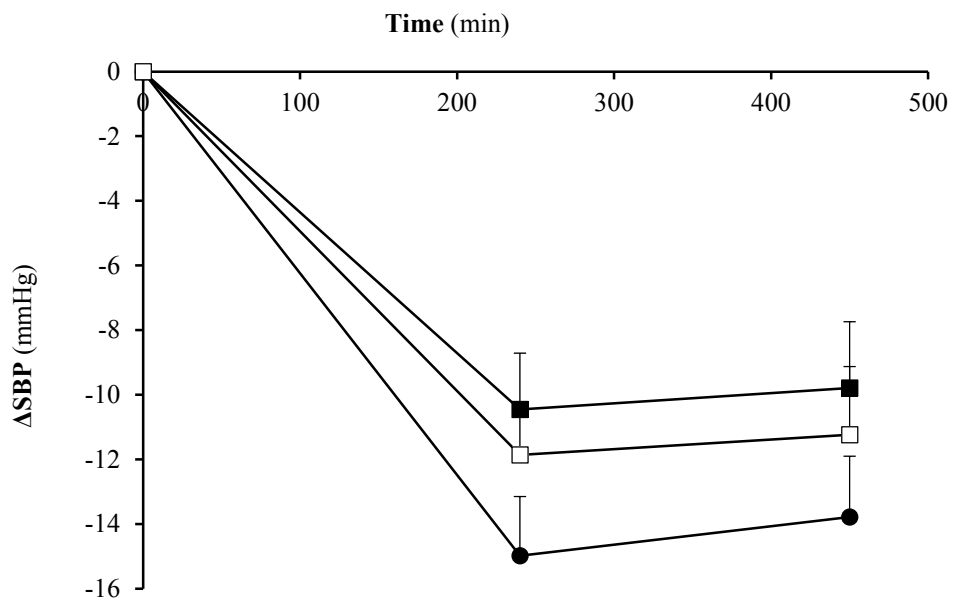
B

FIGURE 3.3
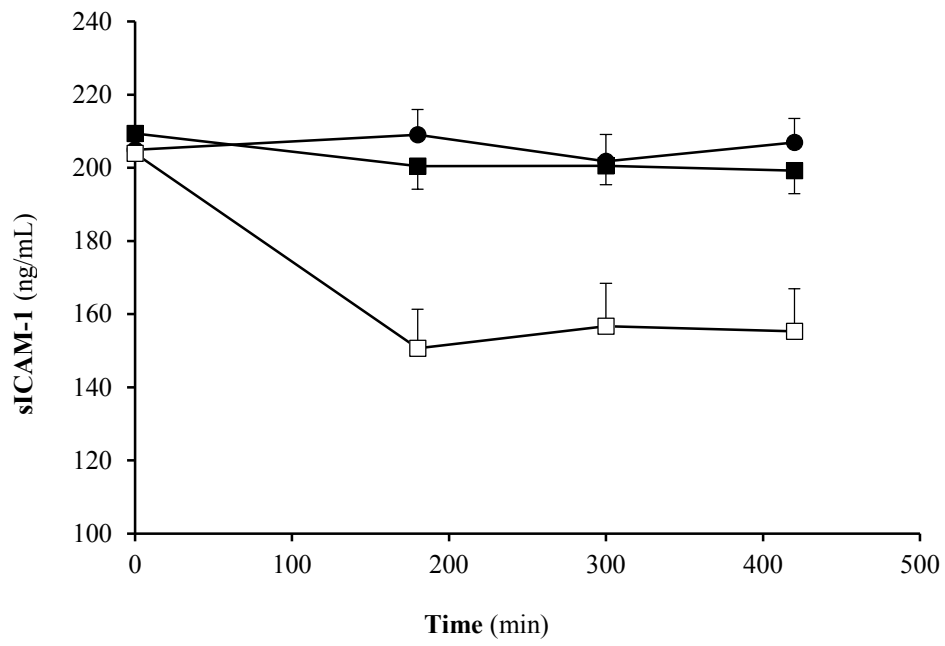
Chapter 4: Greater *ex-vivo* IL-1β production after sequential saturated than unsaturated fat-rich meals in postmenopausal women

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Manuscript prepared for submission to Cytokine Journal

Contribution towards the manuscript

In addition to my responsibilities from Chapter 3 with regards to conducting the DIVAS-2 study, I also collected the additional blood samples at the designated time points for the monocyte count analysis (determined by the Pathology Department at the Royal Berkshire Hospital) and whole blood culture (WBC) analysis. After each study visit, I prepared the blood samples for the WBC and collected the supernatant after 24 h. I was also responsible for analysing the samples using the *Luminex* method to determine cytokine production. Furthermore, I performed all of the statistical analyses for this chapter, and was responsible for writing the manuscript, which was modified with feedback from the other authors.

Greater *ex-vivo* IL-1β production after sequential saturated than unsaturated fat-rich meals in postmenopausal women

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Running title: Saturated fat increases *ex vivo* IL-1β production

Abbreviations: AUC, area under the curve; CVD, cardiovascular disease; DIVAS-2, Dietary Intervention and Vascular Function-2; IAUC, incremental area under the curve; IL, interleukin; LPS, lipopolysaccharides; MUFA, monounsaturated fatty acid; NF-κB, nuclear factor kappa B; PA, palmitic acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; WBC, whole blood culture.

4.1 ABSTRACT

High fat meals have been shown to induce postprandial inflammation but little is known about the response to meal fat composition. The Dietary Intervention and Vascular Function-2 study investigated the effects of sequential meals rich in saturated (SFA), n-6 polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids on *ex-vivo* cytokine production in postmenopausal women. In this acute, double-blind, randomised, cross-over study, 32 subjects consumed mixed test meals at 0 min (50 g fat) and 330 min (30 g fat) enriched in different fatty acids on three separate occasions, each 4-6 weeks apart. Blood samples were collected at 0, 180, 300 and 420 min to measure *ex-vivo* cytokine production using whole blood culture (WBC). Cytokine concentrations were determined in WBC supernatant samples after stimulation for 24 h with bacterial lipopolysaccharide (LPS, 0.05 µg/mL). Since whole blood monocyte numbers were influenced by test fat composition, all cytokine data were corrected for the monocyte count. There was a significant test fat*time interaction IL-1β response ($P<0.001$), with a greater postprandial production and area under the curve for the response after the SFA than MUFA and n-6 PUFA rich meals ($P=0.001$). IL-1β concentrations still remained elevated compared with the unsaturated fatty acid-rich meals at the end of the postprandial period. *Ex-vivo* production of IL-6, IL-8, IL-10 and TNF-α did not differ between the test fats. In conclusion, our study in postmenopausal women has revealed marked effects of meal SFA, but not unsaturated fatty acids, on postprandial IL-1β production by *ex-vivo* WBC LPS-stimulated blood samples, and warrants further investigation.

4.2 INTRODUCTION

Menopause induced inflammation, characterised by higher plasma tumour necrosis (TNF)-α and interleukin (IL)-1β concentrations, is considered to contribute to the greater cardiovascular disease (CVD) risk in postmenopausal (257). Diet composition, in particular dietary fat intake, has been identified as an important modulator of low-grade inflammation (258). In general, high intakes of saturated (SFA) and n-6 polyunsaturated fatty acids (PUFA) are considered to be pro-inflammatory, whereas long chain n-3 PUFA exert anti-inflammatory effects (259). However, a large body of this evidence has been derived from animal and *in vitro* studies, with limited consistent data obtained from human studies in response to chronic dietary fat intake (260). Changes in pro-inflammatory cytokines have also been observed after high fat than low fat meals (261), suggesting that a transient state of postprandial inflammation may contribute to the impact of augmented lipaemia on CVD

risk (254, 262-264). To date, very few studies have compared the effects of meals of varying fat composition on postprandial inflammation, especially in postmenopausal women.

Determination of cytokines produced by monocytes is recognised as a more sensitive and meaningful marker of local inflammation (265) than circulating biomarkers. Production of pro-inflammatory (such as IL-1, IL-6, and TNF-α), and anti-inflammatory (IL-10) cytokines (266) can be measured *ex vivo* using lipopolysaccharide (LPS) stimulated whole blood culture (WBC), and this technique has shown a strong correlation with cytokine production from monocyte cultures (267). As part of the Dietary Intervention and VAScular function (DIVAS)-2 study, we determined the acute impact of sequential meals rich in SFA, n-6 PUFA and MUFA on postprandial inflammation using *ex-vivo* LPS stimulated WBC cytokine production in postmenopausal women.

4.3 SUBJECTS AND STUDY DESIGN

The details of the recruitment criteria, study design and test meals for the DIVAS-2 study have been described elsewhere (Chapter 3). Briefly, this was an acute, double-blind, randomised, cross-over study conducted in 36 postmenopausal women aged ≤65 y, who were non-smokers, not using hormone replacement therapy and with no previous diagnosis of CVD (QRISK®2 score for developing CVD within the next 10 y was 5.1 ± 0.4 %).

On three separate occasions, participants attended the Hugh Sinclair Unit of Human Nutrition (University of Reading, UK) where they consumed sequential mixed test meals at breakfast (0 min, 3.8 MJ, 50 g fat, 98 g carbohydrate and 19 g protein) and lunch (330 min, 30g fat, 3.0 MJ, 98 g carbohydrate and 19 g protein) rich in SFA (butter), MUFA (refined olive oil and MUFA-rich spreads) or n-6 PUFA (safflower oil and n-6 PUFA-rich spreads), each 4-6 weeks apart. The corresponding fatty acid contributed approximately 65% and 62% of the total fat content in the breakfast and lunch, respectively. This study was registered at www.clinicaltrials.gov (NCT02144454), and conducted in accordance with the Declaration of Helsinki. The University of Reading Research Ethics Committee (project reference number 14/16) gave a favourable ethical opinion for conduct and all participants provided written informed consent prior to taking part.

WBC analysis

WBC analysis was performed as described by Koutosis et al. (268). Briefly, at each study visit 4 mL blood samples were collected via cannulation into K2EDTA blood collection tubes (VACUETTE) at 0 (baseline), 180, 300 and 420 min. For each time point, one sample was diluted 1:9 with RPMI-1640 medium (Sigma, UK) containing 1% L-glutamine-penicillin-streptomycin and 1% MEM non-essential amino acids (Sigma, UK). Diluted blood samples were incubated for 24 h at 37°C with 0.5 μg/ml LPS from *E. coli* 026: B6 (Sigma, UK), at a final concentration of 0.05 μg/ml. The WBC samples were then centrifuged at 1000 rpm (700 x g) for 5 min to collect the supernatant, and stored at -20°C until the cytokines were analysed. The monocyte count was determined in the second blood sample by the Pathology Department at the Royal Berkshire Hospital, Reading, UK.

Determination of cytokine production using the *Luminex* method

A human cytokine premixed magnetic Luminex performance assay determined IL-1β, IL-6, IL-8, IL-10 and TNF-α concentrations (R & D Systems Europe Ltd.) using the Luminex200 (Thermo Fisher Scientific). The WBC supernatant samples were diluted 1:3 with the calibrator diluent prior to the Luminex analysis.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 24. Two-way repeated measures ANOVA determined the effects of meal fat composition on the postprandial time course profile for the WBC cytokine production using the within-subject factors of 'test fat' and 'time', where $P{\leq}0.05$ was considered significant. Summary measures for the postprandial cytokine responses following the sequential meals were expressed as area under the time curve (AUC) and incremental AUC (IAUC). When a significant effect of test fat was found, a paired sample t-test was performed, with the application of Bonferroni's correction (where $P{\leq}0.017$ was considered significant). Non-parametric one-way repeated measures ANOVA were used for IAUC which could not be normalized. Data are presented in the text, tables, and figures as mean ± SEM.

4.4 RESULTS

Of the 36 postmenopausal women recruited for the DIVAS-2 study, 32 completed all three study visits but complete blood samples were only available from 27/32 participants for the WBC analysis. These volunteers had a mean ± SEM age of 60 ± 1 y and BMI of 26.0 ± 0.7 kg/m^2

Monocyte numbers differed postprandially (time effect $P<0.001$) and according to meal fat composition (test fat*time interaction; $P=0.045$) (**Figure 4.1A**), with a significant test fat effect also observed for the IAUC for this response (P=0.034). The IAUC was lower after the PUFA (mean ± SEM, 8 ± 4 x 109/l cells x 420 min) than MUFA-rich (23 ± 5 x 109/l cells x 420 min) meals ($P=0.007$), with a tendency for a similar response compared with the SFA-rich meals (19 ± 4 x 109/l cells x 420 min) ($P=0.056$). Cytokine responses were therefore corrected according to the monocyte count since monocytes are the main site of cytokine production following LPS stimulation in whole blood.

Baseline cytokine concentrations following LPS-stimulation were not different between the three acute visits (**Table 4.1**). There was a significant test fat*time interaction for the postprandial IL-1β response time course profile ($P=0.008$; **Figure 4.1B**), with higher concentrations observed after the SFA than MUFA and n-6 PUFA-rich meals ($P<0.0001$). Following the SFA-rich meals, IL-1β remained significantly elevated at the end of the postprandial period (420 min; $P<0.0001$) compared with the unsaturated fatty acid-rich meals, which had returned to baseline concentrations or just below. These findings were associated with a significantly greater AUC for the IL-1β response after the SFA than MUFA and n-6 PUFA-rich meals ($P=0.0001$), with only a tendency for a difference in the IAUC between test fats (($P=0.093$; **Table 4.1**). *Ex-vivo* cytokine production of IL-6, IL-8, IL-10 and TNF-α were not modulated by the type of fat given in the sequential meals.

4.5 DISCUSSION

Our WBC analysis showed a greater sensitivity of the pro-inflammatory cytokine IL-1β to meal fat composition, with greater LPS stimulated production after consumption of sequential meals rich in SFA than unsaturated fatty acids. Furthermore, IL-1β concentrations remained elevated at the end of the postprandial period after SFA intake suggesting that transient increases in IL-1β on a daily basis could have implications for long-term cardiovascular health in postmenopausal women.

Although there are a lack of human studies which have compared the effects of meals rich in SFA, n-6 PUFA and MUFA on cytokine production, our findings are in agreement with an *in vitro* study (269) which reported increased secretion of active IL-1β from dendritic cells following treatment with palmitic acid (PA), with a lack of effect on IL-6, IL-8, IL-10, IL-12 and TNF-α. IL-1β is an important pro-inflammatory multifunctional cytokine closely associated with the role of inflammation in atherosclerosis (270). A review by Teng et al. (80) proposed that SFA may mediate inflammation by two mechanisms, one dependent on, and the other independent of, the binding to Toll-like receptors (in particular TLR4) on the surface of immune cells such as monocytes. SFA is a ligand for TLR4 (although unsaturated fatty acids may inhibit this pathway) (269, 271, 272), promoting translocation of nuclear transcription factor (NF-κB) to the nucleus and increased expression of pro-inflammatory cytokines. Postprandial activation of NF-κB has been demonstrated in peripheral mononuclear cells following a single meal rich in SFA (butter, 35% SFA, 22% MUFA and 4% PUFA) than MUFA (extra virgin olive oil, 22% SFA, 38% MUFA and 4% PUFA) in healthy men (273), and also following culture of dendritic cells with varying concentrations of PA (269). The lack of effect of meal fat composition on other cytokines measured in our study suggests that SFA may stimulate production of IL-1β by a TLR4 independent mechanism involving generation of reactive oxygen species (80). NF-κB is a redox sensitive transcription factor suggesting that some cross talk between the two pathways may exist following SFA intake, a finding supported by Nicholas et al. (269). Interestingly, the lipid A moiety of LPS which possesses the biological activity (of LPS) is comprised of SFA (predominately lauric, myristic and PA) (80, 272) suggesting the possibility of enhanced stimulation of IL-1β production in the *ex-vivo* LPS stimulated WBC samples collected following the sequential SFA enriched meals. Further studies are warranted to determine the mechanisms underlying the effects on fatty acids on innate immunity, in the presence and absence of LPS stimulation.

A strength of this study was the use of a sequential test meal protocol incorporating two high fat meals which has a greater capacity to reveal differences in postprandial metabolic responses than a single high-fat meal. Furthermore, our study compared meals rich in SFA, n-6 PUFA and *cis*-MUFA which is timely given that current dietary fat guidelines recommend replacement of dietary SFA with unsaturated fatty acids for CVD prevention. This study has some potential limitations. It was powered to detect a significant difference in the primary outcome of the DIVAS-2 study (flow mediated dilatation) and so our sample size may not be sufficient to detect changes in ex-vivo cytokine production. Postmenopausal women were only included in this study so our findings may not be representative of responses in premenopausal women or men.

In conclusion, meal fat composition was shown to have marked effects on postprandial IL-1 β production by *ex-vivo* WBC LPS-stimulated blood samples, with a greater response following SFA than n-6 PUFA and MUFA meals. Further studies are required to examine the mechanisms underlying the effects of dietary fatty acids on cell signalling and pro-inflammatory cytokine production.

4.6 ACKNOWLEDGMENTS

FIGURE LEGEND

Figure 4.1: Mean ± SEM (n=27) for the A) whole blood monocyte count (x 10^9/L) and B) *ex-vivo* lipopolysaccharide stimulated IL-1β production (expressed as µg per 10^3 monocytes) following sequential meals (0 min and 330 min) enriched with SFA (■), MUFA (●) and n-6 PUFA (□).Two-way repeated measures ANOVA showed a significant effect of test fat ($P \leq 0.034$) and test fat*time interaction ($P \leq 0.045$) for the monocyte count and IL-1β production.

Table 4.1 Effect of meal fat composition on *ex-vivo* cytokine production (µg per 10^3 monocytes) following stimulation of whole blood cultures with lipopolysaccharide (0.05 µg/ml) for 24 h

	SFA	MUFA	n-6 PUFA	P value[1]
IL-1β				
Baseline	24.3 ± 2.0	22.7 ± 1.8	22.2 ± 1.9	0.276
AUC	10832 ± 773[a]	9357 ± 806[b]	9385 ± 792[b]	<0.001
IAUC	621 ± 362	-175 ± 285	77 ± 291	0.093
IL-6				
Baseline	62.9 ± 4.4	56.3 ± 3.1	60.7 ± 5.1	0.170
AUC	21499 ± 1426	20614 ± 1357	21788 ± 1535	0.545
IAUC	-4906 ± 780	-3026 ± 635	-3724 ± 966	0.097
IL-8				
Baseline	185.0 ± 22.0	169.0 ± 16.8	161.7 ± 14.1	0.547
AUC	63190 ± 7224	57776 ± 5371	58776 ± 5166	0.796
IAUC	-14509 ± 4080	-13197 ± 3040	-9157.4 ± 3550	0.104
IL-10				
Baseline	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.2	0.421
AUC	557 ± 59	567 ± 48	621 ± 59	0.457
IAUC	-9.7 ± 21.3	-26.4 ± 32.1	-19.4 ± 28.6	0.618
TNF-α				
Baseline	9.3 ± 0.8	8.6 ± 0.8	9.5 ± 1.2	0.502
AUC	2851 ± 268	2788 ± 276	2978 ± 291	0.719
IAUC	-1055 ± 135	-809 ± 139	-997 ± 245	0.495

Values are mean ± SEM, for 27 postmenopausal women. Abbreviations: AUC: area under the curve, IAUC: incremental AUC, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids. The units for baseline concentrations are µg per 10^3 monocytes and the postprandial summary measures (AUC and IAUC) as µg per 10^3 monocytes*420 min.

[1]Data were analysed using one-way repeated-measures ANOVA (non-parametric for data that could not be normalized). Post-hoc analysis (paired sample t-test) was performed with Bonferroni correction ($P \leq 0.017$) when there was a significant test fat effect. Different superscript letters within a row indicate significant differences between test fats ($P < 0.05$).
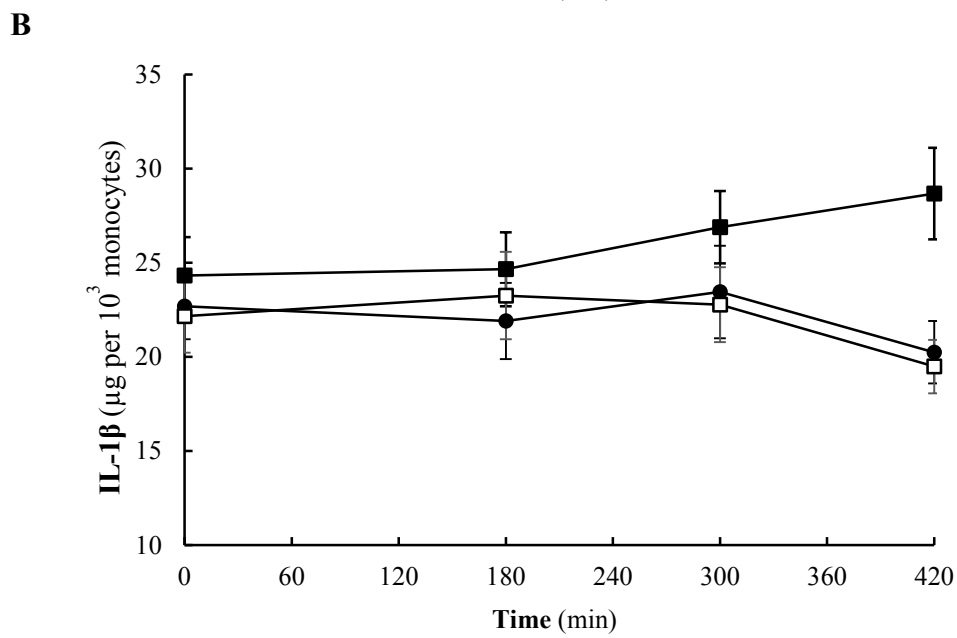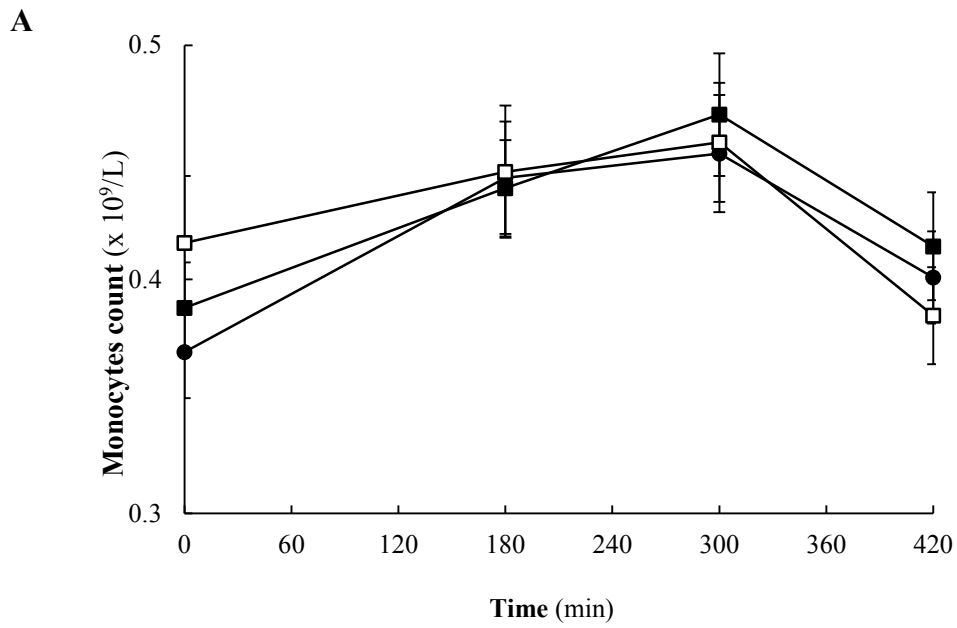
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Chapter 5: Glu298Asp (rs1799983) polymorphism influences postprandial vascular reactivity and insulin response following meals of varying fat composition in postmenopausal women: findings from the randomized, controlled DIVAS-2 study

Kumari M Rathnayake, Michelle Weech, Julie A Lovegrove & Kim G Jackson

Contribution towards the manuscript

In addition to my responsibilities from Chapter 3 with regards to conducting the DIVAS-2 study, I also extracted the DNA from the buffy coat samples and assisted with the eNOS genotype analysis using real time RT-PCR.

