The effects of whole-diet interactions on vascular health and inflammatory and fatty acid status.

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Abbreviations

AA	Arachidonic acid
ABI	Ankle brachial index
ACN	Anthocyanin
αLNA	α-Linolenic acid
ANOVA	Analysis of variance
AUC	Area under the curve
BHT	Butylated hydroxytoluene
BMD	Bone mass density
BMI	Body mass index
BPM	Beats per minute
CAD	Coronary artery disease
CAMs	Cell adhesion molecules
CAVI	Cardio-ankle vascular index
CHD	Coronary heart disease
CRP	C-Reactive protein
CRTU	Clinical Research and Trials Unit
CVD	Cardiovascular disease
D5D	Delta-5-desaturase
D6D	Delta-6-desaturase
DASH	Dietary approaches to stop hypertension
DART	Diet and reinfarction trial
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EDI	Elderly Diet Index
EFSA	European Food Safety Authority
eNOS	Endothelial Nitric Oxide synthase
EPA	Eicosapentaenoic Acid

ET	Endothelin
ETAR	Endothelin A receptor
ETBR	Endothelin B receptor
FADS	Fatty acid desaturase
FAMEs	Fatty acid methyl esters
FBDGs	Food based dietary guidelines
FFQ	Food frequency questionnaire
FMD	Flow mediated dilatation
GC-FID	Gas chromatography flame ionisation detection
GP	General Practitioner
HDL-C	High density lipoprotein cholesterol
HEI	Healthy eating index
HR	Hazard ratio
iNOS	Inducible Nitric Oxide synthase
KASP	Kompetitive Allele Specific PCR
LA	Linoleic Acid
LC-PUFA	Long chained polyunsaturated fatty acids
LD	Linkage disequilibrium
LDL-C	Low density lipoprotein cholesterol
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony stimulating factor
MDS	Mediterranean Diet Score
MI	Myocardial infarction
MSDPS	Mediterranean-Style Dietary Pattern Score
MUFA	Monounsaturated fatty acids
NO	Nitric Oxide
OA	Oleic acid
PA	Palmitic acid
PAD	Peripheral arterial disease
PAT	Peripheral arterial tonometry

RCT	Randomised controlled trial
RH	Reactive hyperaemia
RHI	Reactive hyperaemic index
SA	Stearic acid
SBP	Systolic blood pressure
SEM	Standard error of the mean
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
TC	Total cholesterol
TG	Triglyceride
UEA	University of East Anglia
UN	United Nations
USDA	United States Department of Agriculture
VCAM	Vascular cell adhesion molecule

Declaration

I confirm that the work presented in this thesis is my own. Contributions from other individuals and institutions are fully acknowledged below.

NU-AGE is a European multicentre trial led by Prof Claudio Franceschi at the University of Bologna. Other team members that contributed to NU-AGE at the University of East Anglia include Dr Amy Jennings (senior postdoctoral fellow), Ms Rachel Gillings (research assistant) and placement students from University College of Dublin; Ms Carol Huet and Ms Sophie Millar.

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Abstract

Introduction

The proportion of people over 65 years in Europe is predicted to increase from 25 to 40% by 2030. Diet has an important modifiable influence on ageing and it is therefore, important to identify realistic dietary strategies that will contribute to healthy ageing. The NU-AGE project (EU FP7) aims to examine the impact of a year-long whole-diet intervention (including advice on intakes of the n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) on chronic low grade inflammation (primary end-point) in 1250 older adults (aged 65-79 years) in five EU centres. As part of this thesis, the impact of intervention on vascular 'health' was established. In addition, a diet score for older adults was designed based on the NU-AGE diet.

The intake and status of EPA and DHA is being increasingly recognised as an important modulator of the risk of chronic disease. Potential determinants of fatty acid status, including other dietary components and genotype were investigated and represent the second major component of the thesis. The genotyping focussed on the fatty acid desaturase (FADS) enzymes which are responsible for the desaturation steps in the synthesis of EPA and DHA from α-linolenic acid (αLNA).

Methods

Diet, vascular and inflammatory health in NU-AGE; the effects of the one year NU-AGE intervention on vascular function and inflammatory and fatty acid status was investigated in 140 participants from the Norwich centre of NU-AGE. Vascular function was clinically measured using EndoPAT, Pulse Wave Velocity (PWV) and Cardio-Ankle Vascular Index (CAVI). The NU-AGE diet score was designed and validated using both the TWIN UK cohort and the NU-AGE baseline data.

EPA and DHA status; retrospective analysis of plasma samples from two completed rodent studies and one human clinical trial, which all included polyphenol-rich interventions, were used to investigate the impact of a range of polyphenols on plasma and tissue fatty acid status. In the NU-AGE cohort, the impact of individual *FADS* gene variants on plasma fatty acid status was examined. 10 tagging single nucleotide polymorphisms (SNPs) were selected and haplotypes were statistically reconstructed.

Results

There was no significant effect of the NU-AGE intervention on any measured outcomes. However, subgroup analysis showed that the NU-AGE diet ameliorated the significant increase in the stiffness of arteries (as assessed by CAVI) in the control group over the 1 year intervention period in females ($p=0.024$). A higher NU-AGE diet score was associated with significantly higher CRP in the TWIN UK cohort ($p=0.028$), but not in the NU-AGE cohort at baseline.

In relation to the impact of various polyphenols on LC-PUFA status, we observed no significant differences in any of the three (two rodent and one human) studies. In the NU-AGE cohort, it was observed that participants with the homozygous minor genotype for several of the *FADS* SNPs had significantly ($p<0.05$) higher plasma linoleic acid (LA) and significantly lower arachidonic acid (AA), EPA and DHA status, as well as significantly lower desaturase activity (measured by a product-to-precursor ratio of AA/LA) compared with participants with either the homozygous major genotype or the heterozygous genotype. Furthermore, the most common haplotype (containing mostly major alleles and occurring in 26.6% of the cohort) was associated with significantly lower LA plasma levels (up to 9% increase) and significantly higher EPA (up to 38%) and DHA (up to 14%) status compared with haplotypes with a higher frequency of minor alleles. This work also showed that the NU-AGE dietary intervention may be successful in overcoming the negative effect of the minor allele on EPA and DHA status.

Conclusion

Although there was no significant effect of the NU-AGE intervention on any measured outcomes, the NU-AGE diet did appear to attenuate the expected progression of arterial stiffness in females. This work also suggests that the health benefits of polyphenols are unlikely to be the result of any impact on EPA and DHA status. Furthermore, common *FADS* genotypes emerged as significant determinants of habitual EPA and DHA status in older adults, the impact of which may be influenced by habitual EPA and DHA intake.

Chapter 1

Literature review; the effects of whole-diet interactions on vascular health, inflammatory and fatty acid status

1 Literature Review

1.1 Ageing

1.1.1 The definition and process of ageing

Ageing is an inevitable process which occurs at many levels (physiological, cellular and molecular), making it difficult to define [1]. Another reason for the difficulty in defining the process of ageing is because it is poorly understood. This has resulted in the formation of many different definitions of ageing, as well as theories on how and why ageing occurs [2]. Mitnitski et al. considered ageing as a process of accumulation of deficits, which were shown to increase with chronological age, which affects different people in different ways [3]. It is clear that ageing is a complex process and is influenced by genetic factors, along with chronic exposure to environmental factors, such as nutrition. However, in general, it can be said that the process of ageing involves an overall decline in function and health, along with an increase in likelihood of disease and disability.

Inflammation is a component of the innate immune response. It is a 'normal' physiological process which helps restore homeostasis following infection or other forms of tissue damage. However, there is evidence that unresolved inflammation is one of the major processes contributing to ageing. A low-grade chronic inflammatory status can be seen in elderly populations and in ill-health [4]. Chronic inflammation can be defined as inflammation that may have a rapid or slow onset but that can be characterised primarily by its persistence and lack of clear resolution; it occurs when the tissues are unable to overcome the effects of the injuring agent. Chung et al. reviewed the evidence and suggested that a number of major age-related diseases may be related to chronic inflammation, including atherosclerosis, arthritis, dementia, osteoporosis, and cardiovascular disease (CVD) [4].

A number of studies have shown that nutrition can influence inflammation, both positively and negatively. However, many of these studies have focused on specific dietary components which often have subtle effects [5-7]. Data from interventions on the effects of the whole-diet on inflammation and ageing are lacking. It is important that more research is carried out on healthy eating patterns in order to clarify whether an overall healthy diet can reduce the processes of inflammation and ageing, especially with some shift in policy towards food-based rather than nutrient-specific recommendations.

1.1.2 The demographics of ageing

The proportion of older persons has been steadily increasing worldwide for many decades. According to figures from the United Nations (UN), the percentage of older persons (over 60 years) has increased from 8% in 1950 to 12% in 2013, and is expected to reach 21% in 2050 [8]. By 2050, 2 billion older persons are projected to be alive [8]. The current proportion of older persons is particularly high for Europe; persons aged 60 years and over make up 22.9% of the population, while persons over 80 make up 4.5% [8]. These currently high figures and predicted increases are often referred to as the "ageing" or "greying" of Europe which could give rise to a number of issues, such as increases in the costs of providing social and health care, as well as decreases in productivity due to a reduced workforce [9]. Therefore it is important that realistic strategies are identified which could contribute to healthy ageing and reduced age-related medical costs.

1.1.3 Compression of morbidity

The compression of morbidity is a hypothesis which was first conceptualised in 1980 by James Fries who envisioned a "reduction in cumulative lifetime morbidity through primary prevention by postponing the age of onset of morbidity to a greater amount than life expectancy is increased, largely by reducing the lifestyle health risks which cause morbidity and disability" [10]. Compression of morbidity therefore refers to the ability to increase the average age at which people become chronically ill or disabled so that older people are healthier for longer. Due to the increasing proportion of older persons in Europe, it is important that population-wide strategies to increase healthy life years are determined in order to reduce the health-care burden of chronically ill elderly people on society. A number of studies have shown that the time between the two points; morbidity onset and mortality, may be reduced by implementation of a number of lifestyle changes, such as a healthier diet, smoking status, or increased physical activity [11, 12].

The majority of chronic illnesses that cause a loss in healthy life years are diseases that can be strongly influenced by nutrition, such as CVD, hypertension, diabetes, cancer, stroke, musculoskeletal diseases. Previous research has shown that diets high in salt and saturated fat and lacking fibre, fruit and vegetables, adequate vitamins, minerals and particular fatty acids are a major risk factor for many of these age-related chronic illnesses [13-15]. It is thought that changes in nutrition at all stages of life will result in significant health benefits;

the acceleration in the decline of health, as a result of external factors, has shown to be reversible at any age [15].

1.2 Cardiovascular Disease (CVD)

1.2.1 Definitions, risk factors and prevalence

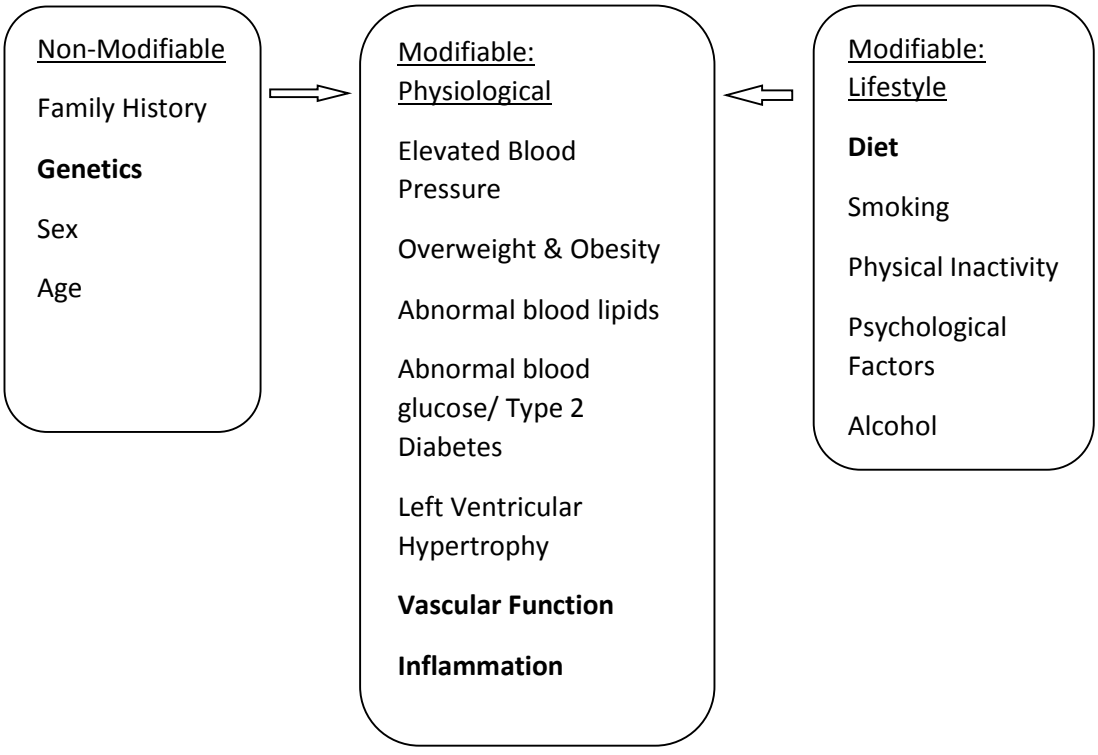
CVD is a class of diseases of the heart and circulatory system (Table 1.1) which includes cerebrovascular disease (stroke), coronary heart disease (CHD) and peripheral vascular disease. CVD was responsible for 17.3 million deaths worldwide in 2008, which was 48% of all non-communicable diseases [16]. Of these 17.3 million deaths, an estimated 7.3 million were due to CHD and 6.2 million were due to stroke [16]. Figure 1.1 describes the various risk factors which can contribute to the development of CVD and their modifiable and non-modifiable determinants. This chapter will focus specifically on vascular function and inflammation, diet and genotype as these were the focus of my PhD programme.

Table 1.1. Definitions of different types of CVD [17]

Term	**Definition**
Atherosclerosis	Artery walls become thicker and harder due to the build-up of fat, cholesterol and other substances forming a plaque
Coronary Heart/Artery Disease	Atherosclerosis of the blood vessels supplying the heart muscle
Cerebrovascular Disease/Stroke	A blood vessel supplying the brain bursts or becomes blocked
Peripheral Arterial Disease	Narrowing of peripheral arteries. Most common in arteries of pelvis and legs
Rheumatic Heart Disease	Damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria
Congenital Heart Disease	Heart or blood vessels near the heart do not develop normally before birth

Deep Vein Thrombosis and Pulmonary Embolism	Blood clots in the leg veins, which can dislodge and move to the heart and lungs
Arrhythmias	An arrhythmia is an abnormal heart rhythm, which can sometimes result in an increase or decrease in heart rate
Heart failure	Occurs when the pumping action of the heart cannot provide enough blood to the rest of the body as it is needed, resulting in fatigue and breathlessness
Ischemic heart disease	A partial blockage of one or more of the coronary arteries can result in a lack of enough oxygenated blood (Symptoms; Angina, Dyspnea). A complete blockage of an artery causes necrosis or a myocardial infarction (MI)

Figure 1.1. Traditional CVD risk factors, modifiable and non-modifiable [13, 16]



1.2.2 The pathology of atherosclerosis

Atherosclerosis is a process that is characterised by the build-up of plaque in the arterial intima and is a key defining pathological feature of CVD. Atherogenesis is thought to occur throughout life, eventually leading to complications such as stroke and myocardial infarction (MI) [18]. Endothelial cells make up the majority of the inner layer of the artery called the tunica intima, and are in direct contact with the blood. The development of an atherosclerotic plaque begins when the endothelial cells are exposed to an unfavourable environment, for example oxidative or inflammatory stress. This can result in the expression of cell adhesion molecules (CAMs) on the surface of the endothelial cells which increases binding of leukocytes to the endothelium. Leukocytes then migrate into the intima, a process which is accelerated by chemoattractant molecules such as monocyte chemoattractant protein-1 (MCP-1). Macrophage Colony Stimulating Factor (M-CSF) is an inflammatory mediator which can be produced by endothelial and smooth muscle cells and can induce the differentiation of monocytes into macrophages. M-CSF may stimulate proliferation of macrophages and modified lipid uptake in the macrophages, leading to the development of foam cells [19]. The foam cell can also become a major source of cytokines, which when secreted can amplify the inflammatory cascade [20]. T lymphocytes are also present in the intima during atheroma formation and can secrete inflammatory cytokines and growth factors which can promote the proliferation and migration of smooth muscle cells (SMCs) from the tunica media to the intima. The SMCs can strengthen the extracellular matrix by producing molecules such as elastin and collagen, which results in advancing the lesion from a fatty streak into a more fibrous lesion [18]. The atherosclerotic plaque now consists of a core containing lipids and macrophages, many of which are dead, surrounded by a fibrous cap. Cardiac and stroke events are often triggered as a result of plaque rupture and subsequent thrombosis due to exposure of the necrotic core of the plaque to the blood. The formation of a thrombus can cause obstruction of the affected artery, resulting in an acute cardiac or cerebral event in addition to atherosclerosis. Inflammation also plays a role in the acute vascular complications by promoting plaque instability [21]. The stability of the plaque depends on the thickness of the fibrous cap which can be disrupted as a result of inflammatory processes, for example infiltration of the cap by macrophage foam cells [22]. This process can be viewed diagrammatically in figure 1.2 (section 1.5). The endothelium and inflammatory processes are therefore potential target areas in the prevention and treatment of CVD.

1.3 Endothelial dysfunction and CVD risk

1.3.1 Endothelial dysfunction

Vascular function is partly determined by the endothelium which is the major source of vasodilators and vasoconstrictors present in the vasculature [23]. Chronic exposure to cardiovascular risk factors, such as hyperlipidaemia and smoking, can overwhelm the vascular endothelium and compromise its integrity, resulting in the initiation of endothelial dysfunction [23]. Endothelial dysfunction is characterised by the loss of sufficient dilation of the arteries in response to an endothelial-derived stimulus [23]. Measuring endothelial function is important because it has been shown to predict cardiovascular events and is a useful prognostic tool which precedes the development of atherosclerosis [24-30].

1.3.2 Nitric Oxide

Endothelial dysfunction can be characterised by reduced bioavailability of Nitric Oxide (NO) [23]. NO is one of the major vasodilators produced by the endothelium and is derived from L-Arginine by endothelial Nitric Oxide Synthase (eNOS). eNOS is activated to produce NO in response to a number of stimuli, an important one being shear stress in the vasculature. NO is an important substance in maintaining, not only vascular tone and elasticity but, overall vascular health. In addition to acting as a vasodilator it also reduces smooth muscle cell migration and growth, monocyte/macrophage adhesion, inflammation and platelet aggregation [31]. For example, a study in which eNOS deficient and non-deficient mice were fed a Western diet, accelerated development of atherosclerosis was seen in the eNOS deficient mice [32]. NO has also been shown to prevent transformation of low density lipoprotein cholesterol (LDL-C) into oxidised LDL-C which is more deleterious in the vasculature as it inactivates eNOS and can be taken up by the macrophages [33]. In addition to the production of NO by eNOS, inducible NOS (iNOS) can also contribute to NO production in the endothelium.

1.3.3 Endothelins

As well as the numerous vasodilators, vasoconstrictors are also important in maintaining vascular homeostasis. For example endothelins (ETs), particularly ET-1 in humans, are vasoconstricting proteins which act as the natural counterpart of the vasodilator NO [34]. There are 3 ET isoforms which are formed by ET-converting enzymes. These 3 isoforms bind to 2 types of receptors; ET receptor subtype A (ETAR) and subtype B (ETBR). ET receptors are

expressed abnormally in diseases associated with vasoconstriction [35]. ET-1 is the most potent vasoconstrictor agent currently identified and has been implicated as an important factor in the development of vascular dysfunction and CVD [34]. ET-1 also plays a role in endothelial dysfunction by modulating the inflammatory responses in macrophages; it activates adhesion molecule expression and regulates release of free radicals [35]. It has been shown that ET-1 is increased in rats on a high-salt diet [36]. Human studies have shown that ET-1 levels can be influenced by dietary change [37, 38].

1.3.4 Clinical measures

Numerous techniques are available to measure various aspects of micro and macro function of the vasculature, including elasticity and stiffness. In relation to the assessment of endothelial dysfunction, Flow Mediated Dilation (FMD) is considered one of the gold standards. This involves the use of ultra-sound imaging and reactive hyperaemia (RH), usually performed on the brachial artery [39]. RH is a transient increase in organ blood flow that occurs following a brief period of ischemia and produces shear stress which stimulates the endothelium induction of NO release. However this method is technically demanding, expensive to set up and there is considerable variation in the methodology used across different studies [40].

Another less onerous non-invasive way of measuring endothelial dysfunction is by using the EndoPAT. It is based on a measurement of Peripheral Arterial Tone (PAT) at the finger via a pneumatic finger bio sensor which measures pulsatile blood volume changes in peripheral arterial beds. PAT is a physiological signal that mirrors changes in autonomic nervous system activity and related vascular events. It examines Reactive Hyperaemia Index (RHI). A study on the assessment of endothelial function by PAT showed that EndoPAT could predict cardiovascular events beyond the Framingham Risk Score [26]. The results showed that the rate of a major adverse cardiac events in patients who tested positive for endothelial dysfunction was 39% vs. compared with 25% in those with normal endothelial function ($p=0.024$). There is evidence which shows that EndoPAT can detect vascular improvements in response to dietary change, both acutely and chronically. For example, an intervention involving supplementation with lycopene for 8 weeks showed a 23% increase in RH-PAT index from baseline (1.45 ± 0.09 vs. 1.79 ± 0.12; $P = 0.032$) in the 15 mg/day group [41]. Given that FMD was not available in Norwich at the initiation of my PhD, the EndoPAT was used to

examine endothelial dysfunction in the University of East Anglia (UEA) subset of Nu-Age participants.

1.3.5 Dietary manipulation of endothelial dysfunction

There is accumulating evidence which demonstrates that diet can modulate endothelial function, as reviewed by Brown et al. and Ceuvas et al. [42, 43]. For example, results of in vitro studies show a consistent beneficial effect of long chain n-3 fatty acids on endothelial function; this effect is thought to be induced by increasing the production and release of NO by an activation of NOS [42, 43]. Results from human intervention studies using n-3 fatty acids (4-5 g/d) have also shown beneficial effects, such as improved vasodilation as assessed by FMD [42, 43]. However, results from these studies have been less consistent, which is most likely due to differences in duration (varying from 3 weeks to 7 months) and subject characteristics (CHD patients, smokers etc.). Consumption of n-3 fatty acids may also benefit endothelial function by a transcriptional level reduction in cell surface expression of adhesion molecules [44].

Various flavonoids and flavonoid-rich foods, such as cocoa, fruit and vegetables, have also been shown to improve endothelial function, possibly due to their effects on plasma concentrations of vascular cell adhesion molecule (VCAM), E-selectin and NO [45-47]. The FLAVURS Study Group showed that a high flavonoid diet improved endothelium-dependent microvascular reactivity ($p = 0.017$), E-selectin ($p = 0.0005$) and VCAM ($p = 0.0468$) in men and NO ($p = 0.0243$) in both men and women [45]. Anti-oxidant vitamin supplementation (vitamins A and E) may also have an beneficial impact on endothelial function, possibly as a result of preserving endothelium-dependent vasodilation during exposure to cardiovascular risk factors [42, 43]. Plantinga et al. showed that supplementation with vitamin C (1g) and vitamin E (400 IU) for 8 weeks has beneficial effects on endothelium-dependent vasodilation, as assessed by FMD ($p < 0.001$) [48]. A recent meta-analysis by Montero et al. concluded that prolonged vitamin E and/or C supplementation could be effective in improving endothelial function in non-obese type-2 diabetes mellitus subjects (standardised mean difference = 1.02, $p < 0.05$) [49]. Folic acid, found in many green leafy vegetables, can have positive effects on the endothelium, possibly by reducing plasma homocysteine levels [42, 43, 50]. A recent meta-analysis indicated that 5 mg/d folic acid for over 4 weeks significantly ($p < 0.005$) improved FMD and lowered homocysteine concentrations in patients with coronary artery disease (CAD) [51]. Dietary nitrate, found in green leafy vegetables and beetroot, may also have beneficial vascular effects due to improving NO availability and preserving and/or

improving endothelial function [52]. As discussed, NO is involved in the inhibition of platelet aggregation, smooth muscle cell proliferation and adhesion of monocytes to endothelial cells. Furthermore, various dietary patterns have also been shown to have an effect on endothelial function, as will be discussed in section 1.8.