Furthermore, I performed all of the statistical analyses for this chapter, and was responsible for writing the manuscript, which was modified with feedback from the other authors.

Glu298Asp (rs1799983) polymorphism influences postprandial vascular reactivity and insulin response to meals of varying fat composition in postmenopausal women: findings from the randomized, controlled DIVAS-2 study

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Running title: Glu298Asp genotype, meal fats and CVD risk markers

Abbreviations: ANOVA: analysis of variance, apo: apolipoprotein, BMI: body mass index, CVD: cardiovascular disease, DIVAS: Dietary Intervention and VAScular function, DVP: digital volume pulse, eNOS: endothelial nitric oxide synthase, FMD: flow-mediated dilatation, HDL-C: high density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment-insulin resistance, IAUC: incremental AUC, LDI: laser Doppler imaging, LDL-C: low density lipoprotein cholesterol, MaxC: maximum concentration, MUFA: monounsaturated fatty acids, NEFA: non-esterified fatty acids, NO: nitric oxide, NOx, both nitrite and nitrate; PUFA: polyunsaturated fatty acids, RI: reflection index, rQUICKI: revised quantitative insulin sensitivity check index, SFA: saturated fatty acids, SI: stiffness index, TAG: triacylglycerol, TC: total cholesterol

Registered at www.clinicaltrials.gov (NCT02144454).

5.1 ABSTRACT

Background: Previous studies have suggested the Glu298Asp polymorphism (rs1799983) may influence vascular reactivity in response to dietary fat intake. However, the effects of this genotype on postprandial vascular function following high-fat meals rich in saturated (SFA), monounsaturated (MUFA) and n-6 polyunsaturated fatty acids (PUFA) are unclear. Objectives: To determine the impact of the Glu298Asp polymorphism on vascular function and cardiovascular disease (CVD) risk markers in response to sequential meals of varying fat composition.

Methods: In a randomized, double-blind cross-over, acute study, 32 postmenopausal women consumed mixed test meals (breakfast: 0 min, 50 g fat; lunch: 330 min, 30 g fat) rich in SFA, MUFA and n-6 PUFA on separate occasions. Blood samples were collected for measurement of CVD risk markers and real-time measures of vascular reactivity (including flow-mediated dilatation (FMD, primary outcome)) were performed before and at regular intervals for 480 min after the breakfast. Participants were retrospectively genotyped for the Glu298Asp polymorphism.

Results: A significant test fat*genotype interaction was observed for the postprandial %FMD response area under the curve (AUC; $P=0.019$), with a greater AUC after MUFA than n-6 PUFA ($P=0.001$), and tendency compared with SFA-rich ($P=0.021$) meals in the Glu298 homozygotes only. Significant test fat*genotype interactions were also evident for the postprandial insulin summary measures ($P≤0.011$), with higher AUC, incremental AUC and maximum concentration after MUFA than SFA-rich (only AUC/incremental AUC) and n-6 PUFA-rich meals in Asp298 carriers. Genotype did not influence any other study outcome measure in response to the test fats.

Conclusion: Our findings indicate the responsiveness of postprandial FMD and insulin responses to sequential meals rich in MUFA to be dependent on Glu298Asp genotype in postmenopausal women. Further studies are required to confirm these observations and to investigate mechanisms underlying the interactive effects of genotype and meal fat composition on vascular function and insulin metabolism.

Keywords: monounsaturated fat, insulin clearance, n-6 polyunsaturated fat, postprandial lipemia, saturated fat, flow-mediated dilatation

5.2 INTRODUCTION

Endothelial dysfunction, characterized by a reduction in the bioavailability of nitric oxide (NO), is now considered to be an early modifiable step in the development of cardiovascular disease (CVD). The potent vasodilator NO is synthesized in the vascular endothelium by the enzyme endothelial NO synthase (eNOS) through the oxidation of L-arginine to L-citrulline (274), and plays a key role in the maintenance of vascular homeostasis. A common single nucleotide polymorphism in the eNOS gene, Glu298Asp (rs 1799983), is regarded to have functional effects on NO production and bioavailability, with a 30% higher CVD risk in minor allele Asp298 carriers attributed to impaired vascular function (119, 275) and elevated blood pressure (116, 276, 277) compared with Glu298 homozygotes. Previous diet-genotype studies have suggested this polymorphism may interact with dietary fat composition, with higher habitual intakes and meals rich in long chain n-3 polyunsaturated fatty acids (PUFA) associated with beneficial effects on flow-mediated dilatation (FMD) (121, 278) and fasting triacylglycerol (TAG) concentrations (122) in Asp298 carriers. However, very little is known about the effects of saturated (SFA), monounsaturated (MUFA) and n-6 PUFA fatty acids on vascular function and CVD risk outcomes in this genotype group. This is particularly important since current dietary fat recommendations for population CVD risk reduction advise replacement of dietary SFA intake with unsaturated fatty acids.

Elevated levels of TAG-rich lipoproteins during the postprandial phase are an independent CVD risk factor (83-85), and are proposed to induce a temporary state of endothelial dysfunction following meal ingestion (47, 250, 279). Interestingly, the effects of long chain n-3 PUFA on the postprandial FMD response appear to be gender and Glu298Asp polymorphism dependent, with a two-fold higher FMD response observed in female compared with male Asp298 homozygotes (121). Although postprandial lipemia is considered to be a more important risk factor in women (83-85), very little is known about the effects of high fat meals on macro- and micro-vascular reactivity, particularly in postmenopausal women who have increased CVD risk (197) yet represent an understudied sub-group of the population. To address this knowledge gap, we performed retrospective genotyping of the Glu298Asp polymorphism in the Dietary Intervention and Vascular Function-2 (DIVAS-2) study (performed in postmenopausal women) to determine the impact of sequential meals rich in SFA, MUFA and n-6 PUFA on postprandial vascular reactivity and CVD risk biomarkers. The main effects of meal fat composition will not be the focus of the current manuscript as these data have been previously published (280).

5.3 METHODS

Study participants and design

The DIVAS-2 study was an acute, double-blind, randomized, cross-over study conducted at the Hugh Sinclair Unit of Human Nutrition (University of Reading, UK) between June 2014 and September 2015. The study recruited 36 non-smoking postmenopausal women aged ≤65 years without diagnosed CVD or type II diabetes, fasting lipid, glucose and markers of kidney and liver function within the normal range and not taking HRT or medication that could influence vascular function and CVD risk biomarkers. The study design, inclusion/exclusion criteria, test meal composition and study measurements have been described in detail elsewhere (Chapter 3). Briefly, participants were randomized using a random assignment program (http://www.randomizer.org/form.htm) (KMR) to consume sequential mixed test meals consisting of a warm chocolate drink with toasted white bread and jam given at breakfast (0 min, 3.8 MJ, 50.0 g fat, 19.2 g protein and 98.0 g carbohydrate) and lunch (330 min, 3.0 MJ, 30.0 g fat, 19.2 g protein and 98.0 g carbohydrate) on three separate occasions, each 4-6 weeks apart. The test fats included in the meals were either rich in SFA (butter; 52.0 g SFA, 20.1 g MUFA and 3.1 g n-6 PUFA), MUFA (refined olive oil and MUFA-rich spreads; 15.5 g SFA, 54.6 g MUFA and 8.5 g n-6 PUFA) or n-6 PUFA (safflower oil and n-6 PUFA-rich spreads; 13.0 g SFA, 10.8 g MUFA and 56.2 g n-6 PUFA). Neither the researchers who performed and analyzed the study measurements (KMR and MW) nor the participants were aware of the meal fat composition at each visit. Habitual dietary intake was assessed using a 4-day weighed food diary completed prior to visit 1, and analysed using the Dietplan7 software (Forestfield).

Following a 12-h overnight fast, anthropometric measurements were conducted before an indwelling cannula was inserted into the forearm and two fasting blood samples were collected (-30 and 0 min) to determine the mean baseline concentration. Vascular function (FMD (primary outcome), laser Doppler imaging (LDI), digital volume pulse (DVP)) and clinic blood pressure measurements were then performed before the breakfast was provided. This meal was consumed within 15 minutes. Blood samples were collected at regular intervals until lunch (every 30 min until 180 min and then every 60 min until 300 min), with further blood samples collected (every 30 min up to 420 min), with the final sample taken at 480 min after the breakfast meal. In addition to the test meals, no other food or drink except for low-nitrate drinking water (Buxton) was allowed during the study visit. Postprandial

measurements of FMD were conducted at 180, 300 and 420 min and blood pressure, LDI and DVP at 240 and 450 min after the breakfast meal.

The study was registered at www.clinicaltrials.gov (NCT02144454), conducted in accordance with the Declaration of Helsinki and approved by the University of Reading Research Ethics Committee (project reference number 14/16). All participants provided written informed consent prior to participation, including consent for genotyping for the eNOS Glu298Asp polymorphism.

Vascular reactivity measurements and blood pressure

To assess vascular reactivity, a single trained researcher conducted endothelial-dependent vasodilation of the brachial artery (FMD) and microcirculation (LDI) as previously described (232). In the peripheral arteries, DVP (Pulse Trace PCA2; Micro Medical Ltd.) determined the stiffness index (DVP-SI; m/s) and reflection index (DVP-RI; %) as measures of arterial stiffness and vascular tone, respectively (232). Clinic blood pressure (systolic blood pressure and diastolic blood pressure) and heart rate were measured in triplicate using an OMRON M6 automatic digital blood pressure monitor (OMRON). Vascular reactivity and clinic blood pressure measurements were performed after participants had rested for 30 min in a supine position in a quiet, temperature controlled room ($22 \pm 1^{\circ}$C).

Biochemical analysis

Blood samples were collected and centrifuged at 1700 x g for 15 min at 20°C (for serum) or 4°C (for plasma and buffy coat) and stored at -20°C and -80°C, respectively. Serum was used to determine lipids (total cholesterol (TC, fasting only), HDL cholesterol (HDL-C, fasting only) and TAG), apolipoprotein (apo)B, glucose, non-esterified fatty acids (NEFA) and high sensitivity C-reactive protein (fasting only) using an ILAB600 autoanalyzer (reagents: Werfen (UK) Ltd.; NEFA reagent: Alpha Laboratories; apoB reagent: Randox Laboratories Ltd). LDL cholesterol (LDL-C) in the baseline fasting samples was estimated using the Friedewald formula (234). ELISA kits were used to analyze circulating serum insulin (Dako Ltd.; Denmark), plasma soluble intercellular adhesion molecules (sICAM-1 and sVCAM-1), E-selectin and P-selectin (R & D Systems Europe Ltd.; UK & Europe). The mean intra-assay and inter-assay CVs were <5% for the automated assays and <10% for the insulin ELISA assay. Plasma nitrite and nitrate levels were analyzed using the HPLC based

approach, Eicom NOx Analyzer ENO-30 (Eicom; San Diego; USA) as described elsewhere (235), and summed to calculate total nitrates and nitrites (NOx). Quality controls with low and high levels were run per 12 samples to check for CV% compliance (<20%) in nitrate and nitrite analysis.

Using baseline fasting measures, insulin resistance was determined by homeostatic model assessment of insulin resistance (HOMA-IR), and insulin sensitivity was estimated with the use of revised quantitative insulin sensitivity check index (rQUICKI) derived using standard equations (236). The QRISK®2-2016 online calculator was used to evaluate the risk of developing CVD in next 10 y (http://www.qrisk.org/index.php).

DNA extraction and genotyping

DNA was extracted from the buffy coat using the Qiagen DNA Blood Mini Kit (Qiagen Ltd., Crawley, UK). The Glu298Asp polymorphism (rs1799983) was determined using the Applied Biosystems RT-PCR 7300 instrument and Assay-on-Demand single nucleotide polymorphism genotyping assay (Life Technologies, UK).

Statistical analysis

To detect a 1.5% (SD 2.0%) intergroup difference in FMD response (the primary outcome measure) with a power of 80%, and 5% significance level, this study required 28 participants. To allow for a 30% dropout rate, 36 volunteers were recruited onto the study. All statistical analysis was performed using IBM SPSS statistics version 24 and results are presented as mean ± SEM. All data were checked for normality and log transformed if necessary to normalise these data. To determine the interactive effect of test fat and genotype on the postprandial time profile responses for all outcome measures, a mixed factor repeated measures ANOVA was used with 'test fat' and 'time' as within subject factors and 'genotype' as a between subject factor. Summary measures for the postprandial responses following the sequential meals were expressed as area and incremental area under the time response curve (AUC and incremental AUC (IAUC), respectively), and maximum concentration (maxC). For NEFA, AUC and IAUC were computed from the time of suppression until end of postprandial period (120-480 min). For summary measures, an ANOVA with repeated measures was used with 'summary measure' as the within subject factor and 'genotype' as a between subject factor. When a significant test fat*genotype interaction was found, data were split according to genotype group and further analyzed using repeated measures

ANOVA with Bonferroni correction to correct for significant pairwise differences ($P \leq 0.017$). Non-parametric one-way repeated measure ANOVA was applied for IAUC with negative values and for the data that could not be normalized by transformation (AUC for the % FMD response). An independent t-test was used to compare average baseline data between genotype groups. $P \leq 0.05$ was considered as significant.

5.4 RESULTS

Study participation

A total of 36 postmenopausal women were randomly allocated to a meal sequence but only 32 completed all three study visits. These participants had a mean age of 57 ± 1 y and BMI of 25.9 ± 0.7 kg/m^2. The genotype distributions of the eNOS single nucleotide polymorphism were in line with the reference data for the Caucasian populations in HapMap, with the frequency of the Glu298 homozygotes 53% (n=17), Asp298 carrier heterozygotes 44% (n=14) and Asp298 homozygotes 3% (n=1). The Asp298 carrier groups were combined for the data analysis.

Table 5.1 shows the average baseline characteristics of the participants for the three study visits according to the Glu298Asp polymorphism. BMI was found to be significantly different between genotype groups, with a 12.2% higher BMI in the Asp298 carriers than Glu298 homozygotes (P=0.040). Other baseline anthropometric, CVD risk markers and estimates of insulin sensitivity and CVD risk were similar between genotype groups. There were also no differences in habitual dietary energy or macronutrient intakes, although intakes of cholesterol were 36% greater in the Asp298 carriers than Glu298 group (P=0.049) (**Table 5.1**).

Vascular function and blood pressure

At baseline, the % FMD response was not found to be different between the genotype groups. There was a significant test fat*genotype interaction (*P*=0.019) and genotype effect (*P*=0.015) for the % FMD response AUC, with differences between test fats only evident in the Glu298 homozygotes (*P*=0.011) but not Asp298 carriers (**Table 5.2**). A greater AUC for the % FMD response was observed in the Glu298 homozygotes after consumption of the MUFA than n-6 PUFA-rich (*P*=0.001) meals, with only a tendency for a difference between the MUFA and SFA-rich meals (*P*=0.021) (**Figure 5.1**). Independent of the meal fat composition, Asp298 carriers had on average a lower AUC for the postprandial %FMD response compared with the Glu298 homozygotes.

No statistically significant test fat*genotype interactions were found for other vascular measurements (LDI and DVP), clinic blood pressure, biomarkers of NO production (NOx) or endothelial activation (cell adhesion molecules). Independent of the meal fat composition, there was an effect of genotype on the IAUC for the DVP-SI, with a tendency for a reduction in postprandial SI after sequential meals in the Asp298 carriers than Glu298 homozygotes (*P*=0.015). On average, fasting plasma nitrate and NOx (nitrite and nitrate combined) tended to be lower in Asp298 carriers (*P*≤0.038), with a minimal change in the IAUC for the postprandial responses compared with the Glu298 homozygotes (*P*=0.015) (**Table 5.2**).

Postprandial CVD risk markers

Fasting insulin concentrations were not different between study visits or genotype groups. Significant test fat*genotype interactions were evident for the AUC (*P*=0.001), IAUC (*P*=0.011) and maxC (*P*=0.005) **(Figure 5.2)** for the postprandial insulin response, with differences between the test fats found in the Asp298 carriers only (*P*≤0.004). In this genotype group, both AUC and IAUC for the postprandial serum insulin response were significantly greater after consumption of MUFA than SFA (*P*≤0.012) and n-6 PUFA-rich (*P*≤0.003) meals. MaxC was also higher after the MUFA than n-6 PUFA-rich meals (*P*=0.0001) with only a tendency for a similar response compared with the SFA-rich meals (*P*=0.024). For the postprandial glucose response, a significant test fat*genotype interaction was also observed for the AUC after the sequential meals (*P*=0.039) **(Table 5.2)**. However, after the data were split according to genotype group, there were no differences between the test fats in either genotype group. The Glu298Asp polymorphism did not influence the

postprandial lipid responses following the sequential SFA, MUFA and n-6 PUFA-rich meals (**Table 5.2**).

5.5 DISCUSSION

This study has revealed the eNOS Glu298Asp polymorphism to be responsive to acute meal fat composition, with differential effects of genotype observed on postprandial FMD and insulin responses to sequential MUFA-rich meals in postmenopausal women. Other measures of vascular function and CVD risk biomarkers were not changed following the SFA, MUFA and n-6 PUFA rich meals in the Glu298 homozygotes and Asp298 carriers.

A genotype specific relationship was found between the AUC for the postprandial %FMD response (our primary outcome) and MUFA intake, with a greater AUC compared with n-6 PUFA in the Glu298 homozygotes only. This was surprising since a previous dietary fat-genotype study had shown a greater impact of meal fat composition in the minor allele carriers, although this study had focused mainly on SFA and long chain n-3 PUFA-rich meals (121). Only one other study has determined the impact of olive oil containing meals on postprandial vascular function according to the Glu298Asp polymorphism but this study did not include a comparator meal of a differing fat composition (120). Although genotype did not influence postprandial microvascular reactivity to the olive oil meals containing varying levels of phenolics, the postprandial vascular response and NOx concentrations were lower (between 4 and 8 h) in the Asp298 carriers than wild-type group. These findings in middle-aged subjects classified with the metabolic syndrome are similar to those in our postmenopausal women, and were both associated with lower baseline and postprandial NOx concentrations in the Asp298 carriers, suggestive of a lower NO bioavailability. Interestingly, in both of these studies, vascular reactivity was not different between genotype groups in the fasting state but only during the postprandial state, a finding also observed after a mixed high-fat meal in healthy men (119). Therefore, the compromised vasodilatory response in the Asp298 carriers may only be expressed during times of metabolic stress, such as the postprandial state.

A number of acute studies have shown vascular function to be modulated by the type of fatty acids consumed (46, 47) but findings have been inconsistent with respect to MUFA-rich oils. A systematic review and meta-analysis by Schwingshackl et al. (281) using data from

chronic randomised clinical controlled trials reported favourable effects of olive oil on the % FMD response, in particular those varieties high in polyphenols. Similar findings have been observed in the postprandial state, with an increase and/or attenuation of the decrease in FMD response to a high-fat meal, evident after meals rich in extra-virgin olive oil (282, 283) and olive oil containing higher amounts of phenolic compounds (120). Polyphenols are considered to mediate their effects on postprandial vascular function by counteracting fat induced oxidative stress and lipid peroxidation, increasing NO bioavailability. However, in the current study, we observed a greater % FMD response with refined olive oil devoid of phenolic compounds. The lack of effect of meal fat composition in Asp298 carriers may shed some light on the mechanisms underlying the increase in the % FMD AUC with MUFA in the Glu298 homozygotes. The glutamate to aspartate amino acid substitution at position 298 of the eNOS protein has been reported to make the enzyme more susceptible to cleavage (causing dysfunction), decreasing eNOS activity and potentially vasodilation (284). Furthermore, Asp298 carriers may also have less eNOS associated with caveolin-1 in the endothelial cell membrane (285). Findings from an animal study (286) and *in vitro* studies (287) including some of our own unpublished findings (288), have shown olive oil (extra-virgin) and oleic acid to induce eNOS activity and gene expression compared with lard (286) and single fatty acids (palmitic, stearic and linoleic acids (286, 288). Therefore, it is possible that during the postprandial state, the greater FMD response in the Glu298 homozygotes may reflect an effect of MUFA per se on eNOS enzyme activity and expression, a finding which requires confirmation in human studies.

Insulin provides a link between metabolic risk markers and cardiovascular health by stimulating eNOS to produce NO, regulating blood flow by inducing blood vessel endothelial dependent vasodilation (289). In support, previous studies have reported associations between the Glu298Asp polymorphism with insulin resistance (120, 290, 291), with a greater prevalence of Asp298 carriers in adults classified with the metabolic syndrome. In the current study, Asp298 carriers showed differential insulin responses to the meal fatty acids, with MUFA ingestion leading to higher postprandial concentrations than the SFA and n-6 PUFA-rich meals. The lack of a genotype effect on glucose handling is suggestive that the sequential MUFA meals may have led to a transient reduction in postprandial insulin sensitivity in this genotype group. However, our findings are in contrast with previous studies which have often reported MUFA-rich meals (containing olive oil and palmolein) to improve beta cell function and postprandial insulin sensitivity compared with SFA-rich

meals (224, 292), but no studies to date have determined the effects according to the Glu298Asp polymorphism. Interestingly, greater insulin but not glucose concentrations were reported in Asp298 carriers than Glu298 homozygotes after an oral glucose tolerance test in Japanese non-diabetic adults (293). Although the authors could not discount differences in insulin sensitivity during the oral glucose tolerance challenge between genotypes, they did speculate that the dysfunctional eNOS enzyme in Asp298 carriers may have reduced insulin mediated blood flow in tissues leading to a decreased insulin clearance. In our postmenopausal women, fasting insulin and estimates of insulin sensitivity (HOMA-IR) and rQUICKI were similar in our wild-type group and minor allele carriers. However, similar differences in blood flow and insulin clearance have also been identified in obese versus lean individuals so we cannot discount that the higher BMI in the Asp298 carriers may have also contributed to the insulin responses observed. Further studies are now warranted to determine the mechanisms underlying the impact of the Glu298Asp polymorphism on postprandial glucose handling and insulin control in response to dietary fat intake.

Our study has some limitations. Genotyping was performed retrospectively and so it was not possible to compare the effects of meal fat composition in Asp298 homozygotes as they represent only 10% of Caucasian populations. The difference in BMI between genotype groups which supports a previous finding (294), may have been a confounding factor influencing the postprandial vascular function and CVD risk factors in the current study. Furthermore, as the participants were postmenopausal women, our findings may not be representative of those in men or premenopausal women, or other ethnic groups.

In conclusion, our study revealed that the Glu298Asp polymorphism is likely to be a determinant of the inter-individual variability in postprandial FMD and insulin responses to acute dietary fat intake in postmenopausal women. However, further studies are warranted using prospective genotyping to investigate the mechanisms underlying the effects of the Glu298Asp polymorphism and MUFA-rich meals on endothelial function and insulin metabolism. Our findings may be important in identifying population subgroups with greater responsiveness to the beneficial effects of targeted dietary fatty acid manipulation through personalized nutrition.