1.4 Arterial Stiffness and CVD risk

1.4.1 Arterial Stiffness

Arterial stiffening is the result of complex interactions between stable and dynamic changes of the vessel wall and has been shown to be a strong predictor of CVD events and all-cause mortality [53]. Arterial stiffness occurs as the ratio of elastin to collagen decreases within the intimal medial layer of the endothelium due to hypertension or cholesterol deposition which results in less compliant arteries. This leads to a requirement for increased cardiac muscle contraction, which over time may promote left ventricular hypertrophy and cardiac failure [54]. Cellular changes (involving substances such as NO and ET) and extrinsic factors (such as hormones and salt) are also involved [54]. Ageing and hypertension contribute to increased arterial stiffness; however it can be reduced in response to lifestyle changes such as diet [54]. This project aimed to determine the impact of a one year whole-diet intervention on arterial stiffness in older adults.

1.4.2 Clinical measures

Arterial stiffness can be measured in a number of ways; major methods include Cardio-Ankle Vascular Index (CAVI) and Pulse Wave Velocity (PWV), both of which were utilised as part of this PhD project. PWV measures the pulse transit time from one part of the body to another, with the gold standard being between the carotid to femoral artery. PWV is dependent on the radius of the vessel through which it is measured. Any increase in radius as a result of an increase in vascular tone is counterbalanced by an increase in medial thickness and an intrinsic alteration of the elasticity of the vessel. PWV has been shown to be a predictor of CVD risk and is strongly reproducible. Blacher et al. showed the presence of a PWV >13 m/s appeared as a strong predictor of cardiovascular mortality [55].

CAVI has been developed as a measure of arterial stiffness that is independent of pulse pressure. A number of studies have shown CAVI to be associated with carotid intima media thickness, homocysteine and the presence and severity of coronary atherosclerosis [56, 57]. CAVI is thought to be a particularly useful indicator of arterial stiffness in elderly cohorts because it is independent of blood pressure and is therefore thought to evaluate arterial

stiffness more accurately than PWV in those taking anti-hypertensive medications or those with masked hypertension [58, 59].

1.4.3 Dietary manipulation of arterial stiffness

Evidence from animal studies has shown that a number of nutrients and non-nutrients can play a role in the progression of arterial stiffness. For example, high fat foods, obesity inducing diets (high fat, high sugar) and vitamin D have all been shown to influence arterial stiffness [60-62]. Human epidemiological studies using PWV and CAVI, have also shown that dietary intake influences arterial stiffness. For example, anthocyanins (ACNs), flavones and calcium intakes have been shown to be inversely associated with arterial stiffness, as measured by PWV [63, 64]. A systematic review, including 38 randomised control trials (RCTs), reported that n-3 fatty acids and soya isoflavones were effective in lowering PWV, while there was also limited but consistent evidence that salt restriction and bioactive peptides could also help improve arterial stiffness [65]. As it is a relatively new measure, there is a lack of data on the utilisation of CAVI in dietary interventions. One example shows that increasing levels of EPA (1.8 g administered daily for 3 months) resulted in a decrease in CAVI in those with metabolic syndrome [66]. Isoflavones and plant stanol esters have also been shown to modulate CAVI in certain subgroups of the population [67, 68]. The impact of dietary patterns on arterial stiffness will be discussed in section 1.8.

1.5 Inflammation and CVD risk

1.5.1 The role of inflammation in atherosclerosis

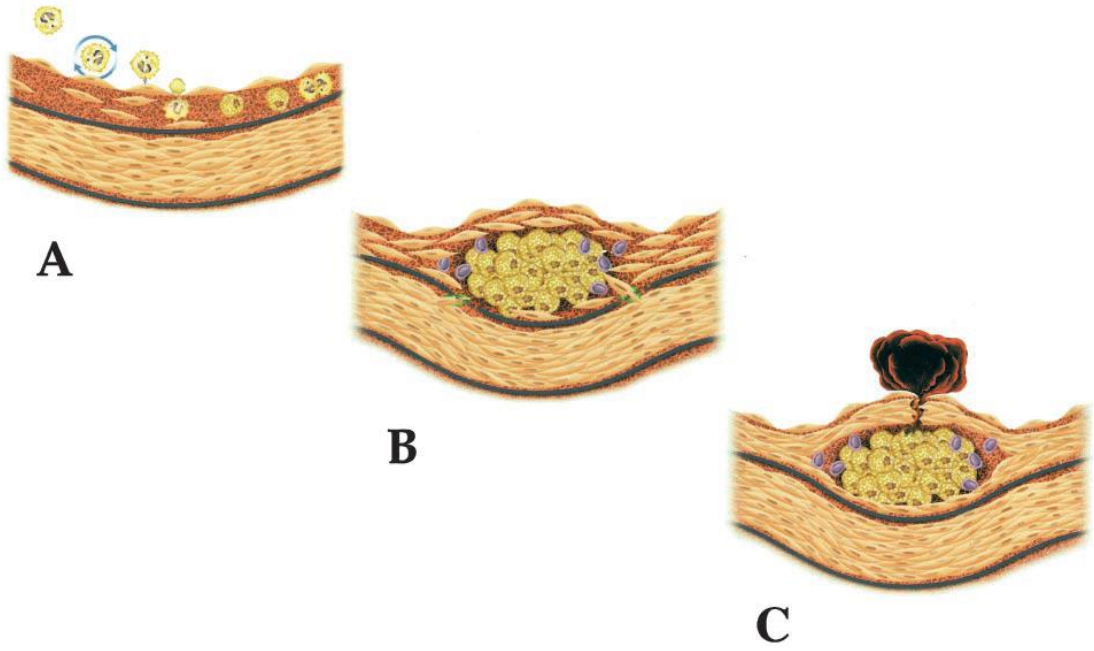
Inflammation is a 'normal' physiological process; however low-grade chronic inflammation can play a major role in the development of atherosclerosis, as noted in section 1.2.2. Inflammation is regulated by a number of processes including T-cell and macrophage function, cytokine production, NO production, C-reactive protein (CRP), eicosanoid, resolvin and protectin production. The role of inflammation in all stages of atherosclerosis is summarised in figure 1.2.

Figure 1.2. Role of inflammation in all stages of atherosclerosis [69]

A. An inflammatory environment in the endothelium causes expression of adhesion molecules that bind ligands on leukocytes. Selectins mediate a rolling interaction while integrins mediate firmer attachment. Chemoattraction of adherent leukocytes directs their migration into the intima.

B. T lymphocytes join macrophages in the intima. Leukocytes and vascular wall cells secrete cytokines and growth factors which can promote migration and proliferation of smooth muscle cells.

C. Alterations in extracellular matrix metabolism thin the fibrous cap, making it weak and susceptible to rupture. When the plaque ruptures the tissue factor triggers the thrombus that causes most acute complications of atherosclerosis.



1.5.2 C-reactive protein as a biomarker for inflammation

CRP is a key inflammatory mediator and an acute phase plasma protein that plays a role in immunity by activating the complement system. During inflammatory states, CRP synthesis is stimulated by IL-6 and other cytokines. As a result, the CRP concentration in plasma increases and is therefore often used as a biomarker for inflammation [70]. Research has focused on the use of the acute phase protein CRP as a biomarker for inflammation, with many studies showing CRP as a potential predictor of heart disease and associated symptoms, such as the metabolic syndrome [71-75]. Albert et al. showed that men in the highest quartile of CRP concentrations had a 2.78 fold higher risk of sudden cardiac death compared with those in the lowest quartile [71]. Sakkinen et al. showed that after 10-15 years follow up, the percentage of men with a MI in the highest quartile for CRP concentrations was 2.5 fold higher than men in the lowest quartile [72]. Ridker et al.

presented data which suggested that CRP is a stronger predictor of cardiovascular events than LDL-C [76] and also made the link, through the use of CRP as a biomarker of inflammation, that the efficacy of statins may be a result of anti-inflammatory effects as well as lipid-lowering effects in the Cholesterol and Recurrent Events trial [77]. Cut-off points have been established with CRP concentrations < 1mg/l meaning low risk and > 3mg/l meaning high risk of future heart attack for individuals [78]. However despite all of this, CRP has not yet been recommended for routine use in clinical practice. The reasons for this were highlighted in a meta-analysis published in 2010 evaluating the quality of research evidence for the association of CRP with fatal and nonfatal events among patients with stable coronary disease, which concluded that due to several types of reporting and publication bias, the strength of any independent association between CRP and prognosis among patients with stable coronary disease remains sufficiently uncertain [79]. Traditionally, CRP has been thought of as a marker of inflammation rather than having a role in the development of CVD. However, research has shown that CRP itself has pro-inflammatory effects and may be a direct cause in the initiation and progression of CVD through a number of potential mechanisms; for example enhanced production of IL-6 is observed in response to the presence of CRP [80]. The exact function of CRP is not yet fully understood.

Furthermore, it has been shown that CRP can be successfully modulated by dietary intervention, for example increased adherence to the Mediterranean diet has been shown to be associated with lower CRP concentrations in a number of studies [81-84]. Therefore, CRP was assessed as a major outcome measure in the NU-AGE intervention and as part of this PhD to determine the effect of a one year whole-diet intervention in older adults on inflammation.

1.6 n-3 fatty acid status and cardiovascular health

1.6.1 Sources, benefits, recommendations and consumption

n-3 fatty acids are polyunsaturated fatty acids which contain the first double bond at the third carbon atom from the methyl end of the fatty acid. There are three major n-3 fatty acids that play essential roles in the human body namely αLNA, EPA and DHA.

Dietary sources of αLNA are seeds and leaves of certain plants; flaxseed is one of the best sources containing 22.8g/100g raw edible portion [85]. Other sources include soybeans, walnuts, chai seeds and oats [85]. EPA and DHA are consumed as fish, and in particular oily fish, but can also be consumed as supplements derived from the flesh of oily fish or the liver of non-oily fish (such as cod and haddock). Oily fish include mackerel which contains 1.8–

5.3% (by weight) n-3 fatty acids, herring (1.2–3.1% by weight), salmon (1.0–1.4% by weight), tuna (0.5–1.6% by weight), and trout (0.5–1.6% by weight) [85]. Although the best way to increase EPA and DHA in tissue is generally considered to be through increased consumption of fish, bioconversion from αLNA is important in people who do not consume fish. For example, vegetarians and vegans have much lower intakes of EPA and DHA, yet their EPA and DHA status is higher than expected which is thought to be due to increased bioconversion [86].

The potential health benefits associated with consumption of EPA and DHA are numerous. The most studied and accepted health benefit is their association with a reduction in CVD risk. However consumption of EPA and DHA has also been linked to many other diseases, for example, autoimmune diseases such as arthritis, cancer, depression, diabetes, respiratory diseases, gastrointestinal diseases, Alzheimer's disease, as well as psychotic disorders, for example schizophrenia [87]. EPA and DHA intakes have also been linked to normal growth and development, particularly in relation to the brain and visual function, as well as improved immune function, throughout life [88, 89].

The current recommended intakes of EPA plus DHA in the UK is ≥450 mg/day [90]. This recommendation is based on the cardiovascular benefits and can be achieved by consuming two portions of fish per week one of which should be oily. However estimated EPA and DHA consumption in adults aged 19-64 years in the UK is approximately 244mg per day, which is about 50% of the recommended minimal intake [90].

1.6.2 n-3 fatty acids and CVD risk

The benefits of the LC n-3 fatty acids, EPA and DHA, in relation to CVD were first suggested when Bang et al. investigated the composition of the diet consumed by the Inuit population in Greenland over 40 years ago [91]. In comparison to the Danish diet, Inuits had a higher dietary intake of LC-PUFAs, particularly EPA, as a result of regular consumption of seal meat and whale blubber. It was thought that this could explain the difference in morbidity from coronary atherosclerotic disease between the Inuit population of Greenland and the Danish population [91]. The Japanese population have lower rates of heart disease compared with other countries and this has also been linked to the consumption of a diet rich in seafood [92]. As a result, numerous subsequent studies assessing the impact of more modest intakes of fish on CVD risk have been conducted. A meta-analysis of 13 cohort studies (up to September 2003) looking at fish consumption and CHD, included 222,364 individuals with an average follow-up of 11.8 yrs. The results showed that mortality from CHD may be reduced

by eating fish once per week or more and that each 20g/d increase in fish intake was related to a 7% lower risk of CHD mortality (P for trend=0.03) [93].

The epidemiological evidence is also supported by numerous RCTs. Burr et al. performed a study in 1989 to investigate the effects of dietary intervention, including an increase in fatty fish intake, in the secondary prevention of MI in 2033 men (DART Study; diet and reinfarction trial). Men who were advised to eat approximately 300g of oily fish per week or were given fish oil (up to three capsules daily (170 mg EPA and 115 mg DHA per capsule)) had a 29% reduction in all-cause mortality over 2 years compared with those not receiving such advice [94]. Since then numerous RCTs have been conducted. The GISSI-Prevenzione study involved the consumption of a supplement containing 850-882 mg/day of EPA and DHA for up to 3.5 years in 2836 men (control group; 2828) who had suffered from MI within the last 3 months. The results showed a 20% reduction in overall mortality and a 30% reduction in mortality as a result of CVD [95]. The results of the JELIS study were released in 2007, which focused on primary prevention, also showed a reduction in death associated with heart disease. This study was carried out in Japan on 18,645 patients with an average total cholesterol (TC) of 6·5 mmol/l who received either 1800 mg EPA daily with statin or statin only with a follow up of 5 yrs. There was a 19% relative reduction in major coronary events (p=0·011) in the group receiving EPA [96]. Since then, a number of other RCTs have shown beneficial effects of n-3 fatty acids in relation to CVD including the GISSI-Heart failure study (2008), the Alpha-Omega study (2010), Omega (2010), and SU.FOL.OM2 (2010) although the majority of these were underpowered to determine effects on CVD mortality due to low event rates [97-100].

Several systematic reviews have pooled the results of a varying number of RCTs to further demonstrate that long chain n-3 fatty acids have the ability to reduce all-cause mortality and cardiovascular mortality [101-105]. However, it should be noted that there have also been a number of meta-analyses studies in which no beneficial effects of n-3 fatty acid consumption on cardiovascular health outcomes and mortality were reported [106-110]. Reasons for lack of beneficial effects in these studies may be attributed to lack of statistical power due to lower than expected death rates, the inclusion of the DART-2 study (considered methodologically flawed), relatively low EPA and DHA doses, as well as a masking of beneficial effects by current effective medications. However, despite these potential reasons for lack of a beneficial effect of n-3 fatty acids on CVD, the results from these meta-analyses suggest that the beneficial effects of n-3 fatty acid supplementation may not be as large as

was originally considered and, in addition, may be only useful for certain subgroups of the population.

There are a wide range of potential mechanisms through which LC n-3 fatty acids may exert their potential beneficial effects on cardiovascular health as listed in table 1.2.

Table 1.2. Potential mechanisms through which n-3 fatty acids may benefit cardiovascular health

Mechanism	Background
Changes in Triglycerides (TGs) and High Density Lipoprotein cholesterol (HDL-C)	Balk et al. conducted a systematic review which showed that fish oil consumption resulted in a change in TGs of −27 mg/dL, in HDL-C of +1.6 mg/dL [111]
Incorporation into cell membranes	Consumption of n-3 fatty acids results in their incorporation into cell membranes, replacing other fatty acids such as arachidonic acid (AA), which in turn can affect eicosanoid and other vasoactive mediator production, resulting in a less thrombotic situation [112]
Anti-arrhythmic effects	n-3 fatty acids have anti-arrhythmic effects due to their electrophysiological impact when present in cardiomyocytes; they directly affect membrane ion channel currents and decrease excitability [113]. This mechanism may also be responsible for the lowering effect of n-3 fatty acids on resting heart rate [114]
Regulation of Transcription Factors	n-3 fatty acids can regulate various transcription factors and therefore modulate expression of a number of genes, producing various effects [115-117]. For example, n-3 fatty acids are ligands for peroxisome proliferator-activated receptor α and δ which regulate lipid homeostasis and inflammation [115]
Eicosanoid Production	Eicosanoids produced during metabolism of n-3 fatty acids are usually less inflammatory compared with those produced during n-6 metabolism. Some are also known to

	be anti-inflammatory, inflammation resolving and can also inhibit vasoconstriction [118]
Increased Nitric Oxide	n-3 fatty acids may increase endothelial bioavailability of vasoactive substances. This is seen in the case of NO which may be a result of gene regulation or a decrease in NO degradation due to reduced oxidation or increased antioxidant activity [119]
Decreased Oxidative Stress	A decrease in oxidative stress is associated with lowering of triglycerides, therefore the hypolipidemic effect of n-3 fatty acids may also result in a decrease in oxidative stress [120]
Anti-Inflammatory Effects	EPA and DHA metabolites, such as resolvins, have anti-inflammatory and inflammation resolving properties
Improved Endothelial Function (see section 1.3.5)	n-3 fatty acids have been shown to improve endothelial function, however the mechanism through which the n-3 fatty acids exert the benefits on the endothelium remains unknown [121]

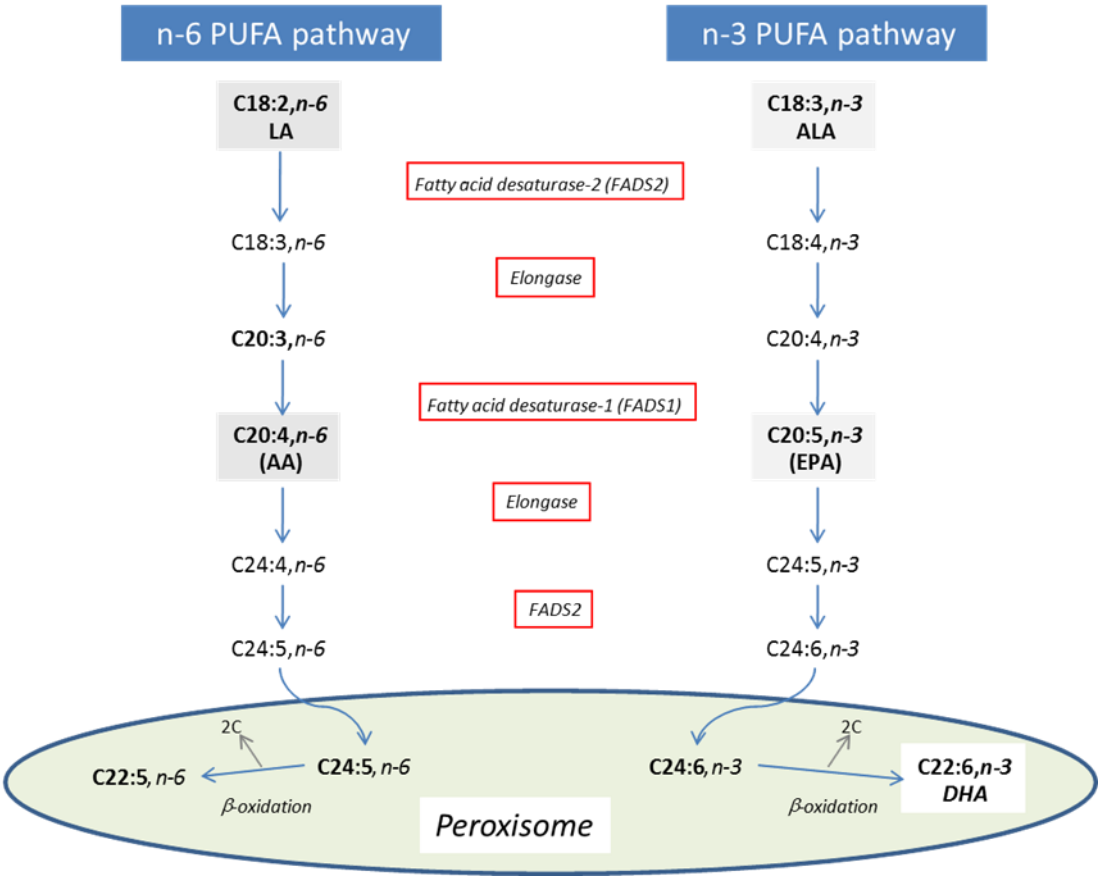
1.6.3 Regulation of LC-PUFA status *in vivo*

As mentioned above, in addition to increased intake, tissue EPA and DHA is also influenced (and in particular in non-fish or fish oil supplement consumers) by the rate of bioconversion from αLNA. Desaturases and elongases are responsible for the conversion of essential fatty acids to long chain PUFAs in humans (Figure 1.3). The delta-5 (D5D) and delta-6 desaturase (D6D) enzymes are the key rate-limiting enzymes in this pathway [122]. The human desaturase complementary DNAs were first cloned in 1999 by Cho et al. [123, 124] and were later identified as *FADS*-1 and *FADS*-2 in the human genome [125]. The *FADS* genes are located in a cluster on chromosome 11 (*11q12*-13.1). D5D and D6D are both found in many human tissues but the liver is the site at which they are most highly expressed [123, 124].

In the n-3 PUFA family, the parent fatty acid is αLNA with the metabolic products of αLNA being EPA, DHA and Docosapentaenoic acid (DPA) as seen in Fig. 1.3 below. This figure shows that LA and αLNA are metabolised by the same series of enzymes. EPA and DHA are produced at limited conversion rates of 0.2-6% for EPA and 0-0.5% for DHA in humans, with the higher rates being in pre-menopausal females rather than males [126]. The higher conversion rates observed in pre-menopausal women is thought to be an evolutionary adaptation so that

younger females have sufficient LC-PUFAs to meet the demands of pregnancy and the developing foetus. As will be discussed in chapter 2, conversion rates could also potentially be influenced by other dietary components, such as flavonoids [127]. Furthermore, the bioconversion rates are also thought to dependent on genetics, as discussed in section 1.7. The impact of the *FADS* genotype on plasma fatty acid status in older adults will be examined in this project (chapter 6).

Figure 1.3. n-6 and n-3 PUFA pathways [128]



Linoleic acid (LA) and α-linolenic acid (αLNA) are desaturated, elongated and β-oxidised by the same enzyme series, including fatty acid desaturase (*FADS*)-1 and *FADS* -2, to form longer chained fatty acids such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

1.7 Impact of common gene variants on LC-PUFA status

1.7.1 *FADS* polymorphisms and the utilisation of haplotypes

As mentioned in section 1.6.3, the *FADS* enzymes are major rate limiting enzymes in the bioconversion of essential fatty acids into longer chained fatty acids. The whole *FADS* gene region appears to be important in terms of its effects on plasma fatty acid status; no

particular SNP has emerged has having a much higher impact on fatty acid status compared with other SNPs. A tag SNP is a SNP in a region of the genome that has high linkage disequilibrium (LD) and can represent multiple genetic variants on one chromosome, known as a haplotype. The selection of tag SNPs and reconstruction of haplotypes allows studies to gain insight into the gene region associated with a particular phenotype without the requirement to analyse every SNP in the gene region. Haplotypes refer to the inheritance of a cluster of SNPs. There are several advantages to the reconstruction of haplotypes, for example the haplotype can represent the biologically functional genetic unit and can therefore provide additional information in relation to association analyses with complex diseases that involve multiple susceptible alleles [129]. Furthermore, haplotype diversity is considered limited, resulting in only a few existing haplotypes and therefore a gain in power for the analyses [129].

1.7.2 The relationship between the *FADS* genotype, fatty acid status and CVD

Numerous studies have shown associations between variations in the *FADS* genotype and fatty acid status in humans [130-135]. In general, carriers of *FADS* minor alleles tend to have increased levels of essential fatty acids, LA and αLNA, as well as decreased levels of longer chained fatty acids, such as AA, EPA and DHA. Various calculations of desaturase activity have also been conducted in the majority of studies and generally tends to be lower for participants carrying the minor alleles. Female carriers of the minor allele have sometimes been considered to be at a disadvantage during pregnancy due to lower levels of EPA and DHA (both of which are particularly important to the foetus and infant) found in blood during pregnancy and in breast milk [135-140]. It has also previously been hypothesised that reduced desaturase activity, resulting in decreased formation of AA, EPA and DHA from LA and αLNA, could lead to an increased risk of atherosclerosis initiation and progression due to inadequate formation of prostaglandin E_1, prostacyclin, lipoxins, resolvins, NO and nitrolipids which are thought to be anti-inflammatory relative to their AA derived metabolites [141].