The authors' responsibilities were as follows: JAL, KGJ, KMR and MW designed the study; KMR conducted the research and carried out DNA extraction, analysed the data, conducted the statistical analysis, and wrote the manuscript under the guidance of KGJ and JAL; MW conducted the research, analyzed the data and provided statistical guidance; KGJ performed the genotyping and provided guidance for statistical analyses; all authors critically appraised the writing of the manuscript at all stages and approved the final manuscript and JAL was responsible for the final content. None of the authors had a conflict of interest with regards to the writing or submission of the manuscript.

FIGURE LEGENDS

Figure 5.1: Mean ± SEM for the area under the curve (AUC) for the % FMD response in Glu298 homozygotes (white bars, n=16) and Asp298 carriers (black bars, n=15) following sequential meals enriched in saturated (SFA), monounsaturated (MUFA) and n-6 polyunsaturated fatty acids (PUFA). A mixed model ANOVA revealed a significant test fat*genotype interaction (P=0.019) and genotype effect (P=0.015) for the AUC for the postprandial % FMD response. *P=0.001 compared with n-6 PUFA-rich meals in the Glu298 homozygotes group only.

Figure 5.2: Mean ± SEM for the A) area under the curve (AUC), B) incremental AUC (IAUC) and C) maximum concentration (maxC) for the postprandial insulin responses in the Glu298 homozygotes (white bars, n=14) and Asp298 carriers (black bars, n=12) following sequential meals rich in saturated (SFA), monounsaturated (MUFA) and n-6 polyunsaturated (PUFA) fatty acids. A mixed model repeated measure ANOVA showed significant test fat*genotype interactions the AUC (P=0.001), IAUC (P=0.011) and maxC (P=0.005). *P≤0.012 compared with the AUC and IAUC for the SFA and n-6 PUFA-rich meals and †P=0.001, compared with the maxC for the n-6 PUFA-rich meals in the Asp298 carriers only.

Table 5.1 Baseline characteristics according to the Glu298Asp polymorphism[1]

	Glu298 homozygotes	Asp298 carriers	P value[2]
N	17	15	
Age, y	59 ± 1	57 ± 1	0.383
BMI, kg/m^2	24.5 ± 1.0	27.5 ± 1.0	**0.040**
Waist circumference, cm	87.6 ± 2.1	93.1 ± 2.2	0.080
Body fat, %	34.9 ± 1.7	38.9 ± 1.4	0.078
Blood pressure (mmHg)			
Systolic	135 ± 4	137 ± 4	0.762
Diastolic	77 ± 2	79 ± 2	0.427
Heart rate, beats/min	60 ± 1	58 ± 1	0.294
Fasting CVD risk markers			
Total cholesterol, mmol/L	5.76 ± 0.13	5.72 ± 0.21	0.769
HDL cholesterol, mmol/L	1.68 ± 0.07	1.56 ± 0.06	0.239
LDL cholesterol, mmol/L	3.52 ± 0.13	3.51 ± 0.19	0.872
Triacylglycerol, mmol/L	1.23 ± 0.07	1.43 ± 0.12	0.199
C-reactive protein, mg/L	0.91 ± 0.20	1.72 ± 0.51	0.146
Glucose, mmol/L	4.98 ± 0.08	5.28 ± 0.15	0.084
Insulin, pmol/L	32.7 ± 4.5	42.7 ± 6.4	0.257
NEFA, μmol/L	588 ± 23	652 ± 48	0.315
Estimates of CVD risk and insulin sensitivity			
QRISK®2[3]	4.8 ± 0.5	4.5 ± 0.6	0.727
HOMA-IR	1.21 ± 0.17	1.74 ± 0.31	0.143
rQUICKI	0.42 ± 0.01	0.40 ± 0.01	0.156
Habitual dietary intakes			
Energy, MJ	7.0 ± 0.4	7.7 ± 0.5	0.331
Total fat, %TE	36.0 ± 2.2	34.8 ± 1.4	0.698
SFA, %TE	13.8 ± 1.1	12.4 ± 0.5	0.306
MUFA, %TE	12.6 ± 0.8	12.6 ± 0.8	0.942
n-6 PUFA, %TE	5.1 ± 0.5	5.3 ± 0.4	0.612
n-3 PUFA, %TE	0.89 ± 0.07	0.89 ± 0.06	0.959
Trans fat, %TE	0.87 ± 0.08	0.92 ± 0.11	0.715
Dietary cholesterol, mg/d	194 ± 18	264 ± 29	**0.049**
Protein, %TE	15.5 ± 0.7	16.4 ± 0.7	0.380
Carbohydrate, %TE	45.2 ± 2.1	45.5 ± 1.8	0.783
Dietary fibre (AOAC), g/d	20.7 ± 1.3	23.6 ± 1.6	0.166

[1]Data represent the average of the three baseline visits, with the exception of the dietary intakes that were determined from a single 4-day weighed food diary completed prior to visit 1. Values are mean ± SEM. NOx represents both plasma nitrite and nitrate concentrations combined.
[2]Independent t-tests were used to compare the outcomes between two genotype groups.
[3]QRISK®2 was used to estimate the 10 y CVD risk (myocardial infarction or stroke).
Abbreviations: %TE: percentage of total energy, AOAC: Association of Official Analytical Chemists, CVD: cardiovascular disease, BMI: body mass index, HDL: high density lipoprotein, HOMA-IR: homeostatic model assessment-insulin resistance, LDL: low density lipoprotein, MUFA: monounsaturated fat, NEFA: non-esterified fatty acids, PUFA: polyunsaturated fat, rQUICKI: revised quantitative insulin sensitivity check index, SFA: saturated fat.

Table 5.2 Effect of Glu298Asp polymorphism on the postprandial summary measures for the vascular function outcomes and circulating CVD risk markers after sequential meals of varying fat composition

	Glu298 homozygotes			Asp298 carriers			P values		
	SFA	MUFA	n-6 PUFA	SFA	MUFA	n-6 PUFA	Diet[3]	Diet* Genotype[4]	Genotype[5]
Vascular function									
% FMD									
Fasting, %	4.9±0.6	6.2±1.0	4.8±0.7	4.6±0.7	3.7±0.5	4.7±0.5	0.994	0.056	0.182
AUC, % x min	2246±149[ab]	2783±217[a]	2252±182[b]	1801±172	1813±178	1973±208	0.086	**0.019**	**0.016**
IAUC, % x min	186±171	161±328	231±238	-126±235	276±160	16±119	0.542	0.556	0.510
LDI-Ach									
Fasting, AU	1582±186	1877±268	1890±284	1687±145	1687±131	1714±171	0.386	0.438	0.735
AUC, AU x min x 10^3	72.1±63.8	86.2±114.8	82.1±84.1	75.1±53.9	68.9±45.7	73.1±39.5	0.468	0.135	0.389
IAUC, AU x min x 10^3	9.2±46.1	17.8±80.6	-29.5±64.1	-7.5±37.5	-70.1±54.0	-39.8±69.2	0.595	0.578	0.600
LDI-SNP									
Fasting, AU	1599±188	1711±154	1858±309	1707±193	1963±201	1436±135	0.177	0.201	0.926
AUC, AU x min x 10^3	685±52	798±94	776±100	810±73	829±86	661±37	0.293	0.247	0.873
IAUC, AU x min x 10^3	-34.4±55.9	28.4±64.3	-60.5±79.0	42.1±75.6	-53.7±64.7	15.3±63.5	0.527	0.233	0.754
DVP-RI									
Fasting, %	61.2±1.4	60.8±1.9	61.8±2.2	62.8±2.8	60.5±2.9	63.8±2.9	0.641	0.836	0.639
AUC, % x min x 10^3	25.6±0.7	25.6±0.8	26.9±1.0	26.4±1.3	24.7±0.9	25.5±1.1	0.374	0.359	0.669
IAUC, % x min x 10^3	-2.0±0.5	-1.8±0.7	-1.0±0.6	-1.9±1.2	-2.5±1.0	-3.2±0.8	0.687	0.355	0.140
DVP-SI									
Fasting, m/s	6.7±0.2	6.9±0.3	6.9±0.3	7.2±0.5	7.8±0.4	7.3±0.3	0.497	0.591	0.118
AUC, m/s x min	3150±95	3215±123	3346±143	3295±177	3083±80	3198±160	0.596	0.169	0.776
IAUC, m/s x min	115±99	128±90	233±134	60±129	-404±121	-71±111	0.666	0.063[2]	**0.015**
SBP									
Fasting, mm Hg	134.9±4.1	136.2±3.9	134.3±4.5	133.2±4.4	138.2±3.7	138.5±4.4	0.207	0.232	0.785
AUC, mm Hg x min x 10^3	56.6±1.5	55.8±1.4	56.2±1.6	57.4±2.0	58.1±1.7	58.9±1.9	0.297	0.221	0.395
IAUC, mm Hg x min x 10^3	-4.1±0.7	-5.5±0.7	-4.2±0.8	-2.6±0.8	-4.1±0.9	-3.4±0.7	0.053	0.803	0.180
DBP									
Fasting, mm Hg	76.2±2.0	77.2±1.8	76.2±2.1	77.1±2.1	79.4±2.1	79.5±2.1	0.139	0.372	0.434
AUC, mm Hg x min x 10^3	32.8±0.8	32.4±0.8	32.3±0.9	33.3±1.0	33.5±0.9	34.3±1.0	0.699	0.111	0.333
IAUC, mm Hg x min x 10^3	-1.5±0.4	-2.3±0.3	-1.9±0.3	-1.4±0.4	-2.2±0.5	-1.5±0.5	0.007	0.843	0.585
Nitrite									
Fasting, µmol/L	0.12±0.01	0.12±0.01	0.12±0.01	0.14±0.01	0.13±0.01	0.13±0.01	0.243	0.449	0.259

AUC, µmol/L x min	49.8±3.4	50.0±3.5	52.6±3.7	56.1±3.7	56.3±4.5	55.1±3.9	0.661	0.275	0.510
IAUC, µmol/L x min	-0.7±0.4	-0.4±0.6	2.2±2.6	-1.8±1.2	1.3±1.3	-0.8±0.8	0.010	0.244	0.579
Nitrate									
Fasting, µmol/L	22.3±2.4	20.7±2.5	19.9±3.3	15.5±1.1	16.7±2.5	13.8±1.5	0.127	0.806	**0.037**
AUC, mmol/L x min	6.9±0.7	6.3±0.7	6.3±0.9	5.3±0.3	5.8±0.7	4.9±0.5	0.381	0.612	0.090
IAUC, mmol/L x min	-2.5±0.4	-2.4±0.5	-2.0±0.5	-1.3±0.2	-1.2±0.3	-0.9±0.2	0.054	0.970	**0.015**
NOx									
Fasting, µmol/L	22.5±2.4	20.9±2.5	20.0±3.3	15.7±1.1	16.8±2.5	13.9±1.5	0.127	0.805	**0.038**
AUC, mmol/L x min	6.9±0.7	6.4±0.7	6.4±0.9	5.3±0.4	5.8±0.7	5.0±0.5	0.384	0.609	0.091
IAUC, mmol/L x min	-2.5±0.04	-2.4±0.5	-2.0±0.5	-1.3±0.2	-1.2±0.3	-0.9±0.2	0.054	0.968	**0.015**
Biochemical measures									
TAG									
Fasting, mmol/L	1.29±0.11	1.16±0.05	1.24±0.11	1.42±0.14	1.50±0.12	1.62±0.19	0.735	0.297	0.063
AUC, mmol/L x min	968±107	912±56	966±114	996±82	1145±111	1165±147	0.546	0.233	0.264
IAUC, mmol/L x min	350±65	353±47	369±80	314±34	423±72	386±70	0.135	0.258	0.841
MaxC, mmol/L	2.91±0.33	2.89±0.21	3.04±0.32	2.83±0.24	3.44±0.35	3.36±0.43	0.140	0.311	0.526
Glucose									
Fasting, mmol/L	5.07±0.13	4.94±0.09	4.99±0.07	5.33±0.19	5.42±0.17	5.33±0.17	0.929	0.211	0.062
AUC, mmol/L x min	2886±78	2863±108	2972±93	3032±102	3129±145	2990±140	0.933	**0.039**[2]	0.341
IAUC, mmol/L x min	454±82	491±93	576±96	472±68	529±90	429±78	0.304	0.195	0.778
MaxC, mmol/L	8.79±0.32	8.77±0.44	9.18±0.33	9.00±0.57	9.53±0.66	9.07±0.54	0.641	0.279	0.646
NEFA									
Fasting, µmol/L	545±28	636±41	585±25	649±58	606±64	595±64	0.609	0.135	0.605
AUC$_{120-480}$, mmol/L x min	128±10	132±11	131±8	145±11	126±13	125±11	0.404	0.052[2]	0.926
IAUC$_{120-480}$, mmol/L x min	-68±11	-97±13	-79±12	-88±17	-92±14	-90±17	0.089	0.528	0.573
MaxC, µmol/L	725±46	724±40	706±44	784±62	695±67	689±63	0.223	0.331	0.946
Apo B									
Fasting, mg/mL	978±34	953±49	938±40	1023±51	1051±65	1062±65	0.850	0.164	0.195
AUC, mg/mL x min	469±15	456±21	451±18	492±26	509±29	510±31	0.893	0.080	0.163
IAUC, mg/mL x min	-0.8±5.4	-1.1±5.5	0.5±6.4	0.4±5.9	4.1±4.6	-0.1±3.8	0.891	0.805	0.740
MaxC, mg/mL	1038±34	999±44	999±42	1094±66	1132±66	1138±69	0.908	**0.045**[2]	0.141
Insulin									
Fasting, pmol/L	43.2±13.8	32.5±3.3	27.9±3.1	41.6±8.0	44.7±7.5	44.7±9.9	0.491	0.474	0.339

Values are mean ± SEM. Total n=26-32, with n=13-17 Glu298 homozygotes and n=12-15 Asp298 carriers per outcome.

Time intervals for AUC and IAUC: 420 min for FMD, NOx and circulating markers of endothelial activation; 450 min for DBP, SBP, DVP measures (stiffness index and reflection index) and LDI; 480 min for all other biochemical markers; 120-480 min for NEFA.

[1]LDI-Ach and LDI-SNP were expressed as AUC for the 20-scan protocol. IAUC was also determined for the 20-scan protocol but differences between test fats for subsequent AUC and IAUC were not significant (data not shown).

[2]Post-hoc tests were not significant after Bonferroni correction.

[3]Data was analyzed using one factor repeated-measures ANOVA with 'summary measures' as the within subject factor.

[4]Data was analyzed using mixed model repeated-measures ANOVA with 'summary measures' as within subject factor variable and 'genotype' as between subject factor; if the effect of test fat was significant, post-hoc analysis was performed with a paired sample t-test for AUC summary measures and non-parametric test for IAUC summary measures both with Bonferroni correction ($P \leq 0.017$). Different superscript letters within a row indicate significant differences between test fats within genotype group.

[5]Independent t-test was performed to compare the outcomes between two genotype groups.

Abbreviations: Ach: acetylcholine, AU: arbitrary units, apoB; apolipoprotein B, DBP: diastolic blood pressure, DVP: digital volume pulse, FMD: flow-mediated dilatation, IAUC: incremental AUC, LDI: laser Doppler imaging, NOx: total nitrite and nitrate, MUFA: monounsaturated fat, PUFA: polyunsaturated fat, SFA: saturated fat, SBP: systolic blood pressure, sICAM-1: soluble intercellular cell adhesion molecule-1, NEFA; non-esterified fatty acids, SNP: sodium nitroprusside, TAG; triacylglycerol, sVCAM-1: soluble vascular cell adhesion molecule-1.

FIGURE 5.1
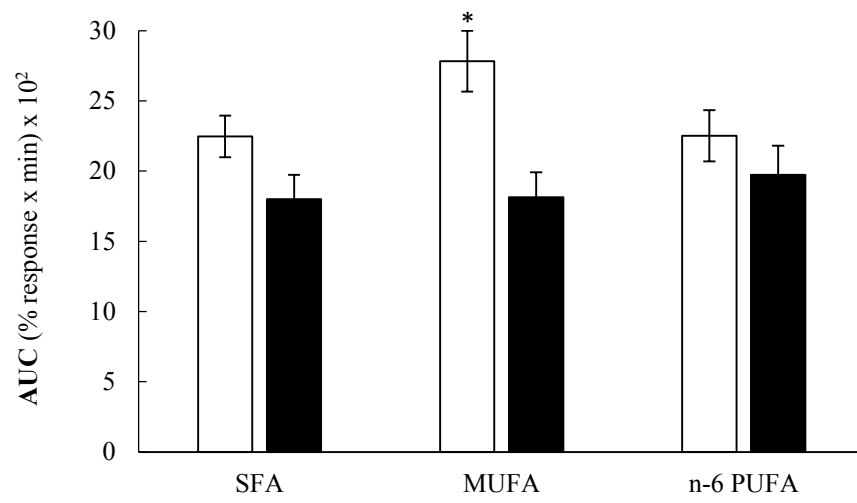
FIGURE 5.2

A
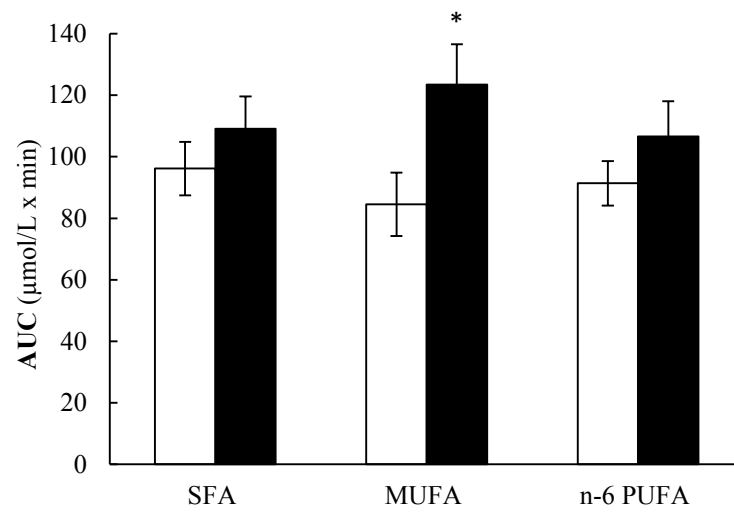
B
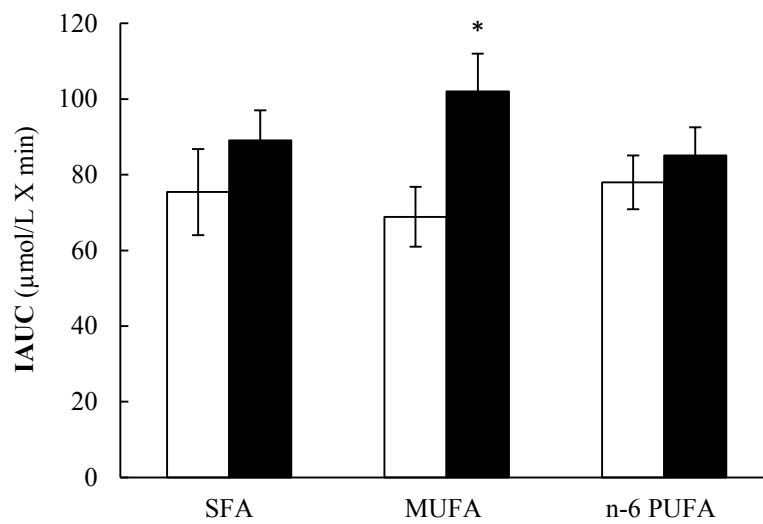
C
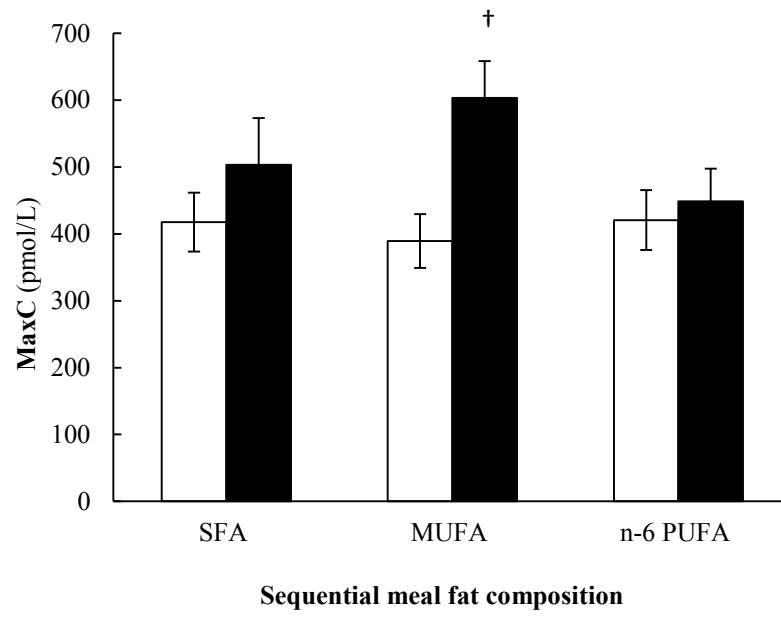
y-axis: **MaxC (pmol/L)** with values 0, 100, 200, 300, 400, 500, 600, 700

†

x-axis categories: SFA, MUFA, n-6 PUFA

Sequential meal fat composition

Chapter 6: Impact of the *APOLIPOPROTEIN E* (epsilon) genotype on cardio-metabolic risk markers and responsiveness to acute and chronic dietary fat manipulation

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Contribution towards the manuscript

In addition to my responsibilities from Chapter 3 with regards to conducting the DIVAS-2 study, I also extracted the DNA from the buffy coat samples for the DIVAS-2 volunteers and performed PCR to determine *APOE* genotypes. I also analysed the data from the DIVAS study in relation to the *APOE* genotype interactions. For this study, DNA was extracted (M. Hasaj and M. Weech), and genotype for *APOE* genotypes (K. Jackson and R. Rothwell) by other researchers

I performed all of the statistical analyses for this chapter, and was responsible for writing the manuscript, which was modified with feedback from the other authors.

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Impact of the *APOLIPOPROTEIN E* (epsilon) genotype on cardio-metabolic risk markers and responsiveness to acute and chronic dietary fat manipulation

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Disclaimer: JAL is a member of the Scientific Advisory Committee on Nutrition (SACN) and SACN's Saturated Fats Working Group

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Running title: *APOE,* dietary fat and cardio-metabolic risk markers

Abbreviations: %TE, total energy; ANCOVA, analysis of covariance; ANOVA: analysis of variance, apo: apolipoprotein, AU, arbitrary units; AUC, area under the curve; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; DIVAS, Dietary Intervention and VAScular function; DVP, digital volume pulse; DVP-RI, DVP reflection index; DVP-SI, DVP stiffness index; FMD, flow-mediated dilatation; HDL-C, high density lipoprotein cholesterol; HOMA-IR, quantitative insulin resistance index; IAUC, incremental AUC; LDI, laser Doppler imaging; LDL-C, low density lipoprotein cholesterol; MUFA, monounsaturated fatty acids; NEFA, non-esterified fatty acids; NOx; sum of nitrite and nitrate concentrations; PUFA, polyunsaturated fatty acids; rQUICKI, revised quantitative insulin sensitivity check index; SBP; systolic blood pressure; SFA, saturated fatty acids; sICAM-1; soluble intercellular cell adhesion molecule-1; SNP; sodium nitroprusside; sVCAM-1; soluble vascular cell adhesion molecule-1; TAG, triacylglycerol; TC, total cholesterol

6.1 ABSTRACT

Background: *APOLIPOPROTEIN E* (*APOE*) (epsilon) genotype is considered to play an important role in plasma lipid responses to dietary fat manipulation. However, little is known about the impact on novel cardio-metabolic risk markers, such as vascular function.