However, the majority of studies to date suggest that *FADS* minor alleles, associated with decreased desaturase activity, are in fact also significantly associated with reduced inflammation, TC, LDL-C, and CAD risk [131, 142-146]. Martinelli et al. also stated that a higher AA/LA ratio was an independent risk factor for CAD [131]. Mathias et al. recently summarised some of the potential reasons for such findings [147]; for example the ratios of fatty acids entering the fatty acid metabolic pathways through the diet are likely to influence

findings. LA and αLNA compete at the early stages of the biosynthetic pathway and in the last 50 years there has been a dramatic increase in LA levels in the Western diet [147] resulting in a shift in the ratio of LA to αLNA in these pathways and therefore reduced synthesis of n-3 LC-PUFAs. The corresponding increase in n-6 conversion leads to increased levels of AA which is a direct precursor of many pro-inflammatory eicosanoids [147, 148]. Hester et al. recently showed that subjects with the major allele for *FADS* SNP rs174537 had significantly higher levels of pro-inflammatory eicosanoids, LTB4 and 5-HETE, compared with minor allele carriers [148]. However, a few studies reported contradictory results [149-151] which could potentially be due to differences in the n-6 to n-3 PUFA dietary environment or racial differences. Furthermore, these studies each examined either only one or two *FADS* SNPs, whereas many of the larger studies selected numerous *FADS* SNPs. Therefore a comprehensive overview of the association between genetic variation across the entire *FADS* gene locus and fatty acid status is relatively lacking.

1.7.3 The relationship between *FADS* genotype and diet

There have been a number of studies that show that diet composition can influence the relationship between *FADS* genotype and fatty acid and lipid status. In 2012, Hellstrand et al. published an epidemiological study in which 4,635 individuals were genotyped and analysed for rs174547 and cholesterol [152]. The paper reports that the minor allele was associated with lower LDL-C only among individuals in the lowest tertile of n-3 LC-PUFA intakes ($p < 0.001$). A significant interaction between rs174547 and the ratio of αLNA and LA intakes on HDL-C was also observed ($p=0.03$). However another paper published in 2012 by Standl et al. reported that, although carriers of the *FADS* minor alleles had lower cholesterol, LDL-C, HDL-C and higher TG concentrations, the associations between genotype and PUFA intakes did not interact [145]. This study involved 2,006 children and examined the effects of 6 *FADS* SNPs. More recently, a study with a 14 year follow-up examined the impact of rs174546 and fatty acid intakes in 24,032 participants on CVD risk [153]. αLNA -to-LA intake ratio was found to be inversely associated with CVD risk only among participants homozygous for the minor allele. ALA was also inversely associated with ischemic stroke among participants homozygous for the minor allele.

In terms of intervention studies, Gillingham et al. showed that subjects with minor allele variants had decreased desaturase activity but an increase in αLNA intakes resulted in increased plasma EPA beyond that of major allele homozygotes consuming a typical Western diet [154]. This was a randomized crossover trial carried out in 36 hyperlipidemic subjects in

which three diets (diets enriched with flaxseed oil or high-oleic acid canola oil compared with typical Western diet) were consumed for four weeks and 5 *FADS* SNPs were analysed. Cormier et al. conducted a study in 208 subjects involving fish oil supplementation (5g/d containing 1.9-2.2 g/d EPA and 1.1 g/d DHA) for 6 weeks. This study examined the impact of fish oil supplementation and 19 *FADS* SNPs on desaturase activity and reported that gene-diet interactions were potentially responsible for modulating enzyme activities post-supplementation.

In terms of whole-diet interventions, one study to date has examined the interaction of fatty acid genotype and the Mediterranean diet on changes in serum and colonic fatty acids [155]. This was a 6 month intervention study involving 108 participants and the examination of 4 *FADS* SNPs. Following intervention, there was a significant diet by genotype interaction for AA concentrations in the colon; subjects with *FADS* major alleles following the Mediterranean diet had 18% lower AA concentration in the colon than subjects on the control diet (Healthy Eating Diet) suggesting that the Mediterranean diet could be especially favourable for reducing the colon cancer risk, associated with high levels of AA, in the subset of people with the major alleles [155]. There was no significant diet by genotype interaction for serum fatty acids.

Overall, results are inconsistent and it is clear that further research is required to determine the impact of the *FADS* genotype on health outcomes. Further research is also necessary to determine the potential of the diet to modify the relationship between the *FADS* genotype and fatty acid status.

1.8 Dietary compounds which may potentially influence fatty acid status; polyphenols

1.8.1 Background

Previous research has suggested that other dietary components, such as polyphenols, may have an impact on the metabolism of fatty acids in humans, resulting in improved LC-PUFA concentrations in blood and cells. It has been shown that alcohol consumption is positively associated with n-3 fatty acids, EPA and DHA, in human blood cells and plasma [156]. Furthermore, it has also been shown that this association is stronger when alcoholic beverage consumed is wine relative to beer or spirits, and therefore it has been hypothesised that the beneficial effect could be a result of non-alcoholic compounds present in wine, such as polyphenols [156]. More recently, two animal studies have published results which further

suggest that specific dietary polyphenols, known as flavonoids, have the potential to improve plasma fatty acid composition [127, 157].

1.8.2 Polyphenols

Polyphenols are molecules that are secondary metabolites of plants, and are highly abundant in the diet; approximately 8,000 have been identified [158]. They provide colour to plants which aids the attraction of pollinators and also play a role in defence against insects and microbes [158]. Polyphenols are classified into different groups based on the number of phenol rings that they contain, as well as the structural elements that bind these rings to one another. The main polyphenolic groups are the phenolic acids, flavonoids, stilbenes, hydroxybenzoic acids, hydroxycinnamic acids and lignans [158].

As mentioned previously, consumption of wine has been shown to be associated with n-3 fatty acids, EPA and DHA, in human blood cells and plasma [156]. This effect is thought to be due to non-alcoholic components of wine, such as polyphenols. Although white wines tend to be lower in polyphenols, particularly flavonoids, compared with red wines, Champagne wine has been shown to contain relatively high amounts of phenolic compounds [159]. Champagne wine has approximately 19 phenolic compounds including hydroxybenzoic acids (gallic acid, protocatechuic acid), hydroxycinnamic acids (caffeic acid), flavonoids (catechin, epicatechin, quercitin), phenolic alcohols (tyrosol, trans-resveratrol) and phenolic aldehydes (vanillin) [160]. Studies have shown numerous benefits of moderate champagne intakes; neuroprotective effects against oxidative neuronal injury [161], improvements in spatial memory via modulation in hippocampal signalling and protein expression [162], and the promotion of endothelium-independent vascular reactivity [163]. Although we did not have access to blood or tissue samples of animals that had been fed red or white wine, we did have access to a number of previously collected blood and tissue samples of animals that had been fed a control diet, a diet supplemented with champagne wine, and a diet supplemented with alcohol. Therefore, we aimed to investigate the potential effects of champagne, as well as alcohol alone, on fatty acid status in animals.

1.8.3 Flavonoids

Research has particularly been focused on the potential health effects of the flavonoid subclass of polyphenols; flavonoids share a common structure of two aromatic rings connected by three carbon atoms that contain an oxygenated heterocycle ring [158]. Flavonoids make up over two thirds of the polyphenols and the main subclasses of flavonoids are flavonols, flavones, flavan-3-ols, ACNs, flavanones and isoflavones. In general, flavonoids

are found as monomers or polymers in fruits, vegetables and plant based food products such as tea and cocoa [158].

ACNs are naturally occurring water soluble pigments that contain sugar and are responsible for the red and blue colours of many dark plant based foods, for example blackcurrants, blackberries, and blueberries which are major dietary sources of ACNs in the diet, along with red wine [158]. ACNs are glycosides of anthocyanidins. Anthocyanidins are also plant based compounds but differ from ACNs as they are made up of various polyhydroxy or polymethoxy derivates of 2-phenylbenzopyrylium or flavilium salts [164]. It is estimated that 400 different ACNs have been found in nature, such as cyanidins, hirsutidin and malvidin. It is the number of hydroxyl groups, the nature, number and position of sugars attached to the molecule, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule that makes each ACN different [165]. A number of studies have estimated dietary intake which has been reported to vary widely, from 1-215mg/d [166-168]. There is also a large amount of variation in the area of bioavailability of ACNs and although bioavailability of ACNs was originally thought to be low, recent research shows that levels have been underestimated due to the number of metabolites, some of which were not quantified in earlier bioavailability studies [169]. Many studies have been conducted in order to determine health benefits of flavonoids, including ACNs, in relation to CVD, and evidence suggests that moderate intake of ACNs may be protective against CVD [47, 164, 170, 171]. However, the mechanisms by which ACNs may produce cardioprotective effects are not fully understood. It has been hypothesised that ACNs and/or their metabolites may be beneficial to health as a result of their indirect antioxidant activity, regulating various signal pathways or gene expression, or perhaps by protecting against damage to DNA [172-174]. As previously mentioned, two animal studies suggested that ACNs have the potential to improve plasma fatty acid composition [127, 157], which may be a mechanism which partly explains their reported health benefits. However the impact on tissue fatty acid status has not been investigated. In my thesis, I investigated the effect of ACNs, as well as blueberry and elderberry supplementation (rich sources of ACNs) on plasma/serum and tissue fatty acid status.

Flavan-3-ols, another subgroup of the flavonoids, are derived from flavans and exist in both monomer (catechins) and polymer (pro-anthocyanidin) forms [158]. Flavan-3-ols are abundant in teas, cocoa, fruits, vegetables and red wine [158]. Flavan-3-ols are thought to make up over 80% of total flavonoid intakes but due to low absorption and rapid elimination, plasma concentrations rarely exceed 1 μmol/l [158, 167]. Research suggests that flavan-3-

ols may also play an important role in human health, particularly in relation to cardiovascular health which may, for example, be the result of effects on vascular function and inflammation [175]. The European Food Safety Authority (EFSA) have approved health claims that cocoa products (containing >200mg flavan-3-ols per daily serving) “help maintain elasticity of blood vessels, which contributes to normal blood flow” [176]. Although limited research has suggested that ACNs have the potential to alter plasma fatty acids, there is no research on the potential of flavan-3-ols to have such an effect [127, 157]. Therefore, we aimed to investigate the impact of both ACN and flavanol supplementation on fatty acid composition for the first time.

1.9 Dietary patterns and the use of diet scores

1.9.1 Whole-diet interactions and cardiovascular health

Research from epidemiological studies, RCTs and meta-analysis has shown that dietary components such as fatty acids and flavonoids, can influence health outcomes. However, research has also shown that the alteration of dietary patterns may be much more effective than intervening with individual components at improving health outcomes [177-181]. Whole dietary interventions may be a more beneficial approach due to the multiple potential bioactives which can have additive and/or synergistic effects when consumed in combination [182-184].

In the last two decades research has focused on the Mediterranean diet and has shown it be effective in improving many different health outcomes, particularly those related to cardiovascular health. Kesse-Guyot et al. conducted a 6 year prospective study on 3232 participants which showed that adherence to the Mediterranean diet was inversely associated with systolic blood pressure (SBP), triglycerides (TGs) and positively associated with HDL-C [185]. Centritto et al. analysed 7646 healthy participants in an Italian cross-sectional study and identified three dietary patterns [186]. The "Olive Oil and Vegetables" pattern, characterized by high intake of olive oil, vegetables, legumes, soups, fruits and fish, was associated with lower glucose, lipids, CRP, blood pressure and CVD risk score. Other epidemiological studies have shown that the Mediterranean diet is positively associated with n-3 fatty acids and inversely associated with saturated fatty acids [187, 188]. It is also associated with lower CRP concentrations across all age groups, including older adults [189-191], an improved lipid profile [186, 192, 193], and improved fatty acid profile in older adults. Furthermore, a number of intervention studies have also shown the

Mediterranean diet to be beneficial. The PREDIMED study was a primary prevention trial conducted in Spain that looked at the effects of a low fat diet, a Mediterranean diet supplemented with extra virgin olive oil, and a Mediterranean diet supplemented with nuts on a wide range of outcomes. 7,447 subjects, aged between 55 and 80 years, were recruited and followed up every year for 4 years. All 3 diet groups exerted beneficial effects on blood pressure overall, while participants allocated to either of the two Mediterranean diets had significantly lower diastolic blood pressure (DBP) than the participants in the control group [194]. The PREDIMED Mediterranean diet supplemented with extra-virgin olive oil or nuts also had reduced incidence of major cardiovascular events, reduced peripheral arterial disease (PAD) incidence, improved plasma NO and improved fatty acid status [178, 195-197]. Participants consuming the Mediterranean diet with olive oil were shown to have significant increases in palmitic acid (PA) and oleic acid (OA), with decreases in margaric, stearic and LA. Participants consuming the Mediterranean diet plus nuts had significantly higher PA, LA and αLNA, and significantly lower myristic, margaric, palmitoleic and dihommo-γ-linoleic acid [197]. This study also showed that these changes were beneficially associated with the incidence, reversion and prevalence of the metabolic syndrome.