Objectives: Using data from the Dietary Intervention and VAScular function (DIVAS) studies, we investigated the relationship of *APOE* genotype with fasting cardio-metabolic risk markers and their responsiveness to acute and chronic dietary fat intake.

Methods: Associations between genotype with fasting (baseline) outcome measures (n=216) was determined using data from the chronic DIVAS (n=191 men and women at moderate cardiovascular disease risk) and acute DIVAS-2 (n=32 postmenopausal women) studies examining the effects of diets/meals rich in saturated (SFA), n-6 polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids. Participants were retrospectively genotyped for *APOE* (rs429358, rs7412).

Results: For baseline cardio-metabolic outcomes, *E4* carriers had higher fasting total (TC) and low density lipoprotein cholesterol (LDL-C), TC: LDL-C ratio and LDL-C: high density lipoprotein cholesterol ratio, but lower C-reactive protein (CRP) than *E3/E3* and *E2* carriers (*P≤0.003*). The digital volume pulse stiffness index was higher in *E2* carriers than *E3/E3* group (*P=0.011*). Following chronic dietary fat intake, significant diet*genotype interaction was found for fasting triacylglycerol (*P=0.010*), with indication of a differential responsiveness to MUFA intake between the *E3/E3* and *E4* carriers (*P=0.006*). Test fat*genotype interactions were observed for the incremental area under the curve for the postprandial apolipoprotein B (*P=0.022*) and digital volume pulse reflection index (*P=0.030*) responses after the MUFA-rich meals, with a greater reduction in *E4* carriers than *E3/E3* group respectively.

Conclusion: Our baseline associations between *APOE* genotype with fasting lipids and CRP confirm previous findings, although a novel interaction with digital volume pulse arterial stiffness was observed. The reported differential impact of *APOE* genotype on cardio-metabolic markers in the acute and chronic state requires further confirmation.

6.2 INTRODUCTION

The *APOLIPOPROTEIN (APO)E* (ε) genotype is the most widely researched single nucleotide polymorphism in relation to cardiovascular disease (CVD) risk, with the *APOE4* allele linked with increased total (TC) and low density lipoprotein cholesterol (LDL-C), CVD risk and mortality (126, 130, 295-297). *APOE* genotype has also been shown to influence the fasting lipid profile in response to dietary fat intake. Studies to date have focused on high-fat, high saturated fat (SFA), low-fat and high-fat, high-SFA with fish oil interventions (135-138, 298-300), whilst little is known of the interactions between the *APOE* genotype with n-6 polyunsaturated (PUFA) and monounsaturated (MUFA) fat intakes. This is particularly important and timely given that population dietary recommendations for CVD prevention advise reducing intakes of SFA to ≤10% of total energy (%TE) via replacement with n-6 PUFA or MUFA. Although fasting lipids contribute to the increased CVD risk they do not seem to solely explain this increased CVD risk in *APOE4* carriers, with limited information available on other cardio-metabolic risk markers. In particular, endothelial dysfunction is now recognised as a critical modifiable event in coronary atherosclerosis but limited data are available on the impact of *APOE* on the responsiveness of vascular reactivity to dietary fat composition (172).

Most of the studies investigating the interaction between *APOE* genotype with dietary fat intake on lipid metabolism have been performed in the fasted state. However, non-fasted lipaemia is now recognised as an independent CVD risk factor (81, 90, 131), which is of particular relevance as individuals spend the majority of the day in a postprandial (fed) state. Previous studies reported polymorphisms in the *APOE* gene to be associated with increased postprandial triacylglycerol (TAG) responses (301-303). However, data is extremely limited on the impact of meal fatty acids on postprandial lipid and vascular outcomes according to *APOE* genotype.

The present analysis explored the interaction of *APOE* genotype with both chronic and acute intake of diets/meals rich in SFA, MUFA and n-6 PUFA on established and novel cardio-metabolic risk markers. This was achieved using data from the chronic Dietary Intervention and Vascular Function (DIVAS) study performed in 186 individuals with moderate CVD risk (223), and the DIVAS-2 postprandial study conducted in 32 postmenopausal women. We hypothesised that *APOE* genotype would influence these risk markers both at baseline and in response to fat manipulation. The overall diet/meal fat effects for both studies will

not be the focus of the current manuscript as these data have been previously reported for each subject group (chronic study: (223); acute study: Chapter 3).

6.3 METHODS

Study participants and design

This paper is based on retrospective *APOE* genotype analysis and previously analysed cardio-metabolic risk markers in participants from two studies (DIVAS and DIVAS-2) performed at the Hugh Sinclair Unit of Human Nutrition (University of Reading, UK). The details of the chronic DIVAS and acute DIVAS-2 studies have been previously published (223) (Chapter 3). Only participants who had provided informed consent for the retrospective genotyping for *APOE* were included in this data analysis (n=191/195 for DIVAS and n=32/32 for DIVAS-2). Both studies were conducted in accordance with the Declaration of Helsinki.

Baseline associations between APOE genotype with established and novel cardio-metabolic risk markers

Baseline data (n=216) was from both the DIVAS (n=84 men and n=107 women) (223) and DIVAS-2 (n=25/32 postmenopausal women; 5 women had participated in both the DIVAS and DIVAS-2 studies, so only baseline data from the DIVAS study for these participants were included) (30) (Chapter 3) studies were combined to investigate the impact of *APOE* genotype on vascular function, blood pressure, biomarkers of endothelial dysfunction, lipids, glucose, insulin and inflammatory markers determined in the fasting state. Habitual dietary intake according to *APOE* genotype was assessed using data extracted from 4-day weighed food diaries and analysed using Dietplan (DIVAS: version 6.6; DIVAS-2: version 7; Forestfield, Horsham, UK).

Impact of APOE genotype on the responsiveness of cardio-metabolic risk markers to:
A) Chronic dietary fat composition

The DIVAS study was a single-blind, parallel-group randomised controlled trial that replaced 9.5-9.6% TE of dietary SFA with MUFA or n-6 PUFA for 16 weeks. Non-smoking men and women (n=191) aged 21-60 y with moderate CVD risk were recruited in three cohorts between November 2009 and June 2012 (304) and allocated to one of three

intervention diets, stratified by sex, age, body mass index (BMI) and CVD risk score. The isoenergetic diets (%TE target compositions of total fat: SFA: MUFA: n–6 PUFA) were rich in SFA (36:17:11:4), MUFA (36:9:19:4), or n–6 PUFA (36:9:13:10), and matched for protein, carbohydrate and n-3 PUFA (304). A flexible food-exchange model replaced sources of exchangeable fats with study foods (spread, oils, dairy products, and snacks) of specific fatty acid composition. Primary sources of exchangeable fats were: butter (SFA-rich diet; Wyke Farm); refined olive oil and MUFA-rich spread (MUFA-rich diet; Unilever R&D); safflower oil and n-6 PUFA-rich spread (n-6 PUFA-rich diet; Unilever R&D). At baseline (week 0) and after the intervention period (week 16), vascular reactivity was measured and a blood sample was collected in the fasting state, as previously described (223). This study was given a favourable ethical opinion for conduct by the West Berkshire Local Research Ethics Committee (09/H0505/56) and University of Reading Research Ethics Committee (project reference number 09/40), and registered at www.clinicaltrials.gov (NCT01478958).

B) Acute meal fat composition

The DIVAS-2 study was an acute, double-blind, randomised, cross-over study conducted between June 2014 and September 2015. Postmenopausal women (n=32) were randomly assigned to consume sequential mixed test meals (0 min, 50 g fat and 330 min, 30 g fat) rich in SFA (butter), MUFA (refined olive oil and MUFA-rich spreads) or n-6 PUFA (safflower oil and n-6 PUFA-rich spreads) on three separate occasions, each 4-6 weeks apart. Details of the test meal fat composition and study procedures are given elsewhere (Chapter 3). Blood samples were collected at regular intervals (every 30 min until 180 min and then every 60 min until 300 min) after breakfast until lunch was given at 330 min. After lunch, blood samples were collected every 30 min up to 420 min, with the final sample taken at 480 min. In this study, flow mediated dilatation (FMD) was performed at baseline (fasting), 180, 300 and 420 min, and clinic blood pressure, LDI and DVP at baseline, 240 and 450 min. This study had a favourable opinion for conduct from the University of Reading Research Ethics Committee (project reference number 14/16), and registered at www.clinicaltrials.gov (NCT02144454).

Vascular reactivity measurements and blood pressure

For both DIVAS and DIVAS-2 studies, macro- and micro-vascular reactivity were assessed by conducting FMD of the brachial artery (primary outcome measure) and laser Doppler imaging (LDI) with iontophoresis respectively (232). In the peripheral arteries, digital volume pulse (DVP) (Pulse Trace PCA2; Micro Medical Ltd.) determined the stiffness index (DVP-SI; m/s) and reflection index (DVP-RI; %) as measures of arterial stiffness and vascular tone, respectively (232). In DIVAS, 24 h ambulatory blood pressure (ABP) and heart rate were recorded every 30 min during the day and 60 min during the night at baseline and week 16 using A/A grade automated oscillometric ABP monitors (A & D Instruments Ltd., UK) as described elsewhere (223). In DIVAS and DIVAS-2, clinic systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate measurements were recorded at each study visit using an OMRON blood pressure monitor. Pulse pressure was calculated as the difference between the average systolic and diastolic blood pressures.

Biochemical analysis, estimates of insulin sensitivity/resistance and CVD risk score

In both studies, serum was used to determine lipids (TC, HDL cholesterol (HDL-C), TAG, apolipoprotein (apo)B (DIVAS-2 only)), glucose, non-esterified fatty acids (NEFA) and C-reactive protein (CRP) using an autoanalyzer (ILAB 600; Werfen (UK) Ltd.; reagents and analyzer: Werfen Ltd.; NEFA reagent: Alpha Laboratories; apoB reagent: Randox Laboratories Ltd). Fasting LDL-C was estimated using the Friedewald formula (234). ELISA kits were used to determine serum insulin (Dako Ltd.; Denmark), and plasma soluble intercellular adhesion molecule-1(sICAM-1), soluble vascular cell adhesion molecule (sVCAM-1), E-selectin and P-selectin (R & D Systems Europe Ltd.; UK & Europe) concentrations. Plasma nitrite and nitrate levels were analyzed with ozone-based chemiluminescence (305) in the DIVAS study and HPLC based approach, Eicom NOx Analyzer ENO-30 (Eicom; San Diego; USA) (235) in the DIVAS-2 study.

Using baseline measures, insulin resistance was determined by homeostatic model assessment-insulin resistance (HOMA-IR), and insulin sensitivity was estimated with the use of revised quantitative insulin sensitivity check index (rQUICKI) derived using standard equations (236). The QRISK®2-2016 online calculator was used to estimate the participant's risk of developing CVD within the next 10 y (http://www.qrisk.org/index.php).

DNA extraction and genotyping

The buffy coat was isolated from 9 mL of blood collected into K2EDTA blood collection tube, and DNA was extracted using the Qiagen DNA Blood Mini Kit (Qiagen Ltd., Crawley, UK). *APOE* genotype (*E2/E4*, *E2/E3*, *E2/E4*, *E3/E3, E3/E4* or *E4/E4)* was determined retrospectively by allelic discrimination using "Assay-on-Demand" single nucleotide polymorphism genotyping assays (rs7412 and rs429358) (Life Technologies, UK).

Statistical analysis

The DIVAS and DIVAS-2 studies were powered to detect a 2% (SD 2.3%, 80% power and 5% significance level) and 1.5% (SD 2.0%, 80% power and 5% significance level) difference in %FMD response (primary outcome), requiring 171 and 28 participants respectively. Secondary outcome measures included microvascular reactivity, arterial stiffness, blood pressure, serum lipid profile, markers of inflammation and endothelial activation, and estimates of insulin sensitivity/resistance. The analysis presented in this manuscript is explorative, investigating the interactions between *APOE* genotype with fat manipulation on the primary and secondary outcome measures.

Analysis of data was carried out with SPSS statistical analysis software version 21 (SPSS Inc., Chicago, IL). Results are presented in the text, tables, and figure as means ± SEMs. $P \leq 0.05$ was considered significant. The data were checked for normality of distribution, and skewed variables were normalized prior to statistical analysis. For the baseline data analysis, which included data from both the DIVAS and DIVAS-2 studies, a univariate general linear model (ANCOVA) was implemented using the baseline outcome measures as the dependant variables, with sex and *APOE* genotype included as fixed factors and age and BMI as covariates, to assess the *APOE* genotype effect. If a significant genotype effect was observed, pairwise comparisons were performed with the application of Bonferroni's correction (where $P \leq 0.017$ was considered significant).

To determine the effects of chronic dietary fat manipulation (DIVAS), a general linear model (ANCOVA) was used to determine the overall effect of diet and *APOE* genotype on the primary and secondary outcome measures. In this model, the post-intervention (week 16) – baseline (week 0) difference was the dependant variable, with genotype, sex, and intervention diet as fixed factors, and baseline value of the variable of interest, age and BMI

included as covariates. The interaction term was added to the model to assess the overall diet*APOE genotype interaction. If a significant interaction was found, a general linear model was performed for the three diets to determine which diets were different within each genotype group separately. When there was no overall diet effect for each genotype group, independent t-tests were performed for the three diet groups separately to determine the differences between the genotype groups.

Acute effects of test fat composition (DIVAS-2) on the time response profiles were analysed using a mixed factor repeated measures ANOVA with test fat and time included as within-subject factors and genotype as the between-group factor. Summary measures for the postprandial responses were expressed as area and incremental area under the curve (AUC and IAUC, respectively) over 420, 450 or 480 min. For NEFA, AUC and IAUC were computed from the time of suppression until end of postprandial period (120-480 min). Non-parametric one-way repeated measure ANOVA was applied for IAUC with negative values and for the data that could not be normalized by transformation. If a significant test fat*genotype interaction was found, a repeated measures ANOVA was performed in the two genotype groups separately, with Bonferroni correction (where $P \leq 0.017$ was considered significant) used to correct for the significant pairwise differences. An independent t-test was used to compare the responses to the test fats separately between genotype groups and $P \leq 0.05$ was considered as significant.

6.4 RESULTS

Baseline associations between *APOE* genotype with established and novel cardio-metabolic risk markers

Table 6.1 presents the baseline subject characteristics and cardio-metabolic risk markers of the 216 participants (84 males and 132 females (self-reported menopausal status: 66 pre-, 8 peri- and 58 postmenopausal women)) according to *APOE* genotype which were presented as *E2* carriers (*E2/E2* and *E2/E3*, n=30), the wild-type homozygous *E3/E3* group (n=128) and *E4* carriers (*E3/E4* and *E4/E4*, n=58). Individuals with the *E2/E4* genotype were excluded from all data analyses due to small subject group (n=5).

At baseline, there was no significant effect of genotype on the primary outcome measure, % FMD response. For TC (*P*=0.0001), LDL-C (*P*=0.0001), TC: HDL-C ratio (*P*=0.002) and LDL-C: HDL-C ratio (*P*=0.0001), a significant genotype effect was evident with lipid

concentrations and ratios increasing in the order: *E2* carriers > *E3/E3* group > *E4* carriers (**Table 6.1**). There was also an influence of genotype on baseline CRP ($P=0.002$), with lower concentrations in *E4* carriers compared with the wild-type group ($P=0.003$) and *E2* carriers *($P=0.002$)*. DVP-SI was found to be different between groups ($P=0.027$), with a 17% higher DVP-SI in the *E2* carriers than *E3/E3* group ($P=0.011$). *APOE* genotype did not influence any of the other baseline characteristics or cardio-metabolic risk markers (**Table 6.1**). Habitual dietary intakes stratified according to genotype (**Supplementary Table 6.1**) showed differences in %TE of trans fat ($P=0.031$), whereby intakes were greater in the *E2* carriers than *E3/E3* homozygotes ($P=0.043$). However, this difference was not significant after correcting for multiple comparisons ($P\geq0.017$).

Effect of dietary fat manipulation and *APOE* genotype on cardio-metabolic risk markers

Since low numbers of *E2* carriers (*E2/E2* and *E2/E3*), were identified by retrospective genotyping, they were excluded from the data analyses for the chronic (SFA diet (n=12), MUFA diet (n=5) and n-6 PUFA diet (n=10)) and acute (n=3 *E2/E3*) fat manipulation.

Chronic dietary fat composition (DIVAS)

A total of 159 subjects (68 men and 91 women) were included in this analysis (E2 carriers were excluded n=27) with a mean age of 44 ± 1 y and BMI of 26.4 ± 0.3 kg/m^2, of which 107 had the *E3/E3* genotype and 52 were *E4* carriers (**Table 6.2**). No diet*genotype interaction was evident for the change in the primary outcome, %FMD response, or other measures of vascular function during the 16-wk chronic intervention. A significant diet*genotype interaction was found for the change in fasting TAG ($P=0.010$) but there was no overall diet effect when the *APOE3/E3* and *E4* carrier groups were analysed separately. However, there was an indication of a differential responsiveness of fasting TAG to MUFA intake (but not SFA and n-6 PUFA) with an increase and decrease in TAG concentration in the *E3/E3* and *E4* carriers, respectively ($P=0.006$) (**Table 6.2**). *APOE* genotype was not found to influence any of the other secondary outcome measures in response to chronic dietary fat intake.

Independent of the 16-wk dietary intervention, genotype effects were observed for the changes from baseline for HDL-C (*P*=0.015), CRP (*P*=0.036), as well as P-selectin (*P*=0.026) (**Table 6.2**) where there was a reduction in HDL-C and CRP in the *E4* carriers compared with an increase in the *E3/E3* group, and an increase in P-selectin in the *E4* carriers relative to the decrease observed in the *E3/E3* group.

Acute meal fat composition (DIVAS-2)

A total of 27 postmenopausal women were included in this analysis (n=22 *E3/E3* and n=5 *E3/E4)*, with a mean age of 58 ± 1 y and BMI of 26.1 ± 0.7 kg/m^2 (**Table 6.3**). *APOE* genotype did not influence the responsiveness of postprandial measures of macrovascular function (%FMD response), microvascular function (LDI) or arterial stiffness (DVP-SI) to the meal fat composition. However, a significant test fat*time**APOE* genotype interaction was observed for the DVP-RI time course profile (*P*=0.014) (**Figures 6.1A** and **6.1B**). This was associated with a significant genotype*test fat interaction for the DVP-RI IAUC (*P*=0.030) (**Figure 6.1C**). When data were spilt by genotype group, there was a significant test fat*time interaction (*P*=0.037) and test fat effect (*P*=0.027) in the *E4* carriers only, in which a reduction in IAUC was observed following the SFA than MUFA (*P*=0.033) and n-6 PUFA-rich (*P*=0.028) meals, although these effects were not considered significant after applying the Bonferroni correction.

There was a test fat*genotype interaction for the postprandial apoB response IAUC (*P*=0.022), with a tendency for higher IAUC after the n-6 PUFA than MUFA and SFA rich meals (*P*=0.068) in *E4* carriers only (**Table 6.3**, **Figure 6.2**). However, there were differential effects on the responsiveness of the genotype groups to the MUFA-rich meals, with a reduction in the apoB IAUC in *E4* carriers compared to an increase in the *E3/E3* group (*P*=0.002). For all other cardio-metabolic risk markers, the postprandial responses to the test fats were not influenced by *APOE* genotype.

6.5 DISCUSSION

It has been suggested that personalised gene-based dietary advice is more useful than general dietary guidelines (306), and more effective at motivating dietary change (307). It is, therefore, important to identify the main genetic determinants that impact on response to key population dietary fat recommendations for CVD risk reduction. To our knowledge, this is the first data analysis that has examined both the impact of chronic and acute dietary fat

manipulation on novel and established cardio-metabolic risk markers according to the *APOE* genotype.

In the present baseline analysis, the higher fasting TC and LDL-C concentrations, and TC: LDL-C and LDL-C: HDL-C ratios were evident in *E4* carriers than *E2* carriers and the wild-type genotype group, confirms previous studies (126-130). There are a number of possible mechanisms that could explain the higher TC and LDL-C concentrations in *E4* carriers (11). ApoE is present on TAG-rich lipoproteins (chylomicrons and very low density lipoproteins (VLDL)) and HDL, but not LDL particles and is involved with lipid transport and receptor mediated clearance. The apoE4 protein isoform has selective affinity for larger TAG-rich lipoproteins, which would be anticipated to result in increased competition with LDL for LDL receptor mediated clearance, increasing circulating LDL-C concentrations (308, 309). However, the lower binding affinity of the E2 isoform to the hepatic LDL receptor, compared with both E3 and E4 would be expected to result in a slower clearance of VLDL and dietary chylomicron remnants, and higher LDL clearance leading to typically lower TC and LDL-C concentrations, yet higher postprandial TAG in *E2* carriers (310). Furthermore, the lipolytic conversion of VLDL remnants to LDL is reported to be faster in *E4* carriers (311). All of these mechanisms could contribute to varying extents to the higher fasted TC and LDL-C concentrations in *E4* carriers in the current study, in the order of *E4 carriers>E3>E2* carriers.

We also observed genotype effects on a biomarker of inflammation, with *E2* carriers having greater CRP compared with *E4* carriers. In agreement with other studies, serum CRP was lower in *E4* carriers (129, 312-319). Although both LDL-C and CRP are recognised as independent CVD risk factors (258), some recent literature has indicated that elevated CRP does not increase the risk of CVD events as much as previously stated (320-322). Therefore, it is possible that *E4* carriers are associated with a greater CVD risk due to increased TC and LDL-C concentrations despite lower CRP (126, 297).

The present study found limited impact of *APOE* genotype on the responsiveness of cardio-metabolic risk markers to chronic fat intake, although there was some evidence that *E4* carriers were more sensitive to the TAG lowering of the MUFA diet. Analysis of data from the LIPGENE study revealed no differences in fasted TAG after a 12-week high MUFA diet according to *APOE* genotype in 442 participants classified with the metabolic syndrome (129). However, analysis of these data was performed according to plasma fatty acids

concentrations, rather than dietary intake, and although circulating fatty acids (particularly PUFA), can reflect consumption, correlations between dietary and plasma SFA and MUFA are weak, due to endogenous *de novo* synthesis of these fatty acids from lipid and non-lipid sources (323). Furthermore, a study in 84 young healthy participants following a high MUFA diet for 4 weeks also reported no difference in TAG according to *APOE* genotype, although there were low numbers of *E4* carriers and could have been underpowered for these comparisons (324). Further research is required to confirm the effects of different unsaturated fats on fasting TAG according to *APOE* genotype.

APOE genotype had a limited impact on postprandial lipid levels in relation to dietary fat manipulation, which is in agreement with our SATgene study (135) which prospectively genotyped according to *APOE* (*E3/E3* and *E4* carriers). However, in the current study a greater reduction in the postprandial apoB response (IAUC) was identified in the *E4* carriers after the MUFA-rich meals, although no difference in TAG responses were evident. This was surprising since TAG is transported predominantly in chylomicrons (containing apoB48) and VLDL (containing apoB100) and postprandial total apoB generally reflect concentrations of these TAG-rich lipoproteins. In contrast, Cardona et al investigated men and women with the metabolic syndrome and found no impact on apoB measured 4 hours postprandially after a MUFA-rich meal, but found non *E3/E3* groups (*E2* and *E4* carriers combined) to have higher postprandial TAG, with *E2* carriers having the highest TAG (303). Postprandial differences in TAG were observed after a SFA-rich test meal in *E4* carriers compared with *E3/E3*, although these were reported to be reflective of the higher baseline TAG concentrations (302). In support of the importance of fasted TAG levels, higher postprandial TAG concentrations in *E4* carriers compared with *E3/E3* was removed after adjustment for fasting TAG (301).