The DASH (dietary approaches to stop hypertension) diet promotes a diet rich in fruits, vegetables, wholegrains, fish and low-fat dairy foods and aims to reduce red meat intakes, salt, sugar, saturated and total fat. The DASH diet has been shown to lower blood pressure in both short and long term feeding studies that looked at the DASH diet alone, the DASH diet and varying intakes of dietary sodium, and the DASH diet in combination with other lifestyle modifications [198-201]. A four month dietary intervention in overweight or obese subjects (n=144, mean age of 52 years), with untreated hypertension, examined the effects of the DASH diet alone and the DASH diet with weight management against a control group (requested to continue with their usual diet) on PWV [200]. Both of the DASH diet interventions resulted in lower PWV compared with the control group ($p= 0.001$), and PWV was significantly lower in the DASH diet combined with weight loss management compared with the DASH diet alone group ($p= 0.045$). A recent systematic review and meta-analysis (including 1917 participants) has reported that the DASH diet significantly reduces blood pressure, TC and LDL-C, particularly in those with an increased cardio-metabolic risk [202].

The Nordic diet also appears to have beneficial effects on cardiovascular health outcomes. The Nordic diet consists of foods that are traditional in Nordic countries, for example fruits (berries, apples and pears), vegetables, legumes, low-fat dairy products and fatty fish, as well

as LDL-C-lowering foods (oats, barley, soy protein) [203]. The Nordic diet has been shown to significantly improve the blood lipid profile, inflammation, insulin sensitivity and blood pressure [203, 204]. The Nordic diet can also result in weight loss and blood pressure reduction in centrally obese adults [205]. An index based on traditional healthy Nordic foods was found to be related to lower mortality among middle-aged Danes, particularly among men [206]. An intervention involving consumption of the Nordic diet for 6 weeks in older adults resulted in decreases in a number of saturated fatty acids and an increase in serum DHA [207]. However, more research is still required to determine the health effects of the Nordic diet as intervention studies are limited and short compared with those investigating the Mediterranean diet.

Overall, there is substantial evidence of the benefits of the whole-diet approach. However, there is no research on the impact of a whole-diet intervention on clinical measures of endothelial function and arterial stiffness in older adults. We aimed to determine the impact of a one year whole-diet intervention, tailored specifically for older adults, on clinical and biochemical measures of vascular function and inflammation in older adults.

1.9.2 The use of diet scores

The utilisation of diet scores/indices has become a popular approach for assessing whole-diet quality and the impact of whole-diets on health outcomes. Diet scores are generally based on a set of dietary recommendations and can be used to evaluate adherence to a dietary pattern. There is currently a wide variety of diet scores which are commonly used to assess the overall quality of diets, some of which have been based on the dietary patterns previously discussed in section 1.8.1.

A number of scores have been designed based on the Mediterranean diet, including the traditional Mediterranean Diet Score (MDS), an updated Mediterranean score (MED) and the Mediterranean style-dietary pattern score (MSDPS), with a high score on all indices associated with cardio-metabolic benefits [185]. The original MDS is a score which measures adherence to the Mediterranean diet by assigning a score of either “1” or “0” to different dietary components [181]. These 9 dietary components were considered either beneficial (fruit and nuts, vegetables, legumes, cereal and fish) or detrimental (meat, poultry and dairy products). For beneficial components, people with intakes that were below the median were assigned a value of 0, and people with intakes that were at or above the median were assigned a value of 1 and vice versa for detrimental components. For alcohol, a score of 1 was assigned to men who consumed between 10 and 50g per day and to women who

consumed between 5 and 25g per day. Therefore the score ranged from 0 to 9, with a score of 9 reflecting maximum adherence to the Mediterranean diet. This diet score has been used in a number of studies. For example, a prospective study involving 22,043 adults, 44 months follow-up (median) and 275 deaths, reported that increased adherence to the Mediterranean diet (2-point increment), as assessed by the Mediterranean diet score, was significantly associated with a reduction in total mortality (hazard ratio (HR): 0.75), death due to CHD (HR: 0.67), and death due to cancer (HR: 0.76) [181].

A diet score has also been created to assess effects of the DASH diet on health outcomes [208]. This score consists of 0-1 ratings. There are several versions of the DASH diet as it has evolved over time; the diet score food groups have included fruits, vegetables, nuts and legumes, low-fat dairy, whole grains, sodium, red and processed meats etc. For example, if 4-5 servings of vegetables are recommended per day; 1= ≥4 servings/day, 0.5= 2-3 servings/day, 0= <2servings/day. Scoring is reversed for food groups where lower intake is recommended. As for alcohol, no score is given and the percentage of people who drink in each group was accounted for. This diet score has been utilised in a study involving 36019 women aged 48-83 years, and results showed that women in the top quartile of the DASH Diet Score had a 37% lower rate of heart failure [208].

The Healthy Eating Index (HEI) is another major diet score and is based on the Food Based Dietary Guidelines (FBDGs) given by the United States Department of Agriculture (USDA). The HEI consists of 12 food groups, which are scored between 0-5, 0-10 or 0-20 points depending on food group. Calories coming from the food group titled “Solid Fat, Alcohol, and Added Sugar” are weighted at least twice as heavily as other food groups. The score for this group can be between 0 and 20. A zero score will be given if ≥50% of energy comes from this food group, while a score of 20 will be given if ≤20% of energy comes from the group. One study shows that men and women with the highest score, and therefore most compliant with the USDA guidelines, have a 23% lower risk of suffering from CHD, and a 16% lower risk of major chronic disease [209].

A diet score for the elderly has previously been designed; the Elderly Diet Index (EDI) [210]. The EDI made up of 10 dietary components, based on a combination of both the modified MyPyramid for Older Adults and the Mediterranean Diet. The validation of the EDI was conducted using data from 668 elderly participants of the MEDIS study. The EDI was designed using food frequency questionnaire (FFQ) data and uses a scoring system ranging from 0 to 4. This study has shown that an increased EDI is associated with lower odds of CVD risk

factors, such as obesity and hypertension, but as of yet this score has not been used in another study.

The diet scores mentioned have proved useful in the examination of population-wide dietary patterns. A diet score specific to the NU-AGE diet recommendations for the elderly could be used in NU-AGE studies to assess adherence to the intervention, and could subsequently be used to assess habitual diet quality in future observational studies and RCTs in older adult populations, and in particular within the European population. Although a diet score designed specifically for the elderly has previously been published, the NU-AGE diet score will be based on substantially different diet goals and will also aim to overcome some of the limitations of the previous score [210]. For example, the NU-AGE diet score will be designed with more dietary components including wholegrains, nuts, eggs, fluid and cheese, many of which are of particular importance to older adults. The NU-AGE diet score will also be suitable for use with both FFQ and food diary data and is designed with a wider ranged scoring system (a range of 0-10) compared with the EDI (0-4) which may result in the NU-AGE diet score being more sensitive.

1.10 Summary, hypothesis and objectives

1.10.1 Summary

Persons aged over 60 now make up 22.9% of the European population, while persons over 80 make up 4.5% [8]. To achieve a compression of morbidity, it is important that realistic dietary strategies are identified that will contribute to healthy ageing. As would be expected, research has shown that whole-diet interventions may be more effective than intervening with individual dietary components at improving health outcomes with foods, nutrients and non-nutrients having additive or perhaps even synergistic effects when consumed in combination [177-184]. For example, the Mediterranean diet, the DASH diet and the Nordic diet have all been shown to improve numerous cardiovascular related outcomes, including major cardiovascular events [194, 202, 203]. Diet scores/indices are a useful approach to analysing diet quality in whole-diet interactions studies.

Low grade chronic inflammation, which can be influenced by diet, is thought to be one of the major influences on the ageing process and it plays a significant role in the pathology and progression of many age-related diseases, for example CVD which causes more than half of all deaths in Europe [211]. Vascular function, which can also be modulated by dietary compounds, is an early indicator of CVD and therefore an important factor to consider in the

ageing process. Vascular function can be analysed by a number of clinical and biochemical measures related to both endothelial function (EndoPAT) and arterial stiffness (PWV and CAVI). However, research on the impact of dietary patterns and whole-diet interventions on these measures is limited, particularly in older adults. No study has specifically examined the impact of a one year whole-diet intervention (tailored specifically for older adults) on clinical, as well as biochemical, measures of both endothelial function and arterial stiffness in 65-79 year olds.

An optimised EPA and DHA status is considered beneficial to ageing in general and in particular in relation to cardiovascular health outcomes. However at a population level, intakes are sub-optimal, with an average EPA plus DHA intake of 244mg per day in UK adults, which is about 50% of the recommended minimal intake [90]. Although increasing EPA and DHA intake is the most effective approach to increasing EPA and DHA plasma and tissue status, EPA and DHA status can potentially be increased by a positive impact of dietary components on the biosynthesis of EPA and DHA from its precursor αLNA. For example, there is an association between moderate wine consumption and n-3 fatty acids, EPA and DHA, in human blood cells and plasma [156]. Two studies in animals have reported results which further suggest that flavonoids have the potential to increase fatty acid bioconversion and improve plasma fatty acid composition [127, 157]. However neither of these studies examined the impact on tissue fatty acid status. Furthermore, no human intervention studies have examined the hypothesis that dietary compounds, such as ACNs, can influence EPA and DHA plasma status, and therefore further research is required.

Fatty acid status can also be influenced by variability in key regulatory genes in the αLNA to EPA and DHA desaturation and elongation pathway. Polymorphisms in the *FADS* 1 and *FADS* 2 gene region have been shown to influence fatty acid status. However, research findings are inconsistent, with little investigation in older adults and little knowledge available on the impact of *FADS* genotype on health outcomes. Further research is also necessary to determine the potential of the diet to modify the relationship between the *FADS* genotype and fatty acid status.

Hypotheses and objectives

- **Hypothesis;** a year-long whole-diet intervention slows the progression of endothelial dysfunction and arterial stiffness, and improves inflammatory and fatty acid status in older adults.
- **Objective;** to examine the impact of a dietary intervention (including advice EPA and DHA intakes) on chronic low grade inflammation and cardiovascular health. NU-AGE (EU FP7) is a five centre trial involving 1,250 older adults (study design described in chapter 3). The objective in the current thesis is to examine the effects of intervention on plasma fatty acid status and vascular function, a major determinant of CVD risk associated with inflammatory status, in Norwich participants. Clinical measures utilised to assess vascular function included EndoPAT, PWV and CAVI. Biochemical measures included the analysis of the lipid and fatty acid profile, CRP, ET-1 and nitrite. A diet score based on the NU-AGE diet (specific to the elderly) was created in order to assess diet quality and to determine if the NU-AGE diet score was associated with vascular function and inflammatory status (as described in chapter 4).

- **Hypothesis**; EPA and DHA status may potentially be altered by specific dietary compounds.
- **Objective**; to investigate impact of ACNs, flavonols, blueberries, champagne and alcohol on fatty acid status. Samples from a number of already completed human and rodent studies were analysed (as described in chapter 2). The rodent studies also involved the examination of the various interventions on fatty acid status in tissues including the liver, cortex, muscle and heart.

- **Hypothesis;** *FADS* gene variants can impact plasma fatty acid status and vascular function in older adults and the NU-AGE diet can influence the relationship between the *FADS* genotype and fatty acid status.
- **Objective;** to investigate the impact of the *FADS* genotype on plasma fatty acid status, as well as the impact on vascular function, of older adults. Fatty acid absolute intake and ratios may determine whether the impact of the *FADS* genotype has a positive or negative effect on health; a diet high with a lower n-6 to n-3 ratio may

help overcome the negative impact. Although the NU-AGE diet was not designed with a focus on n-6 to n-3 ratios, the NU-AGE diet has a strong Mediterranean diet influence (lower in n-6 fatty acids compared with the typical Western diet [155]). Recommendations for participants to consume oily fish (0-2.2: 1 n-6: n-3 ratio) and a spread (4: 1 n-6: n-3 ratio) should help optimise n-6: n-3 ratios which are thought to be as high as 17: 1 in the Western diet [212]. The impact of the NU-AGE intervention on the relationship between the *FADS* genotype and fatty acid plasma status was therefore investigated. (Chapter 6)

Chapter 2

The effect of select dietary compounds on blood and tissue levels of fatty acids in ageing humans and animals

2 The effect of select dietary compounds on blood and tissue levels of fatty acids in ageing humans and animals

2.1 Introduction

The essential fatty acid, αLNA can be converted in the body by FADS enzymes to longer chained fatty acids, such as EPA, DHA and DPA as discussed in Chapter 1. The most efficient means of increasing tissue EPA and DHA in the body is through oily fish consumption, but for vegetarians/vegans/non fish-eaters biosynthesis is the only source. Although bioconversion efficiency is poor (<5% for EPA and < 1% for DHA), these populations have low but stable levels of EPA and DHA [213] as a result of the endogenous production of these fatty acids. An exception to these typically low biosynthetic rates is pre-menopausal women who have higher conversion rates compared with men of the same age [128]. It has been suggested that this increased capacity for EPA and DHA synthesis is in order to have reserves of these fatty acids to meet increased requirements during potential pregnancies; DHA in particular is extremely important during foetal development as it forms part of the central nervous and visual system structures [214]. Specifically, DHA increases membrane fluidity which improves neurogenesis, synaptogenesis and the activity of retinal photoreceptors [214].

Habitual EPA and DHA intakes (244mg/d for UK adults) are below the minimum recommended intakes of 500mg/d with low fish intake attributed to the accessibility, affordability and palatability of fish products [215]. Considering the many beneficial effects of EPA and DHA in human health (as discussed in Chapter 1), the current inadequate oily fish consumption, and ever depleting fish stocks, it is important that we seek new ways of improving the population's EPA and DHA status. Stimulation of endogenous pathways of EPA and DHA synthesis from αLNA could provide an additional approach to ensure that EPA and DHA status in the general population is adequate.

Polyphenols are molecules that are secondary metabolites of plants, and are highly abundant in the diet; approximately 8,000 have been identified with flavonoids making up two thirds of these [158]. Many types of studies have been conducted to determine health benefits of flavonoids, including ACN, in relation to CVD, and evidence suggests that moderate intake of ACNs may be protective against CVD [47, 164, 170, 171]. There is also evidence that supplementation of ACNs to the diet can increase EPA and DHA concentrations in plasma. In one particular rodent study, EPA and DHA plasma concentrations significantly increased from 6.1 ± 0.2% to 7.2 ± 0.2% in the animals that were fed an ACN rich diet for 8 weeks compared with those that were not [127]. In another recent study, rats fed an ACN-rich grape-bilberry

juice for 10 weeks had an increased overall percentage PUFA, as well as a decrease in percentage saturated fatty acids (SFA) in plasma. However there were no significant changes in EPA and DHA specifically [216]. Apart from these two rodent interventions, where the study of fatty acid profiles was not a primary outcome, and EPA and DHA was measured in plasma only, no studies to date have investigated whether ACNs can improve EPA and DHA status in mammals.

In the current work, we aimed to examine, for the first time, the hypothesis that flavonoids could improve LC-PUFA status by conducting plasma and tissue fatty acid analysis on samples from rodent and human studies. Potential mechanisms to explain this could involve increased intestinal absorption, or an effect on the expression/activity of the two desaturase enzymes that play a role in the conversion pathway; FADS1 and FADS2. These enzymes, and FADS2 in particular, are rate limiting steps in the conversion process [217] and it has been shown that they can be regulated by diet composition [218]. For example, Cho et al demonstrated that rats that were fed a diet consisting of 10% oil had only 25% of the level of hepatic mRNA for FADS1 and FADS2 compared with rats fed a fat free diet [218]. It is therefore possible that flavonoids could have a stimulating effect on the FADS enzymes.

Many studies have shown that moderate consumption (1-2 drinks per day) of alcohol-containing beverages is associated with reduced risk of CVD [219-221]. However the exact mechanism of how this occurs has not yet been fully elucidated. It has been shown that alcohol-containing beverage consumption increases EPA and DHA in the blood cells and plasma [156]. Furthermore, it has also been shown that this effect is stronger when the type of alcoholic beverage consumed is red wine, and therefore it has been hypothesised that the beneficial effect could be a result of non-alcoholic compounds present in wine such as ACNs [222, 223]. ACNs and their potential beneficial effects on CVD were discussed in chapter 1.

The samples were derived from completed feeding studies whose primary aim was to investigate the effects of flavonoid/ACN rich sources on cardiovascular and cognitive outcomes. The analyses carried out as part of this PhD (which includes two rodent and one human study) involved investigating the potential effects of ACNs, flavan-3-ols, blueberries, champagne and alcohol on plasma fatty acid status to determine their potential impact on the bioconversion of αLNA to EPA and DHA. The inclusion of the study investigating the effects of both champagne and alcohol could add further insight to the relationship between alcohol-containing beverage consumption and increased EPA and DHA concentrations by confirming whether or not alcohol alone has any effect, as well as determining whether a

relationship can be found for alcohol containing beverages other than wine. However as this study was not designed to test our hypothesis it did not include a group that were fed red or white wine. This could be investigated in future work.

In addition to investigating the effects of these compounds on fatty acid status in the plasma, we also examined a range of metabolically active tissues including the liver, brain, muscle and heart to determine potential distribution changes in tissue. The fatty acid profile in tissues reflects not only the dietary fat intakes, but also fatty acid metabolism in vivo [224]. The liver is particularly important as this is where most EPA and DHA synthesis occurs in humans. The brain can be particularly sensitive to changes in DHA levels, while heart and muscular tissue are also sensitive to changes in EPA and DHA concentrations [224]. Liver samples were unavailable for the study that involved feeding animals a blueberry, ACN, flavanol or a control diet. This limitation was overcome by conducting an additional animal trial at UEA, mentioned in section 2.5 (which was not part of my PhD workload). Another unique aspect of our analyses was the examination of the effects of an ACN intervention on plasma fatty acid status in humans for the first time.

2.2 Methods and study designs

In this section, the methods utilised to analyse the fatty acid profile of plasma and tissues are described. This process involved the extraction of lipids from plasma and tissue, the preparation of fatty acid methyl esters and the analysis of samples via gas chromatography. The study designs of both animal studies, as well as the human intervention study, are also described.

2.2.1 Fatty acid analysis of blood and tissue samples

This section describes the materials and methodology used to analyse blood and tissue fatty acid profiles.

The capillary column used for the fatty acid analysis was a BPX70-0.25 (fused silica, 0.25µm film thickness, 30m x 0.22mm SGE) which was purchased from Fisher Scientific (Leicestershire, UK). Tripentadecanoin was used as the internal standard, PUFA No.2 (Animal Source) was used as the standard, and butylated hydroxytoluene (BHT) as an anti-oxidant, all purchased from Sigma-Aldrich (Dorset, UK). Gases were supplied by BOC, UK. Other glassware and reagents were purchased from Fisher and Sigma.

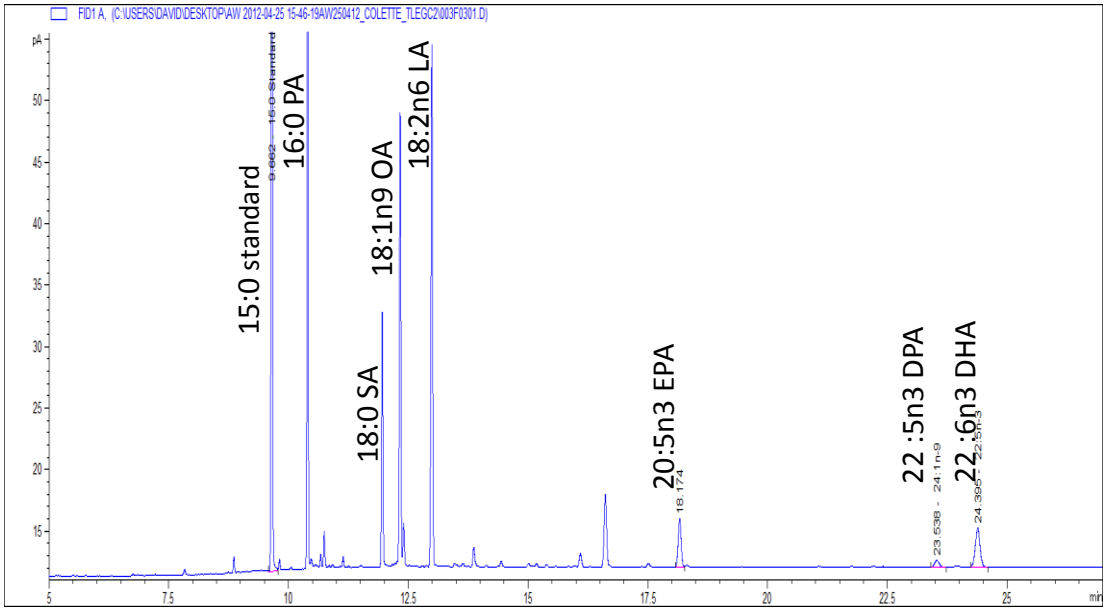
To extract total lipid from plasma samples, samples were vortexed to mix and subsequently centrifuged to remove denatured protein. 800µl of plasma was pipetted into a screw cap glass tube. Tripentadecanoin was used as an internal standard which was dissolved in chloroform: methanol (2:1, v/v) to 1mg/ml. 150µg of this solution was then added to each sample. 5ml of Chloroform: methanol (2:1) containing BHT anti-oxidant (50mg/l) was then added, followed by the addition of 1.0 ml 1M NaCl. The sample was thoroughly mixed by vortexing. The sample was centrifuged at 2,000 rpm for 10 minutes (low brake, room temp) and the lower phase was collected by aspiration into another screw cap glass tube. The sample was dried under Nitrogen at 40 ºC. In the case of total lipid extraction from tissues, approximately 100mg of tissue was cut with a scalpel, stored on ice and weighed. Thawing of sample was avoided. The tissue was then diluted (1 in 10) in 900µl of 0.9% NaCl solution, with the exception of the cortex samples; these were diluted (1 in 20) in 1.9 ml of 0.9% NaCl solution. This is due to the higher fatty acid content of the cortex in comparison to other tissues. This was then homogenised in a glass tube with an Ultraturrax and treated the same as plasma.

Following the extraction of total lipid from either plasma or tissue, fatty acid methyl esters were prepared. 0.5ml of dry toluene was added to the screw cap glass tube and this was vortexed. 1.0 ml of acidified methanol containing 2% (v/v) H_2SO_4 was added to each sample. The samples were heated on a dry block at 50°C for 2 hours and then allowed to cool. 1.0 ml of neutralising solution containing 0.25M $KHCO_3$ (25.03g/l) and 0.5M K_2CO_3 (69.10g/l) was added to each sample. 1.0 ml of dry hexane was added and the sample was mixed using the vortex. The sample was centrifuged at 1,000 rpm for 2 minutes at room temp (low brake, room temp). The upper phase was collected and placed in round bottom glass tube. The sample was dried under Nitrogen at 40 ºC. 150µl of dry hexane was added and the sample was transferred into an autosampler vial.

Prepared samples were then analysed via gas chromatography flame ionisation detection (GC-FID). The column model used was the BPX70 30m x 0.22mm column with 0.25µm film thickness. Agilent GC-FID machines were used to analyse the samples. The initial carrier gas flow rate used was 1.0ml/min and initial temperature used was 115°C, with the maximum temperature set at 250°C. A number of standards were used to identify peaks by their retention time, such as Restek food fatty acid methyl esters (FAMEs), Restek marine FAME, Sigma animal PUFA 2, and Sigma Menhaden oil. The samples were automatically integrated using Chemstation (version B04-02) and each of the fatty acids was identified by retention

times. A chromatogram of the standard is shown in figure 2.1. The area under the curve (AUC) of each peak was used to calculate the percentage of fatty acid in each sample.

Figure 2.1. Example of chromatogram reading from fatty acid analysis



Example chromatogram reading from study 3. PA; Palmitic acid, SA; Stearic acid, OA; Oleic acid, LA; Linoleic acid, AA; Arachidonic acid, EPA; Eicosapentaenoic acid, DPA; Docosapentaenoic acid, DHA; Docosahexaenoic acid.