We found no differences in measures of fasted macrovascular (%FMD) or microvascular (LDI) reactivity, however we provide novel evidence of a difference in baseline (fasting) arterial stiffness according to *APOE* genotype. Vascular dysfunction is considered as an important risk factor for CVD and related components, including hypertension (26), arterial stiffness (44) and endothelial dependent vasodilation (45) are associated with cardiovascular mortality. Arterial stiffness is associated with chronic inflammation and dyslipidaemia, particularly elevated TAG (325), often found in *E2* carriers (309, 312). The higher arterial stiffness (measured by DVP-SI) observed in the *E2* carrier, compared with *E3/E3* in our study, may have reflected the higher inflammatory phenotype (CRP) in our *E2* carriers,

although further confirmation of this association is required. However, there was no diet/test fat and *APOE* genotype interaction observed for arterial stiffness. This is perhaps not surprising as arterial stiffness is a slow progressive process and long term exposure to dietary fat manipulation may be required before differences are observed (326). With regards to vascular function, this study reports for the first time, that the *APOE* genotype had a limited impact on fasting and postprandial measures of vascular function after chronic and acute fat manipulation, respectively. Yet, a reduction in DVP-RI was observed in the *E3/E3* compared with *E4*-carriers after the MUFA-rich meals which indicates differences in postprandial small vessel tone according to genotype, although the mechanism of action and the clinical relevance are unclear.

This study is novel, being the first to investigate the effect of *APOE* genotype on vascular function and cardio-metabolic risk markers at baseline and in response to chronic and acute dietary fat manipulation. Furthermore, there are a number of strengths in the study design of both the chronic and acute interventions. Firstly, the target intakes of SFA in the unsaturated fat diets in the chronic DIVAS study was compliant with the current recommendation for CVD risk reduction of ≤10%TE SFA. Secondly, the diets were followed for a longer duration (16 weeks) than many published dietary fat interventions investigating the effects on vascular function, as the primary outcome. Moreover, the acute study (DIVAS-2) used a two meal sequential postprandial protocol, which is considered superior to a single test meal challenge as it more closely mimics a habitual dietary intake pattern (218, 256). However, there are also some potential limitations. Since the genotyping was performed retrospectively, the number of participants who carried the *E2* allele was low in both the chronic and acute datasets, which necessitated removal of *E2* carriers, and could be considered as a limitation. In addition, as only postmenopausal women were recruited for the postprandial analysis (DIVAS-2), our postprandial findings may not relate to other subgroups of the population, including men and pre-menopausal women. However, both the baseline and chronic data analyses included a wider population, consisting of both men and pre- and postmenopausal women at moderate CVD risk

In conclusion, this study confirmed the previous findings that *APOE* genotype is associated with fasting lipid profile and CRP and novel evidence of association between *APOE* genotype with fasting DVP-SI. Moreover, our findings revealed a limited influence of the *APOE* genotype on the responsiveness of novel and established cardio-metabolic risk markers to chronic and acute fat manipulation in adults at moderate CVD risk. However,

further studies are warranted using prospective genotyping to confirm the findings in relation to the effects of the *APOE* genotype on markers of vascular functions, lipids and inflammation.

6.6 ACKNOWLEDGEMENTS

FIGURE LEGEND

Figure 6.1: Mean ± SEM for the postprandial digital volume pulse reflection index (DVP-RI) response in A) the *E3/E3* group (n=22) B) *E4* carriers (n=5) and C) incremental area under the curve (IAUC) for DVP-RI response according to APOE genotype (*E3/E3* group (black bars) and *E4* carriers white bars) following sequential meals (0 min and 330 min) enriched in SFA (■), MUFA (●) and n-6 PUFA (□). Two-way repeated measures ANOVA revealed a significant test fat*time*genotype interaction (*P*=0.014) for the DVP-RI response. There was a significant genotype*test fat interaction for the DVP-RI IAUC (*P*=0.030). **P*=0.002 compared with the *E4* carriers after the MUFA-rich meal

Figure 6.2: Incremental area under the curve (IAUC) for the postprandial apolipoprotein (apo)B response according to *APOE* genotype in the postmenopausal women following sequential meals (0 min and 330 min) enriched in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and n-6 polyunsaturated fatty acids (PUFA). Data represent mean ± SEM for the APO*E3/E3* group (black bars, n=17) and APOE4 carriers (white bars, n=4). There were significant genotype*test fat interactions for the postprandial IAUC (*P*=0.022). **P*=0.009 compared with the *E4* carriers for the MUFA-rich meals only.

Table 6.1 Baseline characteristics in the study group as a whole and according to *APOE* genotype

	All (n=216)	*E2* carriers (n=30)	*E3/E3* (n=128)	*E4* carriers (n=58)	P (genotype)[1]
Genotype Frequency (%)	-	14	59	27	
Characteristics					
Sex, M/F	84/132	13/17	43/85	28/30	
Age, y	46 ± 1	48 ± 2	45 ± 1	46 ± 2	0.537
Weight, kg	76.5 ± 1.0	78.2 ± 2.8	75.0 ± 1.2	78.9 ± 1.8	0.082
BMI, kg/m^2	26.6 ± 0.3	27.7 ± 0.8	26.4 ± 0.3	26.5 ± 0.5	0.341
Waist circumference, cm	91.3 ± 0.8	96.2 ± 2.7	90.1 ± 1.0	91.3 ± 1.5	0.091
Waist : hip ratio	0.87 ± 0.01	0.90 ± 0.02	0.86 ± 0.01	0.88 ± 0.01	0.152
Blood pressure					
Systolic, mm Hg	119 ± 1	122 ± 3	119 ± 1	118 ± 2	0.432
Diastolic, mm Hg	74 ± 1	77 ± 1	74 ± 1	73 ± 1	0.193
PP, mmHg	45 ± 1	45 ± 2	45 ± 1	45 ± 1	0.873
Biochemical profile and CVD risk					
TC, mmol/L	5.49 ± 0.07	4.77 ± 0.20[a]	5.49 ± 0.09[b]	5.88 ± 0.13[c]	**0.0001**
HDL-C, mmol/L	1.49 ± 0.02	1.43 ± 0.06	1.52 ± 0.03	1.47 ± 0.05	0.606
LDL-C, mmol/L	3.42 ± 0.06	2.73 ± 0.16[a]	3.42 ± 0.07[b]	3.77 ± 0.11[c]	**0.0001**
TC : HDL-C ratio	3.84 ± 0.07	3.49 ± 0.19[a]	3.77 ± 0.09[b]	4.20 ± 0.16[c]	**0.002**
LDL-C : HDL-C ratio	2.41 ± 0.06	2.02 ± 0.15[a]	2.37 ± 0.07[b]	2.72 ± 0.12[c]	**0.0001**
TAG, mmol/L	1.27 ± 0.04	1.34 ± 0.13	1.21 ± 0.05	1.34 ± 0.10	0.551
NEFA, μmol/L	502 ± 12	525 ± 28	509 ± 17	472 ± 22	0.413
Glucose, mmol/L	5.09 ± 0.03	5.12 ± 0.07	5.05 ± 0.04	5.18 ± 0.07	0.527
Insulin, pmol/L	31.2 ± 1.3	36.4 ± 4.6	30.4 ± 1.6	30.2 ± 2.3	0.619
HOMA-IR	1.19 ± 0.05	1.41 ± 0.19	1.14 ± 0.06	1.18 ± 0.10	0.605
rQUICKI	0.45 ± 0.01	0.43 ± 0.01	0.45 ± 0.01	0.46 ± 0.01	0.238
QRISK®2, %	2.8 ± 0.2	3.3 ± 0.7	2.4 ± 0.2	3.3 ± 0.4	0.142
Vascular function					
% FMD response	6.2 ± 0.2	5.8 ± 0.5	6.4 ± 0.3	6.0 ± 0.4	0.698
LDI-Ach, AUC, AU	1548 ± 59	1529 ± 164	1523 ± 77	1601 ± 114	0.588
LDI-SNP, AUC, AU	1464 ± 50	1327 ± 106	1448 ± 64	1557 ± 105	0.370
DVP-RI, %	63.2 ± 0.9	64.8 ± 2.5	62.1 ± 1.1	64.8 ± 1.7	0.649
DVP-SI, m/s	6.9 ± 0.1	7.7 ± 0.5[a]	6.6 ± 0.1[b]	7.2 ± 0.2[ab]	**0.027**
Biomarkers of inflammation and endothelial activation					
C-reactive protein, mg/L	2.23 ± 0.23	3.20 ± 0.70[a]	2.27 ± 0.29[a]	1.66 ± 0.41[b]	**0.002**
sVCAM-1, ng/mL	661 ± 11	653 ± 23	652 ± 16	685 ± 20	0.400
sICAM-1, ng/mL	218 ± 3	228 ± 9	220 ± 4	207 ± 5	0.120
E-selectin, ng/mL	34.2 ± 1.0	31.4 ± 2.5	34.5 ± 1.3	35.0 ± 1.9	0.189
P-selectin, ng/mL	40.7 ± 1.0	37.3 ± 2.5	41.0 ± 1.3	41.8 ± 1.9	0.078

Values represent mean ± SEM, *E2* carriers=*E2E2* and *E2E3; E4* carriers=*E3E4* and *E4E4*. *E2E4* individuals were excluded from the analysis.

[1]Data analysed by univariate general linear model (ANCOVA) adjusted for age, BMI, and sex. If significant, pairwise comparisons were used to determine differences between genotype groups. Different superscript letters within a row indicate significant differences between genotype groups ($P \leq 0.017$).

Abbreviations: Ach, acetylcholine; AU, arbitrary units; DBP, diastolic blood pressure; DVP-RI, digital volume pulse reflection index; DVP-SI, digital volume pulse stiffness index; FMD, flow-mediated dilatation; HDL-C, high density lipoprotein cholesterol; HOMA-IR, quantitative insulin resistance index; LDI, laser Doppler imaging; LDL-C, low density lipoprotein cholesterol; NEFA, non-esterified fatty acids; NOx, sum of nitrite and nitrate concentrations; rQUICKI, revised quantitative insulin sensitivity index; SBP, systolic blood pressure; sICAM-1, soluble intercellular cell adhesion molecule-1; SNP, sodium nitroprusside; sVCAM-1, soluble vascular cell adhesion molecule-1; TAG, Triacylglycerol; TC, total cholesterol.

Table 6.2 Changes in fasting cardio-metabolic risk markers after chronic dietary fat manipulation according to *APOE* genotype (DIVAS study)

| | *E3E3* (n=107) | | | *E4* carriers (*E3E4* and *E4E4*, n=52) | | | *P* value[1] | |
| | | | | | | | | Diet x |
	SFA	MUFA	n-6 PUFA	SFA	MUFA	n-6 PUFA	Genotype	genotype
N	35	36	36	17	17	18		
Age, y	44±1	42±2	43±2	44±3	46±3	47±3		
BMI, kg/m^2	25.8±0.8	26.5±0.75	26.9±0.6	27.5±0.9	26.1±1.0	25.9±0.8		
Biochemical profile and estimates of insulin sensitivity/resistance								
TC, mmol/L	0.42±0.10	-0.03±0.13	-0.01±0.13	0.21±0.19	-0.29±0.17	-0.19±0.20	0.165	0.760
HDL-C, mmol/L	0.06±0.03	0.04±0.03	0.12±0.04	0.03±0.05	-0.05±0.05	-0.02±0.07	**0.015**	0.473
LDL-C, mmol/L	0.35±0.09	-0.09±0.11	-0.11±0.10	0.17±0.15	-0.19±0.15	-0.18±0.14	0.401	0.984
TC : HDL-C ratio	0.20±0.08	-0.11±0.07	-0.25±0.07	0.01±0.13	-0.13±0.17	-0.05±0.08	0.703	0.263
LDL-C: HDL-C ratio	0.19±0.08	-0.12±0.07	-0.23±0.07	0.02±0.11	-0.10±0.15	-0.07±0.08	0.652	0.324
TAG, mmol/L	-0.00±0.05	0.10±0.06[a]	-0.07±0.05	0.06±0.14	-0.23±0.10[b]	0.08±0.18	0.160	**0.010**
NEFA, μmol/L	-17.6±35.1	-13.6±22.1	-11.1±21.5	-64.8±40.2	46.5±66.3	87.7±26.2	0.413	0.082
Glucose, mmol/L	0.04±0.05	0.07±0.04	0.10±0.06	0.08±0.09	0.01±0.06	0.01±0.11	0.957	0.614
Insulin, pmol/L	1.10±2.73	1.23±1.86	2.67±2.31	0.97±2.77	0.49±1.55	0.71±1.67	0.851	0.857
HOMA-IR	0.07±0.11	0.05±0.08	0.12±0.10	0.05±0.12	0.03±0.07	0.06±0.07	0.821	0.930
rQUICKI	0.00±0.01	-0.00±0.01	-0.01±0.01	0.00±0.01	-0.01±0.01	-0.02±0.01	0.620	0.420
Vascular function								
%FMD response	-0.55±0.33	0.33±0.44	-0.09±0.36	-0.40±0.49	-0.20±0.69	-0.95±0.73	0.918	0.368
LDI[2]								
LDI-Ach AUC, AU	-460.3±198.1	-2.1±154.6	91.1±119.9	38.5±164.6	-40.7±232.8	-115.4±142.2	0.438	0.134
LDI-SNP AUC, AU	-283±173	-187±158	131±127	-28±212	-296±287	187±174	0.233	0.601
DVP-RI, %	-1.98±2.34	4.33±2.57	4.29±2.27	-0.27±2.57	1.47±1.91	-4.92±3.52	0.078	0.171
DVP-SI, m/s	0.23±0.32	0.76±0.31	0.22±0.37	0.37±0.37	-0.15±0.32	-0.93±0.43	0.125	0.562
Ambulatory blood pressure[3]								
24-h blood pressure								
SBP, mm Hg	1.7±1.3	-1.1±1.3	0.2±1.8	0.5±2.7	-0.9±2.4	-1.3±2.0	0.681	0.860
DBP, mm Hg	1.6±0.9	-0.4±0.9	-0.3±1.1	1.1±1.9	0.9±1.7	-1.4±1.3	0.921	0.813
PP, mm Hg	0.2±1.4	-0.7±0.8	0.6±1.0	-0.6±1.1	-1.7±1.4	0.1±1.5	0.502	0.974
HR, bpm	0.8±1.0	0.7±1.1	0.6±1.1	2.8±1.3	1.9±1.8	-1.6±1.6	0.565	0.292
Biomarkers of inflammation and endothelial activation								
C-reactive protein, mg/L	0.60±0.60	0.04±0.28	0.024±0.51	-0.14±1.14	-0.25±0.57	-0.79±0.63	**0.036**	0.786

NOx, µmol/L	0.51±3.22	-1.89±1.52	-1.78±1.87	1.70±3.48	4.43±2.97	-2.27±1.62	0.208	0.073
sVCAM-1, ng/mL	-40.0±16.8	11.4±22.2	2.3±13.6	-2.1±16.2	3.9±35.4	25.1±25.2	0.063	0.451
sICAM-1, ng/mL	-1.4±4.2	1.3±3.6	1.5±6.3	5.2±6.6	15.3±8.2	12.8±5.1	0.100	0.836
E-selectin, ng/mL	0.41±1.39	-2.90±1.15	-0.63±1.03	0.70±2.01	-3.03±1.86	-0.24±1.43	0.827	0.881
P-selectin, ng/mL	-0.94±1.70	-1.94±1.06	-3.49±1.23	3.63±1.74	-1.18±2.32	0.21±1.44	**0.026**	0.403

Values represent mean ± SEM, change from baseline after post intervention (week 16). Total n=131-157, with n=89-107 *E3E3* and n=42-52 *E4* carriers per outcome. *E2/E4* and *E2=E2/E2 + E2/E3* individuals were excluded from the analysis.

[1]Data analysed by univariate general linear model (ANCOVA) by using the difference from baseline (Visit 2 (Post-intervention) – baseline (Visit 1)) as the dependant variable, with genotype, sex, and intervention diet as fixed factors and with baseline values of the variable of interest, age and BMI as covariates. The interaction term was added to the model to assess the *APOE* genotype and diet interaction.

[2]LDI-Ach and LDI-SNP were expressed as AUC for the 20-scan protocol. IAUC was also determined for the 20-scan protocol but differences between test fats for subsequent AUC and IAUC were not significant (data not shown).

[3]Day and night ambulatory blood pressure were analysed and no significant effects were found (data not shown).

Abbreviations: Ach, acetylcholine; AU, arbitrary units; AUC, area under the curve, BMI, body mass index; DBP, diastolic blood pressure; DVP-RI, digital volume pulse reflection index; DVP-SI, digital volume pulse stiffness index; FMD, flow-mediated dilatation; HDL-C, high density lipoprotein cholesterol; HOMA, quantitative insulin resistance index; HR, heart rate; LDI, laser Doppler imaging; LDL-C, low density lipoprotein cholesterol; MUFA, monounsaturated fatty acids; NEFA, non-esterified fatty acid; NOx, total nitrites and nitrates; PP, pulse pressure; PUFA, polyunsaturated fatty acids; rQUICKI, revised quantitative insulin sensitivity index; SBP, systolic blood pressure; SFA, saturated fatty acids; sICAM-1, soluble intercellular cell adhesion molecule 1; SNP, sodium nitroprusside; sVCAM-1, soluble vascular cell adhesion molecule 1; TAG, triacylglycerol; TC, Total cholesterol

Table 6.3 Summary measures for the impact of *APOE* genotype on postprandial cardio-metabolic risk markers after sequential meals of varying fat composition (DIVAS-2 study)

	APOE3/E3 (n=22)			*APOE3/E4* (n=5)			*P* value[1]	
	SFA	MUFA	n-6 PUFA	SFA	MUFA	n-6 PUFA	Genotype	Test fat x genotype
Biochemical measures[2]								
TAG, mmol/L								
AUC	942±90	1022±87	1035±124	1063±186	1022±164	1125±299	0.702	0.683
IAUC	320±52	360±53	361±77	402±94	403±126	386±115	0.702	0.828
NEFA, μmol/L								
AUC	143.5±8.4	136.1±11.3	126.9±7.3	108.6±16.1	102.4±13.3	116.9±21.1	0.147	0.382
IAUC	-73.5±12.3	-87.9±12.7	-83.7±12.7	-75.2±29.6	-12.6±16.9	-62.0±29.8	0.802	0.111
Apo B, mg/mL								
AUC	473±18	469±22	480±25	549±32	561±50	512±34	0.329	0.134
IAUC	62.4±4.3	50.7±3.2	-12.4±5.2	-11.6±2.5	-23.3±9.8	31.3±8.3	0.197	**0.022[3]**
Glucose, mmol/L								
AUC	942±90	1022±87	1035±124	1063±186	1022±164	1125±299	0.666	0.299
IAUC	320±52	360±53	361±77	402±94	403±126	386±115	0.342	0.523
Insulin, μmol/L								
AUC	913±8	877±11	821±9	948±19	951±15	952±18	0.640	0.814
IAUC	881±8	831±9	779±6	795±16	783±12	753±14	0.610	0.750
Vascular function[2]								
% FMD response								
AUC	1939±148	2305±191	2131±191	2105±235	2629±486	2329±218	0.510	0.898
IAUC	-149±183	156±261	114±189	345±335	431±279	79±281	0.433	0.683
LDI-Ach[4], AU x 10[3]								
AUC	742±47	766±56	772±50	871±101	973±320	941±160	0.888	0.931
IAUC	7.8±35.1	-8.1±62.2	-11.0±53.9	-46.4±101.6	-70.0±132.3	-21.8±127.9	0.088	0.975
LDI-SNP[4], AU x 10[3]								
AUC	755±57	807±66	652±33	802±99	957±264	113±227	0.690	0.083
IAUC	318±60	-101±59	-331±61	-118±74	-286±117	-505±171	0.754	0.624
DVP-RI, % x 10[3]								
AUC	26.1±1.0	24.7±0.7	25.5±0.9	25.9±0.8	27.6±1.5	27.1±2.7	0.407	0.392
IAUC	-1.7±0.8	-3.2±0.7	-2.4±0.6	-3.3±0.9	1.2±0.7	0.2±1.0	0.055	**0.030[3]**
DVP-SI, m/s								
AUC	3193±121	3074±110	3169±114	3463±178	3505±277	3617±415	0.177	0.735

IAUC	14.6±97.6	-181.2±110.1	40.5±120.4	171.3±178.4	-44.0±241.3	226.5±154.4	0.373	0.987
SBP, mmHg x 10³								
AUC	56.3±1.5	57.1±1.4	57.1±1.6	58.9±3.5	56.7±2.5	58.3±3.5	0.716	0.267
IAUC	-2.5±0.6	-4.8±0.6	-3.3±0.7	-3.6±0.7	-3.2±1.5	-4.2±1.0	0.914	0.178
DBP, mmHg x 10³								
AUC	32.9±3.3	33.1±3.3	33.3±3.3	32.9±1.2	31.9±1.0	32.0±1.4	0.686	0.341
IAUC	-1.1±0.4	-2.2±0.3	-1.5±0.3	-1.0±0.6	-1.7±0.3	-9.7±0.7	0.442	0.867
Biomarkers of endothelial activation²								
NOx, μmol/L								
AUC	5820±482	5962±708	5119±389	6308±258	6278±258	6084±1330	0.288	0.996
IAUC	-1618±231	-1446±370	-1057±219	-2165±521	-3015±695	-1762±393	0.078	0.318
sVCAM-1, ng/mL x 10³								
AUC	263.0±7.1	270.7±9.2	256.9±7.3	263.4±22.2	243.0±14.6	243.5±16.4	0.431	0.118
IAUC	4.2±5.0	3.1±4.6	-4.7±2.9	3.9±4.9	-8.5±9.2	-9.6±8.1	0.360	0.707
sICAM-1, ng/mL x 10³								
AUC	86.3±3.1	876.9±3.8	702.5±5.7	715.3±3.5	717.9±2.9	588.9±6.1	0.141	0.775
IAUC	-3.2±1.3	-0.2±1.5	-18.1±4.1	0.4±6.4	0.6±2.5	-15.9±10.0	0.581	0.881
E-selectin, ng/mL x 10³								
AUC	10.7±0.9	11.0±0.9	10.6±0.8	11.4±1.6	11.9±1.5	10.6±1.6	0.694	0.407
IAUC	-0.4±0.3	-0.01±0.2	-0.2±0.1	-0.3±0.3	0.6±0.4	0.5±0.01	0.065	0.472
P-selectin, ng/mL x 10³								
AUC	13.0±0.7	13.0±0.9	13.1±0.8	14.7±1.8	16.5±2.9	14.8±2.1	0.300	0.131
IAUC	-0.6±0.3	0.01±0.2	-0.01±0.3	-0.07±0.0	1.3±1.3	0.3±0.4	0.078	0.737

Values are mean ± SEM, for the *E3/E3* and *E3/E4* groups. *E2* carriers and *E2/E4* individuals were excluded from the analysis.

[1] *P* value refers to the interaction between summary measures and *APOE* genotype. A mixed factor repeated measures ANOVA was used to analyse effects of the test fats on summary measures. For this, test fat and time were included as within-subject factors and genotype as the between group factor.

[2] Units for AUC and IAUC expressed as biomarker units x time interval. The time interval for AUC and IAUC represents 480 min for TAG, apoB, glucose and insulin; 120-480 min for NEFA; 420 min for FMD and biomarkers of endothelial activation; 450 min for DBP, SBP, DVP-SI, DVP-RI and LDI.

[3] For significant test fat*genotype interactions, independent samples t-test was performed to identify the effects of genotype for each test fat separately.

[4] LDI-Ach and LDI-SNP were expressed as AUC for the 20-scan protocol. IAUC was also determined for the 20-scan protocol but differences between test fats for subsequent AUC and IAUC were not significant (data not shown). Abbreviations: Ach, acetylcholine; apoB, apolipoprotein B; AU, arbitrary units; AUC, area under the curve; DBP, diastolic blood pressure; DVP, digital volume pulse; DVP-RI, DVP-reflection index; DVP-SI, DVP-stiffness index; FMD, flow-mediated dilatation; IAUC, incremental AUC; LDI, laser Doppler imaging; NEFA, non-esterified fatty acids; NOx, total nitrite and nitrate; SBP, systolic blood pressure; sICAM-1, soluble intercellular cell adhesion molecule-1; SNP, sodium nitroprusside; sVCAM-1, soluble vascular cell adhesion molecule-1

Supplementary Table 6.1 Habitual dietary intakes for the study group as a whole and according to *APOE* genotype

	All	*E2* carriers	*E3/E3*	*E4* carriers	
	(n=214)	(n=30)	(n=127)	(n=57)	*P* (genotype)[1]
Genotype Frequency (%)	-	14	59	27	
Energy, MJ	8.58 ± 0.17	8.62 ± 0.39	8.27 ± 0.20	9.27 ± 0.38	0.410
Total fat, %TE	33.3 ± 0.4	34.5 ± 1.5	32.9 ± 0.5	33.6 ± 0.8	0.706
SFA, %TE	11.9 ± 0.2	12.6 ± 0.8	11.7 ± 0.2	12.0 ± 0.4	0.870
MUFA, %TE	11.8 ± 0.2	11.9 ± 0.5	11.8 ± 0.2	11.8 ± 0.3	0.969
n-6 PUFA, %TE	5.25 ± 0.12	5.39 ± 0.35	5.18 ± 0.15	5.33 ± 0.25	0.851
n-3 PUFA, %TE	0.87 ± 0.03	0.93 ± 0.12	0.85 ± 0.03	0.87 ± 0.06	0.752
Trans fat, %TE	0.95 ± 0.03	1.10 ± 0.08[a]	0.89 ± 0.03[b]	0.99 ± 0.05[ab]	**0.031**[2]
Dietary cholesterol, mg/d	265 ± 9	290 ± 29	250 ± 11	285 ± 18	0.322
Protein, %TE	16.1 ± 0.2	16.3 ± 0.7	16.1 ± 0.3	16.0 ± 0.4	0.987
Carbohydrate, %TE	49.5 ± 0.5	48.8 ± 1.7	49.6 ± 0.6	49.5 ± 1.0	0.969
Dietary fibre (AOAC), g/d	22.4 ± 0.5	21.5 ± 1.5	22.1 ± 0.6	23.6 ± 1.1	0.410

Values represent mean ± SEM. Determined from a single 4-day weighed food and drink diary completed prior to the first study visit; dietary assessments not available for n=2. *E2* carriers = *E2/E2* and *E2/E3; E4* carriers = *E3/E4* and *E4/E4*. *E2/E4* individuals were excluded from the analysis.
[1] Data analysed by univariate general linear model (ANCOVA) adjusted for age, BMI, and gender.
[2] Post-hoc analysis identified differences between genotype groups (identified by different superscript letters within a row), although these were not significant following Bonferroni correction (*P*≥0.017).
Abbreviations: %TE, percentage of total energy; AOAC, Association of Official Analytical Chemists; MUFA, monounsaturated fat; PUFA, polyunsaturated fat; SFA, saturated fat.

FIGURE 6.1

A
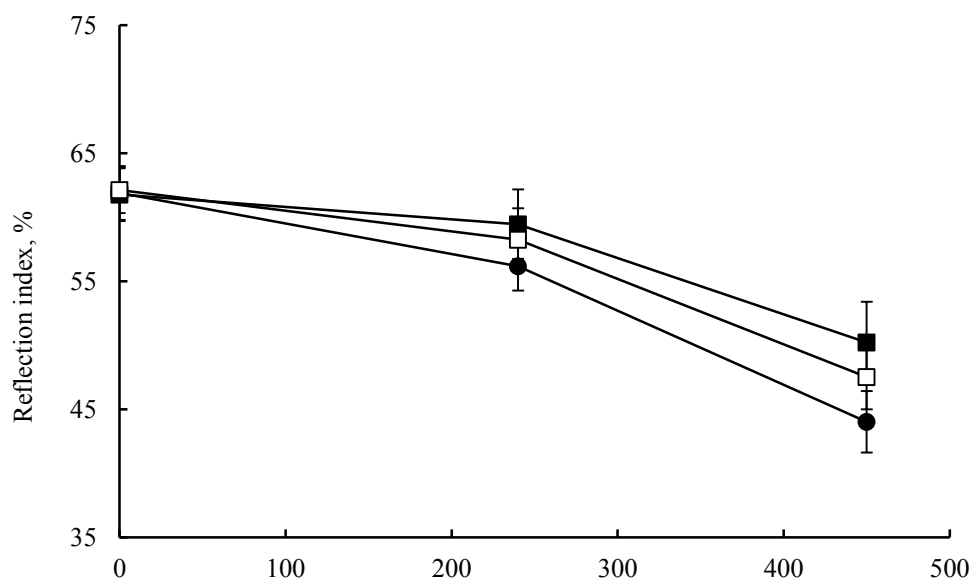
B
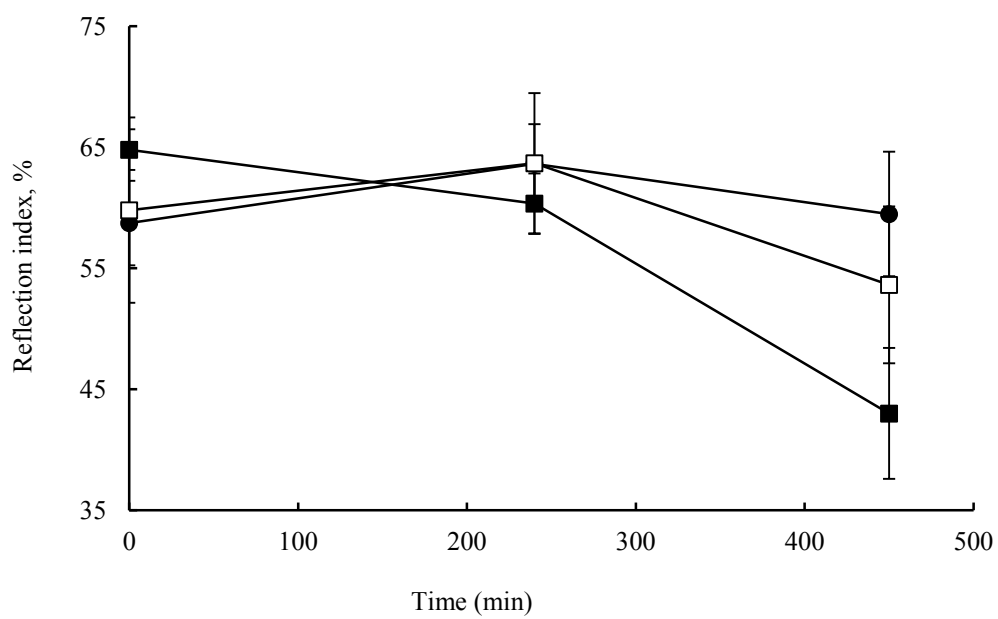
FIGURE 6.1 (continued)

C

FIGURE 6.2

Sequential meal fat composition
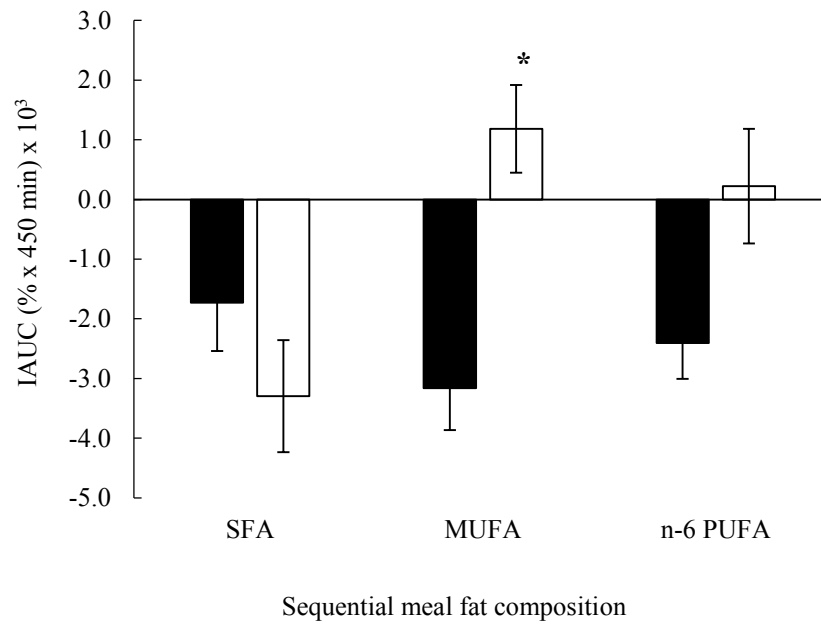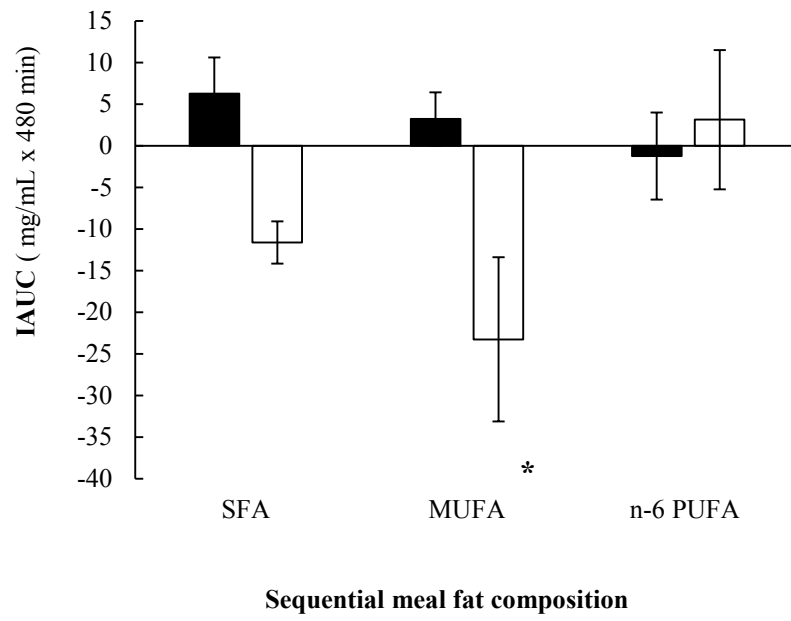
7. GENERAL DISCUSSION

Cardiovascular disease (CVD) is the leading cause of death globally in women (327) and the most common cause of death in the UK for women (28% of all female deaths) (5). The aetiology for CVD is multifactorial and includes several modifiable risk factors, such as cigarette smoking, inactivity, obesity, hypertension, dyslipidaemia, type 2 diabetes mellitus, and non-modifiable factors, such as advancing ageing, sex, family history of heart disease, genotype and ethnicity. Diet is also an important modifiable risk factors for CVD risk. One of the key public health strategies to lower CVD risk is to reduce saturated fatty acid (SFA) consumption to ≤10% of total energy (TE) (221); however, what is not clear is which macronutrient should replace to SFA (196). Previous data from pooled analysis of prospective cohort studies (17) and a meta-analysis of randomised controlled trials (RCT) (196) have indicated that replacement of SFA with carbohydrates was not associated with reductions in CVD mortality, but replacement with total (both n-3 and n-6) polyunsaturated fat (PUFA) was associated with a reduction in CHD risk (17) and CVD events (196). However, there is limited data on the effects of SFA replacement with monounsaturated fat (MUFA) on CVD mortality, or a direct comparison between SFA replacement with MUFA and n-6 PUFA. In the Dietary Intervention and Vascular Function (DIVAS) study, replacement of 9.5-9.6% TE of dietary SFA with either MUFA or n-6 PUFA for 16-wk reduced key CVD risk markers: fasting low density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) to high density lipoprotein cholesterol (HDL-C) ratio, with differential effects of the unsaturated fats on markers of endothelial activation and blood pressure (30).

CVD risk biomarkers, such as fasting lipids (total, LDL, and HDL-C, and triacylglycerol (TAG)), have been routinely measured in dietary studies, but more recently, novel markers such as postprandial lipaemia, vascular function and biomarkers of endothelial activation have been included to provide a more holistic measure of dietary fat composition on CVD risk (30). However, the majority of studies examining the effects of dietary fat composition on lipids and vascular function have been conducted in the fasting state, with very little known about the acute effects of meal fat composition on postprandial vascular function and lipaemia. This is particularly important since individuals spend up to 18 h in the postprandial (fed) state, with non-fasting TAG levels now recognised as an important CVD risk factor particularly in women (85, 328). Endothelial dysfunction is characterised by reduced bioavailability of nitric oxide (NO), which leads to vasoconstriction, increased expression

of adhesion molecules and pro-inflammatory cytokines, platelet activation and increased oxidative stress (48), and has emerged as a critical early modifiable event in the development of coronary atherosclerosis (168). This is also recognised as a novel CVD risk marker in postmenopausal women, in whom the decline in oestrogen is associated with adverse effects on lipid metabolism, vascular function and blood pressure (197).

Meal fatty acids have been shown to impact on both lipaemia and vascular function during the postprandial phase, although the data is limited in postmenopausal women. Therefore, a systematic approach was used to review the literature (Chapter 2) which revealed that there was very weak evidence to suggest a modest reduction in vascular function following a SFA rich meal but there was limited published data on the acute effects of MUFA and n-6 PUFA rich meals on vascular reactivity. It was, therefore, concluded that there was an urgent requirement for suitably powered robust RCT to investigate the substitution of meal fat composition on postprandial lipaemia and vascular function in postmenopausal women. Following on from the chronic DIVAS study, the second DIVAS study (DIVAS-2) was designed to address this knowledge gap and to my knowledge is the first suitably powered study in postmenopausal women to investigate the impact of sequential meals rich in SFA, MUFA and n-6 PUFA on postprandial macro-and microvascular reactivity, blood pressure, lipids and other CVD risk biomarkers.

7.1 Greater impact of acute fat intake on vascular function and markers of endothelial activation than postprandial lipaemia in postmenopausal women

The primary outcome measure of the DIVAS-2 study was postprandial macro-vascular reactivity assessed using flow mediated dilatation (FMD). FMD is a non-invasive ultrasound technique that is widely used to assess endothelial function in conduit arteries in the peripheral circulation and is considered as the gold standard technique (233). FMD of the brachial artery is strongly correlated with coronary endothelial function (59). In DIVAS-2, other measures of vascular function were also determined to give indication of other whole body vascular function and included; micro-vascular reactivity measured by laser Doppler imaging with iontophoresis of acetylcholine (endothelium-dependant) and sodium nitroprusside (endothelium-independent), arterial stiffness measured by digital volume pulse (stiffness index; DVP-SI), vascular tone (reflection index; DVP-RI) and blood pressure.

Endothelial dependant vasodilation is mainly regulated by a number of vasoactive mediators including NO, which is the most potent vasodilator. NO is released from vascular endothelium through the action of the enzyme endothelial NO synthase (eNOS) which converts L-arginine to NO, and FMD of the brachial artery is considered a marker of endothelial dependent vascular function dependent on NO production (227). Although differences in the biomarkers of NO production were evident between the test fats in the current study, with a greater decrease in postprandial plasma nitrite after SFA than MUFA-rich meals, comparable changes in all of the real-time measures of vascular reactivity were not quite significant, suggesting possible indirect effects of meal fatty acids on vascular function. Furthermore, since variations in eNOS genotype could potentially have an influence on the extent of these responses, the interactions between eNOS genotype and meal fat composition were determined retrospectively, the findings of which are discussed in section 7. 3.

Increased arterial stiffness is recognised as a possible mechanism in the initiation/or development of atherosclerosis and hypertension and it has been shown that arterial stiffness predicts the artery's ability to expand and contract with cardiac pulsation and relaxation (329). Furthermore, arterial stiffness is determined by the structural and functional components of the artery which includes the elastin to collagen ratio of the artery walls. Ageing leads to increased arterial stiffness as a result of both elastic fibre degradation and increased production of reactive oxygen species accompanied with decreased bioavailability of NO, which plays an important role in the regulation of vascular tone and function (330). In the current study, we did not observe a meal fat effect on other measures of vascular function including both postprandial DVP-RI and DVP-SI markers of vascular tone and arterial stiffness, respectively, suggesting meal fat composition had little impact on these aspects of vascular function. However, it has been proposed that arterial stiffening is a slow, progressive process and therefore a longer exposure to meal fatty acids may be required to show significant results (326), in which case a long-term chronic intervention of > 6 months would be recommended.

In the current study, each test fat reduced blood pressure over 450 min, with a greater decrease in diastolic blood pressure (DBP) (and to a lesser extent systolic blood pressure (SBP)) observed after consumption of the MUFA than SFA-rich meals. According to the literature review (Chapter 2), only very limited and inconclusive data was identified

regarding the relative effects of acute consumption of meals varying in fat composition on postprandial blood pressure in postmenopausal women. However, findings from previous chronic interventions have shown beneficial effects of replacing SFA with unsaturated fat on BP (39, 223). The DIVAS chronic study found replacing 9.5 %TE of dietary SFA with MUFA for 16 wk significantly attenuated the increase in night SBP in men and women with a moderately increased risk of CVD yet there was no effect of replacing SFA with n-6 PUFA on BP (223). Another 3-month controlled parallel study in which participants followed a high fat diet (37%TE) rich in either SFA (17%TE from SFA) or MUFA (23%TE from MUFA) also reported significant reductions in both fasting DBP and SBP in response to the MUFA-rich diet (39).

High intakes of olive oil are considered as a hallmark of the traditional Mediterranean diet, which results in high intakes of MUFA and low intakes of SFA. Replacement of SFA with MUFA is associated with a reduction in CVD risk, mediated primarily by a reduction in LDL-C, with little impact on HDL-C or TAG concentrations (34). It has been shown that fatty acids in extra virgin olive oil are protected by natural antioxidants, including carotenes, tocopherols, and phenolic compounds. Antioxidants tend to inactivate the effects of the free radicals and lipid peroxidation, which could result in decreased blood pressure (331, 332). Some studies reported that a reduction in blood pressure was attributed to the polyphenols in extra virgin olive oil, which are thought to enhance NO concentrations, leading to the dilation of arteries and reduction in blood pressure. Unlike other studies that are confounded by their use of extra virgin olive oils, refined olive oil was chosen for its low level of polyphenols in the DIVAS-2 study. Therefore, the improvement in blood pressure observed between MUFA and SFA-rich meals was likely to be attributed to the fatty acid content. In animal experiments, it has been shown that olive oil decreased arterial blood pressure more than sunflower oil rich in n-6 PUFA, which is more susceptible to oxidation (333, 334). Another animal study reported oral administration to animals of 2-hydroxyoleic acid, a synthetic derivative of oleic acid, which is a primary MUFA found in olive oil, substantially decreased blood pressure (335). Furthermore, it has been reported that olive oil induced marked and significant reductions of blood pressure after both acute (2 h), and chronic (2 wk) administration in animal models, with blood pressure becoming stable after 3 or 4 days of high olive oil intake (336). Oleic acid is thought to be responsible for the hypotensive effects of olive oil, and Terés et al., 2008 proposed that oleic acid (cis-18:1 n-9) present in olive oil can regulate the activity of the adrenoreceptor signalling pathway, which is

responsible for central and peripheral control of blood pressure. Furthermore, high olive oil intakes have been shown to increase oleic acid levels in vascular smooth muscle cells and endothelial cells (responsible for maintaining the balance between constriction and relaxation of vessels), which regulate membrane lipid structure in such a way as to control G protein mediated signalling, causing a reduction in blood pressure (337). One study reported that daily doses of blood pressure medication were reduced by 48% during adherence to an extra virgin olive oil rich diet and by 4% during the sunflower oil diet (338).

Hypertension is a major risk factor for CVD and a burden on the National Health Service in the UK. According to the 2015 Health Survey for England, high blood pressure was prevalent in 40% of women aged 55-64 y, rising to 58% for those aged 65-74 y. It would be interesting to conduct an acute study in groups suffering from hypertension, to determine whether replacing SFA with MUFA and n-6 PUFA had similar blood pressure lowering effects as reported in the DIVAS-2 study. The results from chronic studies and the DIVAS-2 acute study provide evidence to support the replacement of dietary SFA with MUFA as a potential dietary strategy for lowering blood pressure in older women, although more studies are needed to confirm these findings.

Endothelial dysfunction is associated with an increased expression of adhesion molecules due, in part, to increased endothelial cell activation. During the process of atherosclerotic plaque formation, in addition to the accumulation of inflammatory cells and oxidised low density lipoproteins (LDL), activation of soluble adhesion molecules (such as soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule (sVCAM-1)) and cell surface adhesion molecules (such as E-selectin and P-selectin) occurs (252). As a ligand for $\beta2$ integrins, it is reported that sICAM-1 is involved in leukocyte-leukocyte, leukocyte-endothelial, and leukocyte-epithelial cell interactions and trans-endothelial migration. Furthermore, it has been shown that in cell cultures studies, increased levels of sICAM-1 after stimulation with IL-1β cytokine (339). In the DIVAS-2 study, we observed a lower postprandial sICAM-1 response after the n-6 PUFA than SFA and MUFA-rich meals, with little effect evident on other adhesion molecules (sVCAM-1, E-selectin and P-selectin). One of the strengths in our DIVAS-2 study was the use of three different types of fatty acids to investigate differences in endothelial dysfunction, providing evidence for the optimal type of fatty acid to replace SFA.

Previous extensive reviews have shown that the type of fat consumed in a meal may influence the fatty acid composition of the TAG-rich lipoproteins and the magnitude and duration of the postprandial TAG response (249, 250, 340). Yet, in the current study meal fatty acids did not impact on postprandial lipid, glucose or insulin responses following sequential meals. However, one systematic review and meta-analysis has compared the effects of a single oral fat tolerance tests with differing fatty acid compositions on postprandial TAG responses in men and women (91). The authors concluded that relative to a SFA-rich meal challenge, a PUFA-rich meal significantly reduced the postprandial lipaemic response over 8 h, whereas there was only a trend for a reduced response following a MUFA-rich meal. However, Robertson *et al* (224) reported significantly higher levels of plasma non-esterified fatty acids (NEFA) and lower insulin sensitivity following a SFA-rich meal compared with MUFA and n-6 PUFA oils, which supports detrimental lipid responses to SFA-rich meals. The sequential postprandial protocols used in these studies may provide an explanation for the differences observed on postprandial lipaemia. In the Robertson study, volunteers ingested a high fat breakfast containing 40 g of the assigned test fat followed by a low fat, high carbohydrate lunch (5.4 g total fat) given 5 h later. However, in the DIVAS-2 study volunteers were given a high fat breakfast (30 g total fat) followed by a high fat lunch (50 g of total fat) after 5 h later. Furthermore, in the Robertson study, the SFA meal contained vegetable sources of SFA (palm oil and cocoa butter), whereas the DIVAS-2 study used butter. Therefore, the fat content of the sequential meals and type of SFA may impact on the postprandial outcome measures and warrants further investigation, particularly as individual SFA have been shown to have different effects on plasma lipids (91).

A strength of the current study was the use of a two meal sequential postprandial protocol, which more closely mimics a habitual dietary intake pattern compared with a single test meal challenge (218, 256). However, there are some potential limitations of our study. As only postmenopausal women with moderately raised fasting TAG concentrations were included, these findings may not reflect the responses in men and premenopausal women or in postmenopausal women with greater CVD risk and, as such, further investigation is required using different population groups.

7.2 Effects of acute fat manipulation on *ex vivo* cytokine production

Chapter 4 investigated the impact of acute fat manipulation with different fatty acids on the postprandial *ex vivo* cytokine production in postmenopausal women. A rapidly growing body of evidence has identified markers of inflammation as strong independent predictors of CVD (258). Dietary fatty acids have been identified as potent regulators of inflammation (203). It has been shown that postprandial lipaemia induces the production of pro-inflammatory cytokines (341), and several studies reported increasing concentrations of circulatory pro-inflammatory cytokines after acute consumption of high fat meals, inducing postprandial inflammation (254, 262, 263). A whole blood culture technique (WBC) was used in DIVAS-2 to examine the impact of meal fatty acids on postprandial inflammation modulation. Determination of cytokines produced by monocytes is recognised as a marker of local inflammation in the development of atherosclerosis (265). Activated monocytes produce pro-inflammatory cytokines, interleukin (IL)-1, IL-6, and tumour-necrosis factor (TNF-α), and also the anti-inflammatory IL-10 (266). It has been recognised that monocyte cytokine production can be measured *ex vivo* from WBC, which has shown strong correlation with *ex vivo* monocytic cytokine production (267).

In the DIVAS-2 study, a greater sensitivity of cytokine IL-1β to meal fat composition was observed, with greater production after consumption of sequential SFA than MUFA and n-6 PUFA-rich meals, whilst the production of other lipopolysaccharide (LPS) stimulated cytokines (IL-6, IL-8, IL-10 and TNF-α) did not differ according to meal fatty acid composition. Aligned with our study findings, Nicholas et al also reported an increased secretion of active IL-1β in dendritic cells following treatment with palmitic acid (a SFA), whilst there was a lack of effect on IL-6, IL-8, IL-10, IL-12 and TNF-α (269). IL-1β is an important pro-inflammatory multifunctional cytokine that is closely associated with the role of inflammation in atherosclerosis (270). It is possible that fatty acids circulating after the SFA-rich meals may have stimulated IL-1β via TLR-4 but we cannot discriminate whether this occurred via the dependent or –independent pathway, or a result of cross talk between these two pathways. The increased production of IL-1β after consumption of SFA-rich meals which plays an important role in the formation of atherosclerotic lesions may contribute to the greater CVD risk in postmenopausal than premenopausal women.

7.3 Effects of Glu298Asp (rs1799983) polymorphism and *APOLIPOPROTEIN E (epsilon)* genotype on vascular function and cardio metabolic risk markers

In this study, we considered effects of Glu298Asp (rs1799983) polymorphism and *APOLIPOPROTEIN E (APOE)* genotypes on vascular function and cardio-metabolic risk markers as these have previously been shown to influence the plasma lipid response (*APOE* genotype) and vascular reactivity (eNOS) in relation to dietary fat intake (81). Population-based dietary strategies are known to be highly heterogeneous. There is growing evidence that identifying the molecular loci associated with these variable responses to diet and implementing gene-based personalised nutrition interventions may benefit different subgroups of the population. Although, there has been high expectations regarding use of gene-based disease risk estimates to tailor advice, it has been reported that further RCT are needed to establish such advice on effective dietary behaviour change (342, 343). Since dietary fat has previously been shown to influence postprandial lipaemia and vascular reactivity, it was hypothesised that genetic variations within key proteins involved with these processes (such as apoE and eNOS) would be influenced by meal fat composition. However, little is currently known about the effects of such genetic variations on the responses of CVD risk factors following meal fat manipulation with regards to varying fatty acids composition. Therefore, in chapters 5 and 6, the effects of the Glu298Asp (rs1799983) polymorphism and *APOLIPOPROTEIN E (APOE)* genotypes on vascular function and cardio-metabolic risk markers were determined.

7.3.1 Effect of Glu298Asp (rs1799983) polymorphism

A common single nucleotide polymorphism (SNP) in the eNOS gene, Glu298Asp (rs 1799983), is regarded to have functional effects on NO production and bioavailability, with a 30% higher CVD risk in minor allele Asp298 carriers compared with Glu298 homozygotes, which is attributed to impaired vascular function (275) and elevated BP (116, 276, 277). In the DIVAS-2 study, the Glu298Asp polymorphism was shown to be responsive to acute meal fat composition, with differential effects of genotype observed on postprandial FMD (primary outcome measure) to sequential MUFA rich meals in postmenopausal women. A greater AUC for the % FMD response was found following the MUFA rich meal in the wild-type group, whereas meal composition had no impact on % FMD in Asp298 carriers (or the group as a whole, as previously discussed). A meta-analysis of 8 RCTs showed a significant difference of 0.76 (5% CI 0.27-1.24, P<0.002) in %FMD after olive oil interventions, but these findings were irrespective of eNOS genotype (281). A further study

reported differences between meal fats were only found in the Asp298 minor allele carriers, with a greater response following addition of long chain n-3 PUFA to a SFA-rich meal, although the impact of MUFA and n-6 PUFA-rich meals according to the Glu298Asp polymorphism were not investigated in this study (121) or any other RCT. Therefore, there is a lack of evidence with which to compare these findings and additional RCT are required to confirm or dispute these results. However, in the current study long chain n-3 PUFA test fat was not included yet the differences observed may reflect the functional effect of the single nucleotide polymorphism on eNOS action.

There was also an interaction between the test fats and eNOS genotype for the postprandial insulin responses, with differences observed between the test fats in the Asp298 minor allele carriers only. There is also a strong evidence that β cell function and insulin sensitivity progressively improve in the postprandial state when the proportion of MUFA is increased with respect to SFA (344). It is reported that enhancement of glucose uptake in the skeletal muscle occurs during increased muscle blood flow, which is induced by eNOS expression and by activating the insulin response (345, 346). Moreover, in an animal study, reduced insulin-induced blood flow and glucose uptake was reported in eNOS knockout mice (347). A previous study with non-diabetic subjects (n=247) also showed elevated levels of insulin after a glucose tolerance test with no significant differences in blood glucose levels in the minor allele Asp carriers compared with the wild-type (293). The present observations suggest that eNOS genotype and meal fat composition play an important part in the regulation of insulin induced glucose uptake and macrovascular function. Interestingly, the differential responses to meal fats were observed in opposite genotype groups (i.e. Asp carriers for insulin and Glu homozygotes for FMD), which require further investigation in suitably-powered RCT that include the use of prospective genotyping to determine the effects in Asp/Asp, Asp/Glu and Glu/Glu populations.

7.5 Effect of *APOLIPOPROTEIN E* genotype

The *APOE* (epsilon) genotype is the most widely researched SNP in relation to CVD risk. *APOE* gene has several polymorphic variants, in particular, the *APOE4* allele is linked with increased TC, LDL cholesterol, CVD risk and mortality (126, 130, 295-297) and further in a meta-analysis, it has been shown that *APOE* genotype accounts for up to 7% of the variance occurred in TC and LDL-C (295). A very recent multicentre LIPGENE study, carried out

with 442 adults, suggested benefits of providing personalised dietary interventions for individuals with metabolic syndrome based on *APOE* genotype (129).

Chapter 6 explored for the first time the effects of *APOE* genotype on the responses of vascular function, plasma lipids, and both established and novel CVD risk biomarkers to chronic and acute dietary fat manipulation. In the present analysis, the baseline associations with lipid levels confirmed previous findings (126-128, 130). Higher fasting TC and LDL-C were evident with *E4* carriers relative to *E2* carriers and the wild-type genotype group (*E3/E3*). In addition to that, *APOE* genotype effects on baseline fasting lipids, the genotype effects were evident for arterial stiffness and biomarkers of inflammation (C reactive protein (CRP), with *E2* carriers having greater DVP-SI relative to the *E3/E3* genotype group and CRP relative to *E4* carriers. These observations suggest that the selective affinity of the apoE4 protein isoform for TAG-rich lipoproteins (chylomicrons and very low density lipoprotein) would be anticipated to result in increased competition with LDL for LDL receptor mediated clearance, which may lead to increased circulating LDL-C concentrations (308, 309). In addition, a decreased intestinal cholesterol absorption and higher bile acid synthesis in *E2* carriers than *E3* or *E4* individuals has been suggested, which is thought to contribute to higher LDL-C in *E4* carriers (348, 349). The effects of *APOE* genotype on the chronic changes observed following dietary fat manipulation for 16 weeks were very limited, with little impact of *APOE* genotype on lipid responses. Furthermore, *APOE* genotype also had a limited impact on postprandial cardio metabolic markers, which is in agreement with the SATgene study (135). However, it should be noted that the retrospective genotyping for both genotype analyses produced small group sizes, particularly for the minor alleles, which may have negated identification of significant effects. Furthermore, the combination of groups, e.g. *E3/E4* and *E4/E4* to create '*E4* carriers' but this may have masked differences between *E3/E3* and *E4/E4* groups, further highlighting the importance of retrospective genotyping. Therefore, the purpose of the analysis in Chapters 5 and 6 was hypothesis-testing only. The interactions observed imply that suitably-powered RCT using prospective genotyping are warranted to investigate these hypotheses.

7.6 Conclusion

The primary outcome, % FMD response, and other measures of vascular function were not influenced by the acute consumption of different fatty acids in postmenopausal women. When split according to the eNOS genotype, the Glu298Asp polymorphism influenced

postprandial vascular reactivity (%FMD) as well as insulin responses to sequential MUFA rich meals. Overall, MUFA-rich meals had favourable effects on postprandial DBP, as well as improving plasma nitrite concentrations compared with SFA-rich meals. Furthermore, n-6 PUFA rich meals reduced postprandial sICAM-1 concentrations, a marker of endothelial activation relative to the SFA and MUFA-rich meals. Furthermore, higher fasting TC and LDL-C were evident with *E4* carriers than *E2* carriers and the *E3/E3* wild-type genotype group but lower CRP than *E3/E3* and *E2* carriers at baseline, which confirms previous findings, although there was little impact of *APOE* genotype on the responsiveness of novel and established cardio-metabolic risk markers to chronic dietary fat manipulation in adults at moderate CVD risk. In addition, IL-1β was the only cytokine to be influenced by meal fat composition, with greater production following a SFA-rich meal relative to meals rich in unsaturated fats, which may contribute to higher risk of CVD in postmenopausal women.

The novel data from this research achieved its objective in providing evidence that i) fatty acids have differential effects on CVD risk biomarkers in postmenopausal women and ii) variations in genotype (eNOS and *APOE*) differentially impacted on these responses.

Supporting previous findings from chronic interventions, this research has provided evidence that the acute intake of unsaturated fatty acids relative to SFA though the intakes are higher than what is recommended for CVD risk reduction have beneficial, yet, differential effects on postprandial BP, NO concentrations and biomarkers of endothelial activation in postmenopausal women. These findings will contribute to the evidence base for future dietary fat recommendations for CVD risk reduction. Further studies are necessary to examine the mechanisms underlying the effects of these fatty acids as well as determining the effects of meal fat composition, including the influence of genotype, on postprandial risk factors of CVD in different population groups.

7.7 Future work

The area of fatty acids and cardio-metabolic markers have been researched extensively, however gaps of evidence still remain. Well-powered multi-ethnic clinical trials are required for more consistent evidence to determine the impact of meals with varying fatty acid composition and importantly the underlying mechanisms of action. DIVAS-2 study population was only women and it is interesting to investigate whether DIVAS-2 results could be extrapolated to men. Further investigations should also assess other genotype and diet interactions. Since both the DIVAS dietary intervention and DIVAS-2 acute studies influence blood pressure, it would be interesting to determine whether nutrient-gene

interactions exist for insertion/deletion (I/D) polymorphism of angiotensin I-converting enzyme (ACE) which has been shown to be associated with the risk of myocardial infarction in particular Caucasian population (350). Furthermore, ACE is a major component of the renin-angiotensin system thought to be important in the pathogenesis of hypertension and CVD.

The South Asian population represent one-fifth of the global population and are at higher risk of cardiovascular diseases, in their country of origin and particularly after migration to Western countries. This is demonstrated by higher rates of CVD, insulin resistance and metabolic syndrome among South Asian immigrants in developed countries (351). The influence of genetic variance has been proposed as an important contributory factor for the high prevalence of non-communicable diseases in South Asians. Sri Lanka is a middle-income country in the South Asia with a population about 20 million. CVD is the leading cause of death in Sri Lanka and it accounts for 40% of the total mortality with a high prevalence of hypertension 28% in adults (352). As such, it would be interesting to determine the influence and penetration of common genotypes which contribute to higher CVD risk with a large scale epidemiological study in Sri Lanka and other South Asian countries.

Furthermore, coconut oil is the staple fat source in Sri Lanka. In DIVAS and DIVAS-2 studies, the main SFA source was butter and it would be interesting to compare the effects of coconut fat on vascular function and cardio-metabolic risk factors with unsaturated fatty acids and butter. Although 92% of coconut fat is composed of SFA, the fatty acid profile of tropical oils such as coconut and palm oil differ from animal sources of SFA. Coconut fat predominantly yields the saturated fatty acids lauric acid and monoglycerides, which are directly absorbed from the intestine without undergoing degradation and re-esterification processes in the small intestine. Investigation of different dietary sources of SFA including those derived from animals and plants on cardio-metabolic risk is warranted, as the food matrix of these SFA-rich foods may impact on the relationship between SFA with CVD risk. However, it has been shown in a metabolic diet study that lauric acid raises total and LDL cholesterol (29, 353). Since there is limited and inconsistent data available on the chronic and acute effects of coconut fat on vascular function and other cardio metabolic risk factors, suitably powered RCT are required to investigate these effects in different ethnic groups, such as those who use coconut fat as their staple fat source. When I return to Sri Lanka, it would be interesting to conduct a collaborative study with my colleagues at the University of Reading to compare the effects with the UK population who do not habitually consume coconut oil.

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Appendix I: Abstracts published in conference proceedings

Meal fat composition has differential effects on biomarkers of postprandial endothelial function in postmenopausal women. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *Nutrition Society Summer Meeting, King's College London (London, UK), July 2017 (oral presentation).*

Decline in oestrogen at menopause is associated with adverse effects on lipid metabolism, vascular function and blood pressure [1], markedly increasing cardiovascular disease (CVD) risk in postmenopausal women. As a key public health strategy to reduce the incidence of CVD in the UK, dietary saturated fatty acid (SFA) intake of ≤10% of total energy is recommended [2]. The Dietary Intervention and Vascular Function-2 study aimed to investigate the effects of sequential meals of varying fat composition on postprandial vascular reactivity and associated biomarkers of endothelial function in postmenopausal women.

In an acute, double-blind, randomised, cross-over study, 32 women (mean age of 57 ± 1 y and BMI of 26 ± 0.7 kg/m^2) consumed sequential mixed test meals (0 min, 50g fat and 330 min, 30g fat) rich in SFA, monounsaturated (MUFA) or n-6 polyunsaturated (PUFA) fatty acids on 3 separate occasions, each 4-6 weeks apart. Blood samples were collected and real-time measures of vascular reactivity (flow-mediated dilatation, laser Doppler imaging, digital volume pulse) were performed before and at regular intervals after the breakfast for 480 min.

There were no differences in the real-time measures of vascular function after the SFA, n-6 PUFA and MUFA-rich meals. A significant effect of meal fat composition was evident for the incremental area under the curve (IAUC) for the postprandial plasma nitrite (a biomarker for nitric oxide, an important vasodilator) response ($p=0.010$), with a lower IAUC after consumption of the SFA than MUFA-rich meals ($p=0.007$). There was also a trend for the IAUC for the nitrate and NOx (both nitrite and nitrate combined) responses ($p=0.054$) to be influenced by the meal fatty acids, with a lower IAUC after the SFA than n-6 PUFA-rich meals ($p=0.024$). There were significant test fat* time interactions for the time course profiles for soluble intracellular cell adhesion molecule-1 (sICAM-1) and vascular adhesion molecule-1 (sVCAM-1), with a lower sICAM-1 response after the n-6 PUFA than SFA and MUFA-rich ($p<0.001$) meals whereas the sVCAM-1 response was different after the SFA than MUFA and PUFA-rich meals (Figure). E-selectin and P-selectin responses were not affected by meal fat composition.
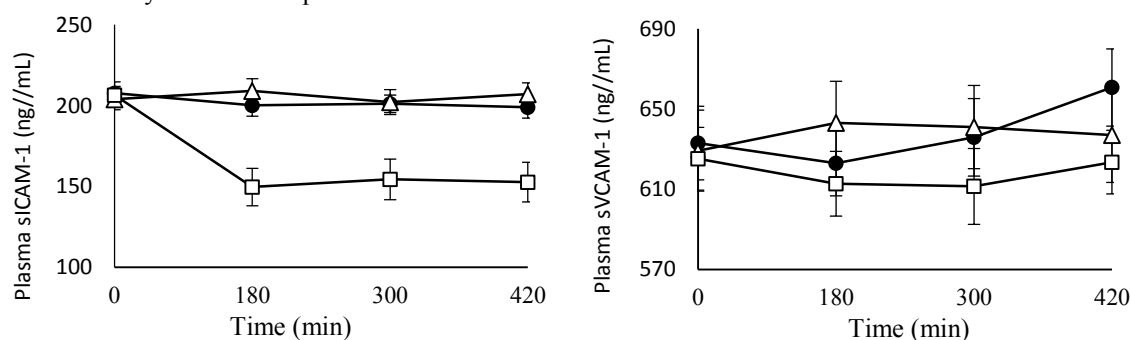

Figure. Mean ± SEM for the sICAM-1 and sVCAM-1 responses following sequential meals (0 min and 330 min) enriched with SFA (●), MUFA (Δ) and n-6 PUFA (□). Repeated measures ANOVA showed a significant test fat*time interaction ($p < 0.001$).

In conclusion, our findings reveal a differential impact of sequential meals of varying fat composition on plasma nitrite and cell adhesion molecules (biomarkers of endothelial activation) in postmenopausal women. Further studies are necessary to examine the mechanisms underlying the effects of meal fatty acids on postprandial endothelial function.

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Meal fat composition has a significant impact on postprandial blood pressure in postmenopausal women: Findings from the DIVAS-2 study. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *The 12th Congress of the International Society for the Study of Fatty Acids & Lipids (ISSFAL), Stellenbosch (South Africa), Sept 2016 (oral presentation). **Awarded young scientist award.***

Background: Elevated postprandial triacylglycerol responses are an important independent risk factor for cardiovascular disease (CVD) in women, but little is known about the effects of meal fat composition on postprandial lipaemia and vascular function in postmenopausal women.

Methods: In a cross-over, double-blind, randomised, controlled postprandial study, 32 postmenopausal women consumed sequential test meals rich in saturated fat (butter), cis-monounsaturated fat (olive oil and spreads) or n-6 polyunsaturated fat (safflower oil and spreads) on three separate study visits, each 4-6 weeks apart. Test fats were coded 1-3 for double-blinding, each consisting of breakfast (0 min; 50g total fat) and lunch (330 min; 30g total fat). Blood pressure was determined at 0, 240 and 450 min. Blood samples were collected at 0 min and regularly until 480 min to measure lipids, and markers of insulin resistance.

Results: Participants had a mean (SD) age 58 (5) years, BMI 25.7 (4.0) kg/m^2, and fasting lipids and glucose were within the normal range. Preliminary results showed no significant differences between study visits at 0 min. There were no significant meal effects or meal*time interactions for plasma triacylglycerol, apolipoprotein B, non-esterified fatty acids, glucose, insulin, systolic blood pressure or diastolic blood pressure (DBP). However, there was a significant meal*time interaction for the incremental change in DBP after the meals (incremental area under the curve) (p=0.007), in which there was a significantly greater reduction after test fat 1 compared with 2 (p=0.012).

Conclusion: Meal fatty acid composition has differential effects on postprandial change in DBP following sequential high-fat meals. These data form part of the DIVAS-2 postprandial study (Clinical Trial.gov reference NCT02144454) which will examine the impact of meal fatty acids on vascular function and other CVD risk biomarkers.

Meal fatty acid composition has a differential effect on postprandial blood pressure in postmenopausal women. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *Nutrition Society Summer Meeting, University College (Dublin, Ireland), July 2016 (oral presentation).*

Elevated postprandial triacylglycerol concentrations are an important independent risk factor for cardiovascular disease (CVD) in women [1], but little is known about the effects of meal fat composition on postprandial lipaemia and vascular function in postmenopausal women [2]. The Dietary Intervention and Vascular Function (DIVAS-2) study aimed to investigate the acute consumption of test meals of varying fat composition on postprandial vascular reactivity and associated CVD risk biomarkers in postmenopausal women.

In a double-blind, randomised, cross-over, postprandial study, 32 postmenopausal women consumed sequential test meals rich in saturated fat (butter), cis-monounsaturated fat (olive oil and MUFA-rich spreads) or n-6 polyunsaturated fat (safflower oil and n-6 PUFA-rich spreads) on three separate occasions, each 4-6 weeks apart. Test meals were coded 1-3 for blinding and the sequential meals consisted of breakfast (0 min; 50g total fat) and lunch (330 min; 30g total fat). Blood pressure (BP) was determined at 0, 240 and 450 min. Blood samples were collected at 0 min and regularly until 480 min to measure lipids, and markers of insulin resistance.

Participants had a mean (SD) age 58 (5) years, BMI 25.7 (4.0) kg/m^2, with fasted lipids and glucose within the normal range. Preliminary results showed no significant differences between study visits at baseline. A significant meal*time interaction for the incremental postprandial diastolic BP (DBP) response (incremental area under the curve) (p=0.007) was observed, with a significantly greater reduction after test fat meal 1 compared with 2 (p=0.012) (Figure 1). No significant effects of meal fatty acid composition were observed for postprandial triacylglycerol, apolipoprotein B, non-esterified fatty acids, glucose, insulin, systolic BP or pulse pressure.
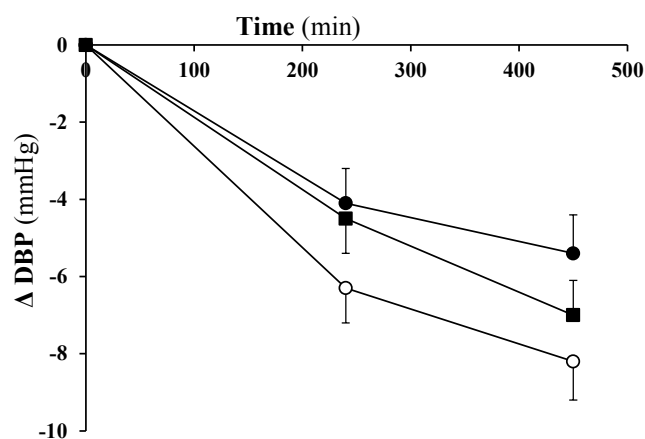

Figure 1: Mean ± SEM for the change in postprandial diastolic blood pressure (ΔDBP) following sequential meals enriched in test fat meal 1 (open circles), test fat meal 2 (closed circles) and test fat meal 3 (closed squares).

In conclusion, meal fat composition was shown to have differential effects on the postprandial change in DBP following sequential high-fat meals. These data from part of the DIVAS-2 postprandial study and this trial was registered at www.clinicaltrials.gov as NCT02144454.

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Impact of meal fatty acids on postprandial vascular reactivity: Overview of the DIVAS-2 study. Kumari M Rathnayake, Michelle Weech, Kim G Jackson and Julie A Lovegrove. *Nutrition Society Postgraduate Conference, Trinity Hall (Cambridge, UK) Sept 2015 (oral presentation).*

Background: Cardiovascular diseases (CVD) are the leading cause of death in women in the UK. After the menopause, the risk of developing CVD increases in women. The Dietary Intervention and Vascular Function (DIVAS-2) study will investigate the acute consumption of test meals rich in saturated (SFA), monounsaturated (cis-MUFA) or n-6 polyunsaturated fat (n-6 PUFA) on postprandial vascular reactivity and associated CVD risk biomarkers in postmenopausal women.

Methods: In a cross-over, double-blind, randomised, controlled postprandial study, participants were randomly assigned to consume sequential test meals rich in SFA (butter), cis-MUFA (olive oil and spreads) or n-6 PUFA (safflower oil and spreads) on three separate study visits, each 4-6 weeks apart. Breakfast (0 min) and lunch (330 min) contained 50g and 30g of total fat, respectively. Vascular reactivity was assessed using flow-mediated dilatation (0, 180, 300 and 420 min) and laser Doppler imaging with iontophoresis (0, 240 and 450 min). Arterial stiffness was measured using digital volume pulse (0, 240 and 450 min). Blood samples were collected at fasting and for 480 min following breakfast to measure lipids, markers of insulin resistance, inflammation and endothelial function.

Results: Thirty-three postmenopausal women were recruited with a mean (SD) age 58 (5) years, BMI 25.7 (4.0) kg/m^2, fasting total cholesterol 6.15 (0.9) mmol/L, triglycerides 1.20 (0.4) mmol/L and glucose 5.45 (0.6) mmol/L. Study visits will be completed in September 2015.

Conclusion: This study will provide further insights on the impact of meal fat composition on postprandial lipaemia and vascular reactivity in postmenopausal women.

Appendix II: Search terms for the literature review

List A Study group search terms

(postmenopausal OR post-menopausal OR post menopausal OR post menopause OR menopause OR menopausal)

List B Exposure search terms (i.e. dietary fat terms)

(unsaturat* fatty acid* OR saturated fatty acid* OR SFA* OR SAFA* OR dietary saturat* OR stearic acid OR palmitic acid OR myristic acid OR monounsaturated fatty acid* OR MUFA* OR dietary monounsaturat* OR oleic acid OR polyunsaturated fatty acid* OR omega 6 polyunsaturat* OR omega-6 polyunsaturat* OR omega-6 PUFA* OR omega 6 PUFA* OR omega-6 fatty acid* OR omega 6 fatty acid* OR n-6 polyunsaturat* OR n-6 PUFA* OR n6 PUFA* OR n-6 fatty acid* OR n6 fatty acid* OR ω-6 fatty acid* OR ω-6 PUFA* OR ω-6 polyunsaturat* OR dietary polyunsaturat* OR linoleic acid)

(butter OR cocoa butter OR milk OR lard OR beef tallow OR beef dripping OR coconut OR margarine OR ghee OR canola oil OR corn oil OR cottonseed oil OR grapeseed oil OR linseed oil OR olive oil OR palm oil OR primrose oil OR rapeseed oil OR safflower oil OR sesame oil OR soybean oil OR sunflower oil OR walnut oil)

(omega 3 polyunsaturated fatty acid* OR omega-3 polyunsaturated fatty acid* OR n-3 polyunsaturated fatty acid* OR n-3 PUFA* OR n3 PUFA* OR n-3 fatty acid* OR n3 fatty acid* OR omega-3 PUFA* OR omega 3 PUFA* OR omega-3 fatty acid* OR omega 3 fatty acid* OR ω-3 fatty acid* OR ω-3 PUFA* OR ω-3 polyunsaturat* OR long-chain n-3 PUFA* OR LCn3PUFA* OR LC n-3 PUFA* OR LC omega3* OR n-3 LC PUFA OR LCPUFA OR eicosapentaenoic acid OR docosahexaenoic acid OR EPA OR DHA OR docosapentaenoic acid OR DPA OR *linolenic acid OR ALA OR ALNA OR fish oil OR cod liver oil)

(trans fatty acid* OR trans fat* OR vaccenic acid OR medium chain triglycerides OR MCT OR CLA OR conjugated linoleic acid)

List C Outcome search terms (i.e. CVD risk factor terms)

Vascular function

(endothelial function OR endothelial dysfunction OR endothelial reactivity OR vascular function OR vasodilatory function OR endothelium OR vascular reactivity OR arterial compliance OR vascular tone)

(skin circulation OR quantitative angiopathy OR venous occlusion plethysmography OR laser Doppler iontophoresis OR laser Doppler imaging OR LDI OR laser Doppler flowmetry OR flow mediated dilatation OR flow mediated dilation OR flow-mediated dilatation OR flow-mediated dilation OR FMD OR digital volume pulse OR DVP OR pulse contour analysis OR pulse wave analysis OR PWA OR augmentation index OR pulse wave velocity OR PWV OR endo-PAT OR endo PAT OR endoPAT OR forearm blood flow OR FBF OR reactive hyperaemia OR reactive hyperemia)

Blood pressure

(blood pressure OR hypertension OR hypotension OR heart rate)

Lipids

(cholesterol OR low density lipoprotein cholesterol OR LDL cholesterol OR OR low-density lipoprotein cholesterol OR LDL-C OR high density lipoprotein cholesterol OR HDL cholesterol OR high-density lipoprotein cholesterol OR HDL-C OR lipoprotein OR apolipoprotein OR lipoprotein(a) OR apoB OR chylomicron remnants OR triglycerides OR triacylglycerol OR TRL* OR lipemia OR lipaemia)

Insulin resistance

(glycaemia OR glycemia OR glycemic OR glucose OR insulin OR insulin sensitivity OR insulin resistance OR non esterified fatty acid* OR nonesterified fatty acid* OR non-esterified fatty acid* OR free fatty acid* OR NEFA OR HOMA OR HOMA-IR OR homeostatic model assessment OR QUICKI OR rQUICKI OR quantitative **insulin** sensitivity check index OR euglycemic clamp OR euglycaemic clamp OR glucose tolerance test OR OGTT OR IVGTT OR adiponectin)

Markers of endothelial dysfunction & inflammation

(nitric oxide OR nitrate* OR nitrite* OR vascular cell adhesion molecule* OR intercellular cell adhesion molecule* OR VCAM-1 OR ICAM-1 OR cell adhesion molecule* OR P-Selectin OR E-Selectin OR blood coagulation factors OR C-reactive protein OR CRP OR interleukin* OR IL-6 OR tumor necrosis factor-alpha OR TNF* OR von willebrand factor OR vWF OR cytokine* OR inflamm* OR inflammation OR endothelial activation OR microalbumin* OR creatinine OR endothelial progenitor cell* OR platelet microparticle* OR endothelial microparticle*)

Searching approach:
(A list) AND (one B list) AND (one C list)

For example:

(postmenopausal OR post-menopausal OR post menopausal OR post menopause OR menopause OR menopausal) **AND** (unsaturat* fatty acid* OR saturated fatty acid* OR SFA* OR SAFA* OR dietary saturat* OR stearic acid OR palmitic acid OR myristic acid OR monounsaturated fatty acid* OR MUFA* OR dietary monounsaturat* OR oleic acid OR polyunsaturated fatty acid* OR omega 6 polyunsaturat* OR omega-6 polyunsaturat* OR omega-6 PUFA* OR omega 6 PUFA* OR omega-6 fatty acid* OR omega 6 fatty acid* OR n-6 polyunsaturat* OR n-6 PUFA* OR n6 PUFA* OR n-6 fatty acid* OR n6 fatty acid* OR ω-6 fatty acid* OR ω-6 PUFA* OR ω-6 polyunsaturat* OR dietary polyunsaturat* OR linoleic acid) **AND** (Endothelial function OR endothelial dysfunction OR endothelial reactivity OR vascular function OR endothelium OR vascular reactivity OR arterial compliance OR vascular tone)

Appendix III: Subject recruitment poster for the DIVAS-2 Study

Appendix VI: Participant Information Sheet for the DIVAS-2 Study

Study Title: DIVAS-2 (**D**ietary **I**ntervention and **Vas**cular Function-2)

Investigators: Dr Michelle Weech, Ms Kumari Rathnayake (PhD student), Professor Sue Todd, Professor Parveen Yaqoob, Dr Kim Jackson and Professor Julie Lovegrove.
Hugh Sinclair Unit of Human Nutrition, Department of Food and Nutritional Sciences, Whiteknights, University of Reading, RG6 6AP

Contact Name: Dr Michelle Weech
Tel: 0118 378 5360; Email: m.weech@reading.ac.uk

Thank you for your interest in the DIVAS-2 study.

Before you decide to participate, it is important that you understand why the research is being completed and what it will involve. Please take your time to read the following information carefully and discuss it with others if you wish. Please ask us if anything is not clear or if you would like further information, and take your time to decide whether or not you wish to take part. Thank you for reading this information sheet.

1. Background of the study
Cardiovascular disease (CVD), which includes coronary heart disease and stroke, is the leading cause of death in women. Premenopausal women have a lower risk of CVD compared with men of a similar age. However, the incidence of CVD increases greatly after the menopause. The risk of heart disease is strongly associated with the health of an individual's blood vessels. It is thought that changes to the type of fat we eat in our diet may affect the normal functioning and elasticity of the blood vessels. There is also much evidence to suggest the types of fat we eat can affect cholesterol levels in the blood. Types of fat in the diet include monounsaturated fats (found mainly in olive oil), n-6 polyunsaturated fats (found mainly in sunflower oil) and saturated fats (found mainly in dairy products, such as butter and cheese). Since we are in the fed (or postprandial) state for up to 18 hours of the day, it is important to see how these different fats affect our blood vessels and blood fats over the course of the day after eating a meal.

2. What is the purpose of the study?
The main purpose of this study is to determine how consuming meals rich in saturated fats, n-6 polyunsaturated fats or monounsaturated fats influence the normal functioning and elasticity of the blood vessels throughout the day in postmenopausal women. A secondary

aim is to determine the effects of these different dietary fats on a range of accepted heart disease risk markers including circulating levels of fats (lipids) and glucose in the blood.

3. Do I have to take part?

It is up to you to decide whether you wish to participate in the study. We will describe the study to you and go through this information sheet, which we will give or send to you by mail. We will then ask you to sign a consent form to show you have agreed to take part. <u>You are free to withdraw at any time without giving a reason</u>; please let one of us know if you would rather not participate and we will not contact you further about the study.

4. Can anyone take part in the study?

We aim to recruit 31 postmenopausal women who have been postmenopausal for at least 12 months and are 65 years of age or under. Participants will be required to have fasting triacylglycerol levels between 0.8 and 4.0 mmol/L, cholesterol levels of less than 8 mmol/L, and glucose levels of less than 7 mmol/L, which will be determined from a small blood sample taken at the screening visit. Participants will also be required to have a blood pressure of less than 160/100 mmHg.

If you have diabetes, heart disease (previous stroke/myocardial infarction), renal or bowel or liver diseases, regularly use certain types of medication (e.g. medication for high blood pressure, high blood lipids and inflammatory conditions or are anaemic, you will not be able to take part in the study. Women taking hormone replacement therapy (HRT) cannot take part. If you take any dietary supplements you will be able to take part in the study, although the supplements cannot be taken for the duration of the study and a washout period will be required before participation. Suitable volunteers should drink no more than 21 units of alcohol (i.e. not more than 10 and a half pints of beer or 21 small glasses of wine) per week and not regularly undertake vigorous exercise or fitness training.

The medical and lifestyle questionnaire that you have already completed over the phone will be used to screen for the above criteria. If you are interested in taking part after reading this information sheet, please contact the Hugh Sinclair Unit of Human Nutrition clinical unit manager, Mrs Jan Luff, on 0118 378 7771 or email: j.e.luff@reading.ac.uk.

5. What will happen to me if I take part?

'Screening visit'

You will be invited to come for a screening visit at the Hugh Sinclair Unit of Human Nutrition in the Department of Food and Nutritional Sciences (University of Reading). The visit will take place in the morning and we would like you to arrive in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). First, your consent for participating in this study will be taken. After that, a small blood sample (~14 ml, volume equivalent to one tablespoon) will be taken and your weight, height and blood pressure will be measured. The screening visit should take no more than 1 hour and you will be provided with a light breakfast before you leave.

You will be informed of the results of your blood tests on blood glucose, blood lipids (cholesterol and triacylglycerol) and haemoglobin levels. If you are found suitable for the study and are willing to participate, we will confirm with you your participation in the study and inform your GP of your wish to take part.

Over three study days, each approximately 4-6 weeks apart, you will be given test meals (breakfast and lunch) in the form of warm chocolate drinks with toasted bread and jam which will contain a different type of dietary fat on each visit. Volunteers will be required to attend each of the three study visits. The type of fat consumed on each visit will be randomly assigned by the researchers and neither you nor the researcher performing the vascular measurements will know this order.

'Run-in period and familiarisation visit'
After we confirm your suitability to participate in the study, you will be asked to come to the Hugh Sinclair Unit of Human Nutrition for a 'familiarisation visit' approximately 1-2 weeks before your first study visit. To familiarise you with the procedure of the study visits, we will explain and demonstrate the various pieces of equipment which we will use to take measurements from you. In addition, we will explain how to complete the diet diary to allow the researchers to assess your usual dietary intake. The familiarisation visit should take about 1 hour.

'Study visits'
The three study visits will be conducted approximately every 4-6 weeks and will last for approximately 9 hours. On each occasion, you will be asked to come to the Hugh Sinclair Unit of Human Nutrition in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). For consistency, we will provide you with meals for the evenings before you come into the unit (these will be given to you at the familiarisation visit) and you will be asked to refrain from drinking caffeinated beverages during and after consuming the evening meal. On arrival at the Hugh Sinclair Unit of Human Nutrition, your weight, waist circumference, blood pressure and body composition will be measured before you are asked to lie down in a temperature-controlled room in preparation for the measurements assessing the normal functioning of the blood vessels ('vascular measurements'). A small flexible cannula will be inserted into a vein in your arm by trained and experienced personnel, and this will remain in place with minimal discomfort to allow us to take blood samples during the study day. A 32 ml blood sample (volume equivalent to two tablespoons) will then be taken. You will rest for a further 30 minutes to ensure that you are relaxed before the vascular measurements are performed, all of which are non-invasive, safe and routinely performed.

The first measurement, called 'Flow Mediated Dilatation' (FMD), uses an ultrasound system to visualise the artery in the upper arm from which its diameter can be measured. A cuff similar to those used to measure blood pressure will be placed just below your elbow and inflated until the pressure or tightness of the blood pressure cuff prevents the blood from

entering your forearm. It will be held at this pressure for 5 minutes. When the pressure is released, the artery widens. The wider the artery after the release of pressure compared to before the pressure is applied implies a healthier artery. The high pressure of the blood pressure cuff is a little uncomfortable and you may experience 'pins and needles' or slight numbness in your fingertips towards the end of the 5 minutes. The level of discomfort will be similar to that associated with having your blood pressure taken. The procedure will take approximately 30 minutes per measurement.

The second technique uses a machine called a 'Laser Doppler Imager' (LDI). Two small perspex rings ('chambers') containing a small amount (~2.5 ml) of two different chemicals will be placed on your forearm and a small current will be applied to deliver the chemical through the skin barrier ('iontophoresis'). The chemicals stimulate your small blood vessels situated under the skin to relax and increase the flow of blood. The LDI will continually monitor this response over 20 minutes by scanning the chambers on your arm. Increased blood flow reflects a healthier artery. The action of the chemicals is only short-term and restricted to the small area of skin where they are applied. The procedure is pain-free and will take approximately 30 minutes per measurement.

After this, the elasticity of your blood vessels will be measured by 'Digital Volume Pulse' (DVP). The DVP is recorded by placing a clip on your index finger which uses infra-red light to measure the volume of blood in the fingertip. This painless measurement will be recorded 3 times over 10 second periods with 3 minute intervals between measurements.
After the measurements are taken, you will be given a breakfast consisting of a warm chocolate drink with toasted bread and jam to provide 50 g of fat. We will ask you to eat all of this meal within 20 minutes. Further blood samples (between 5 and 18 ml, equivalent to up to one tablespoon of blood) will then be taken at regular intervals and two measurements of FMD and LDI will be performed until lunch is provided at approximately 2.30 pm. This will also consist of warm chocolate drink with toasted bread, spread and jam providing 30g of fat. Blood samples will then be collected at regular intervals until 5 pm and during this time a single measure of FMD will be performed. The cannula will be removed and you will be provided with a snack before you go home. You will be asked to remain in the Hugh Sinclair Unit of Human Nutrition for the duration of the study day where you will have unlimited access to drinking water and facilities to watch TV, DVDs, work, read and use the internet in between measurements.

What will be measured in the blood samples collected?
The fasting blood sample collected at the screening visit will be used to measure levels of blood glucose, blood lipids (total cholesterol and triacylglycerol), haemoglobin and levels of liver enzymes. The blood samples taken during the study days will be used to measure blood lipids, markers of heart disease risk factors (such as markers of inflammation and insulin) as well as types of fatty acids present in the blood. You will be supplied with your individual blood results within four months of completion of the study if you are interested. The clinical meaning of your screening results will have to be explained to you by your GP.

6. Will any genetic tests be done?

Genetic tests may be performed in future analysis on blood samples to understand the genetic influences on differences between individuals in response to the test meals given on the study days. The genotypes that will possibly be determined include those that arise from polymorphisms in apolipoprotein E (apoE) and endothelial nitric oxide synthase (eNOS) genes. Additional genetic tests to study the effects of other genotypes on response to diet may also be performed in the future. We will ask you for your consent to genotype your blood sample at the screening visit. You are free to opt out of having your sample stored for this future genetic analysis. This of course will not affect your taking part in the main study.

7. How confidential are the results?

The results will be strictly confidential to the study investigators and each participant will only be identified by means of a random number allocated at the beginning of the study. Information obtained from the study may be published in scientific journals but only in the form of average values for the group; no results for the individual participants will be published or presented in scientific meetings. We will inform you of the broad scientific results of the study, if you interested. If we discover any abnormalities of significance to your health we will inform you and your GP.

8. What will happen if I don't want to carry on with the study?

If you decide to take part, you will be asked to sign a consent form during the screening visit; you will be given a copy of this to keep. However, you are free to withdraw from the study at any time and without giving a reason. We ask that you kindly inform us of this decision and we can amend our records so we do not contact you again regarding the study.

Whilst you are participating in the study it is important for you to attend all visits to the best of your ability. If the appointment or study day is not convenient on a particular date, please contact the study investigators as soon as possible so that an alternative date can be offered to you.

9. Do you have to modify your diet or other activities in any way?

During the study period you will be asked <u>not</u> to change your diet, to exercise normally and to carry out your usual activities. However, for 24 hours before your three study visits, you will be advised not to drink alcohol, to avoid aerobic/intense exercise and to eat the evening meal provided, avoiding caffeinated drinks with the meal. You will also be asked to avoid all food and drink, except water, from 8pm on the evening before your three study visits until we provide you with the study breakfasts the following day. During the study period, please inform us of any newly prescribed medication that you have not mentioned in your initial screening questionnaire or if you are advised to stop any medication that you were taking at the start of the study.

10. Are there any adverse consequences to your health as a result of being a volunteer on this study?

Blood samples will be taken by qualified, experienced investigators (who are not medically qualified but have been trained in venepuncture and first aid) with 'on-call' medical cover

provided throughout the study. The procedure for taking blood directly from a vein in your arm or via a small flexible cannula is routine. The volume of blood collected during each study visit is approximately 109 ml (around 7 tablespoons), and will cause no adverse consequences.

There will be tightness around the forearm when the blood pressure cuff inflates during the Flow Mediated Dilatation technique. Volunteers can opt to stop the inflation or remove the device should they experience too much discomfort.

11. What are the possible benefits of taking part?

Although you will derive no individual benefit, the knowledge gained from this study will help our research into finding the optimal type of dietary fat to reduce the risk of developing heart disease and will contribute to future public health recommendations for the intake of dietary fat.

12. Expenses and Payments

You will be remunerated for your time and travel with a total of £175 on completion of the study. Participants that drop-out of the study before completion of all three study visits will be paid on a pro-rata basis (£50/study day) according to the number of main study visits completed.

13. What if there is a problem?

Complaints

If you have a concern about any aspect of this study, you should ask to speak to one of the study investigators who will do their best to answer your questions (*see contact details at the front of this Participant Information Sheet*).

If you remain unhappy and wish to complain formally, you can do this through the Head of the Department of Food and Nutritional Sciences, Professor Bob Rastall (tel: 0118 378 6726; email: r.a.rastall@reading.ac.uk).

Harm

In the event that something does go wrong and you are harmed during the study, the University of Reading has in place Professional Indemnity Insurances that provide cover against negligence, error or omission for the activities of its employers.

14. Will my taking part in the study be kept confidential?

No. We will inform your GP about your participation in the study only (the actual screening results will not be sent). However, if we discover any abnormalities of significance to your health we will inform both you and your GP. All information about you will be handled in confidence.

15. Who has reviewed the study?

This study has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Appendix V: Subject Recruitment Poster for the DIVAS-2 Study

Appendix V: Favourable ethical opinion for conduct of the DIVAS-2 Study

University of Reading

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Professor Julie Lovegrove
Hugh Sinclair Chair in Human Nutrition
School of Chemistry, Food & Nutritional
Sciences and Pharmacy
University of Reading

29 April 2014

Dear Julie

UREC 14/16: The acute effects of meals rich in SFA, n-6 PUFA and MUFA on vascular function (DIVAS-2 study). *Provisional opinion*

Thank you for your revised application (electronic copy received from Michelle Weech, 8 April 2014, refers). I can confirm that the Chair is pleased to confirm a favourable ethical opinion on the basis of this revised documentation.

Please note that the Committee will monitor the progress of projects to which it has given favourable ethical opinion approximately one year after such agreement, and then on a regular basis until its completion.

Please also find attached Safety Note 59: Incident Reporting in Human Interventional Studies at the University of Reading, to be followed should there be an incident arising from the conduct of this research.

The University Board for Research and Innovation has also asked that recipients of favourable ethical opinions from UREC be reminded of the provisions of the University Code of Good Practice in Research. A copy is attached and further information may be obtained here:
http://www.reading.ac.uk/internal/res/QualityAssuranceInResearch/reas-RSqar.aspx.

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Yours sincerely

Dr M J Proven
Coordinator for Quality Assurance in Research (UREC Secretary)
cc: Dr John Wright (Chair); Professor Bob Rastall, Head of Food and Nutritional Sciences; Miss Michelle Weech, Research Fellow; Mrs Catherine Hale, Research Secretary

This letter and all accompanying documents are confidential and intended solely for the use of the addressee

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