2.2.2 Animal champagne and alcohol intervention (Study 1)

Serum and liver samples were acquired from a male rat study conducted at Reading University in which 18 month old male rats were fed placebo (Rat and Mouse No.3 pelleted standard diet, provided by Special Diets Services, UK), alcohol (12% ethanol) or champagne (Chardonnay, Pinot Noir and Pinot Meunier, 12% alcohol) diets for 6 weeks [225]. The diets were supplemented in-house and were analytically well characterised and contained a defined amount of phytochemicals, and were, as far as possible, macro- and micro- nutrient matched. They were matched for sugars, glycerol and acids. The rats were given the equivalent of 1 glass of designated treatment per day (125ml/70Kg bw), mixed with the standard diet. Although champagne is low in flavonoids compared with red wine, it has a relatively high overall polyphenol content and may have cardio protective potential, for example by improving NO bioavailability [225]. The phytochemical composition of the champagne has been previously published and has been reported to contain 17 different polyphenolic constituents adding up to approximately 60 mg/l [163]. Fatty acid levels were

analysed from serum and liver tissue samples using GC-FID (as described in section 2.2.1) with the end of treatment samples compared.

2.2.3 Animal flavonoid and blueberry intervention (Study 2)

Serum and tissue samples were obtained from a male rat study carried out at Reading University [226, 227]. The 18 month old male rats were divided into 4 groups, each of which were fed a particular diet for 6 weeks. The 4 diets fed to the rats were as follows; placebo (standard rodent diet with 10% kcal from fat, product no. D12450B, supplied by Research Diets Inc., USA.), 2 % (w/w) blueberry, 320µg ACN extract/g feed or 80 µg flavan-3-ols /g feed (containing 16 µg epicatechin/g feed and 64 µg catechin/g feed)**.** The human equivalent dose (HED) for a person weighing 70kg, using allometric scaling, is as follows; 24g blueberry, 390mg ACN and 98mg flavan-3-ols [228].

The blueberry diet was produced by adding blueberry powder at a level of 2% to the standard diet (AIN-76A purified diet for rodents, Research Diets, USA). The blueberry powder was prepared from whole fresh high bush blueberries (A.G. Axon and Sons) that were blended, freeze-dried and powdered. The blueberry supplemented feed contained approximately 253 µg flavonoids/g feed (179 µg ACNs; 74 µg flavan-3-ols). All diets were iso-caloric and matched macro- and micro- nutrients, notably sugars and vitamin C. This study showed that blueberry supplementation can induce spatial memory improvements [226, 227]. Fatty acid levels were analysed from serum, cortex, heart and muscle tissue samples using GC-FID to determine whether ACN, flavanol or blueberry supplementation could affect levels of n-3 fatty acids. The end of treatment samples were compared.

2.2.4 Human anthocyanin intervention (Study 3)

This UEA based study was a parallel, randomised, placebo-controlled study which was designed to examine the effect of chronic consumption of ACNs on biomarkers of CVD risk and liver and kidney function in healthy postmenopausal women [229]. On average, volunteers were 8 years postmenopausal. The groups were matched for age and body mass index (BMI); with a mean age and BMI of 58.3y and 24.3 kg/m^2 in the placebo group at baseline, and 58.1y and 25.1 kg/m^2 in the intervention group. Twenty six plasma samples were acquired from healthy postmenopausal women who were given either 500 mg/d ACNs as cyanidin glycosides (from elderberry) or placebo for 12 weeks [229]. The elderberry extract was given as 4 capsules per day each containing 125mg of ACN. Placebo capsules were distributed to the control group. Fatty acid levels were analysed from these stored plasma samples (collected pre- and post- intervention following a 12 hour fast) using GC-FID

as described in section 2.2.1 in order to test the hypothesis that ACNs can increase LC-PUFA fatty status.

2.2.5 Statistical Analysis

For fatty acids, results were presented as means ± standard error of the mean (SEM) percentage of total fatty acids. Fatty acid levels in plasma, serum and tissues of humans and animals were assessed for normality and outliers by visual inspection of Normal Q-Q plots. Two-way analysis of variance (ANOVA) was utilsed to test the effects of ACN supplementation on fatty acid status in humans. One-way ANOVA, on end of treatment data, was used to test the effect of interventions on fatty acid status in both animal studies. The differences between means were tested using the Tukey's multiple comparison tests. The statistical analysis and graphs were carried out using GraphPad Prism version 5.0 (Graphpad Software, San Diego, CA, USA). Superscripts not sharing a common letter were significantly different ($P < 0.05$).

2.3 Results

The following section reports the results of the fatty acid analysis for both animal studies, in addition to the results from the human intervention study. Overall, fatty acid levels in plasma, serum and tissues of humans and animals were approximately normally distributed in all studies. Furthermore, several outliers were detected but included in the analysis as they did not significantly affect outcomes, as determined using a sensitivity analysis.

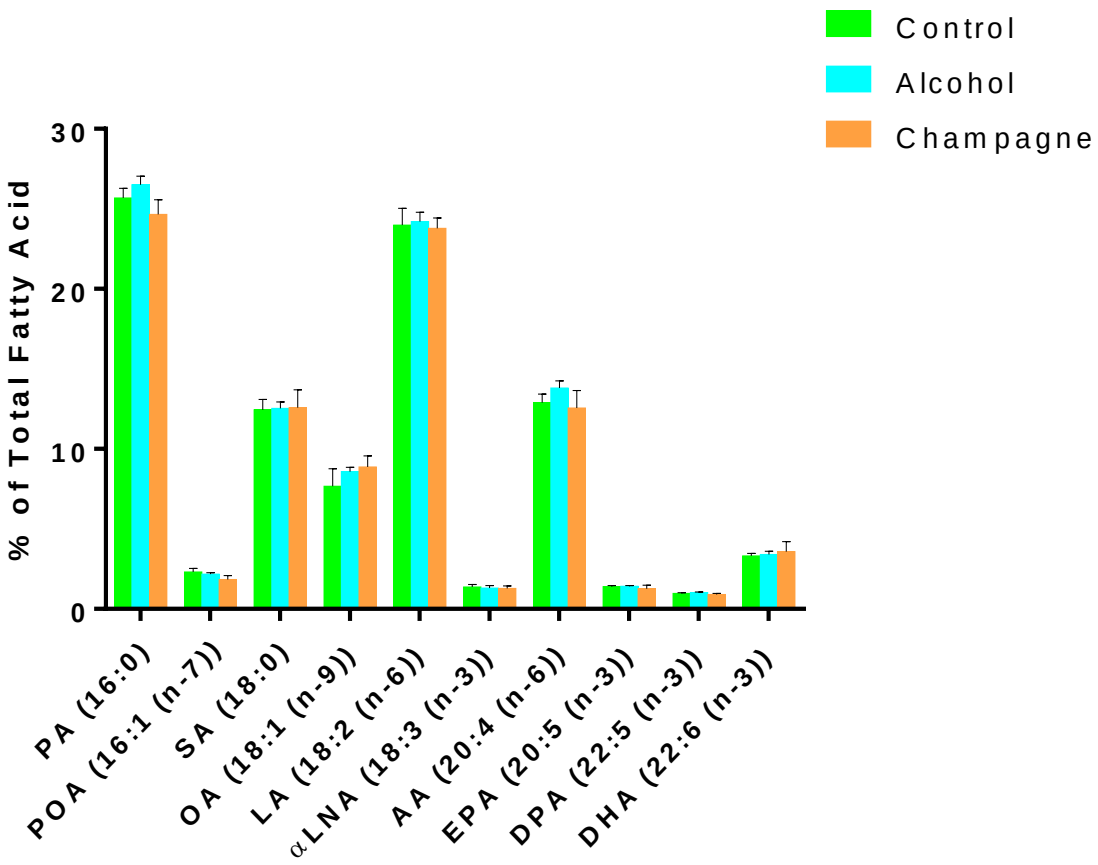
2.3.1 Animal champagne and alcohol intervention (Study 1)

A range of fatty acids were measured in the serum and liver of 18 month old rats (serum n=21, liver n=20). Figure 2.2 shows the relative percentage detected in the serum of rats fed control, alcohol or champagne supplemented diets. As can be seen in the graph, there were no significant differences in levels of fatty acids between the treatment groups ($P>0.05$). PA and LA were the main fatty acids detected in the serum, together making up 49.7 ± 1.7% of the total serum fatty acid content in the control group. Stearic (SA) and AA were also detected in high amounts, 12.5 ± 0.6% and 12.9 ± 0.5% in the control group respectively. The total n-3 fatty acid content was calculated to be 7.0 ± 0.1% in the control group. The champagne and alcohol interventions had no significant effects on these values.

Figure 2.3 shows the effect of the treatments on the fatty acid profile of the liver of these rats. The percentage of PA significantly decreased in the livers of animals given alcohol and champagne, compared with the control group ($P<0.01$). The livers of the control, alcohol and champagne groups contained 30.3 ± 1.1, 26.9 ± 0.8 and 26.2 ± 1.9 % of PA respectively. There

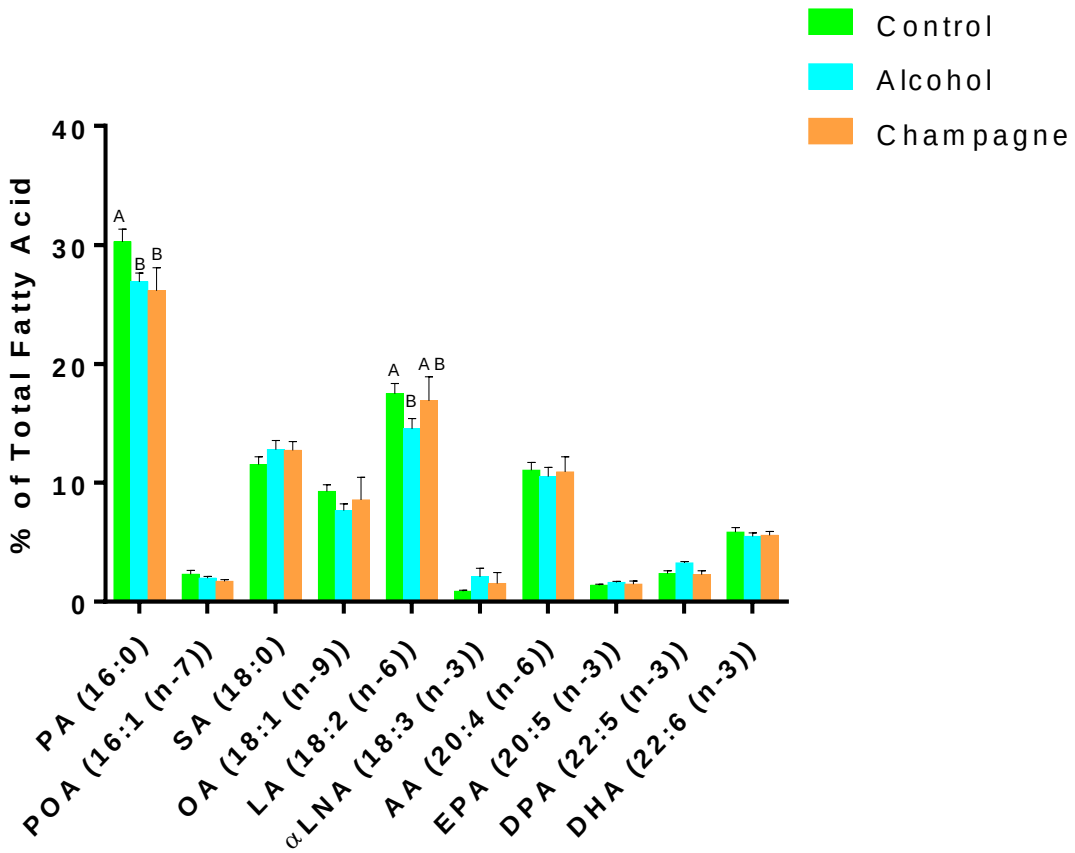
was also a significant decrease from 17.5 ± 0.9 to 14.5 ± 0.9 in the % of LA in the group that were given alcohol compared with a control diet (P<0.05), with no such effect evident in the champagne group. There were no significant differences for any other fatty acids evident (P>0.05).

Figure 2.2. Fatty acids in serum of rats given a placebo, alcohol or champagne intervention



Control group; n=8, Alcohol group; n=8, Champagne group; n=6. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid

Figure 2.3. Fatty acids in liver of rats given a placebo, alcohol or champagne



Control group; n=8, Alcohol group; n=8, Champagne group; n=6. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. Superscripts not sharing a common letter were significantly different ($P < 0.05$).

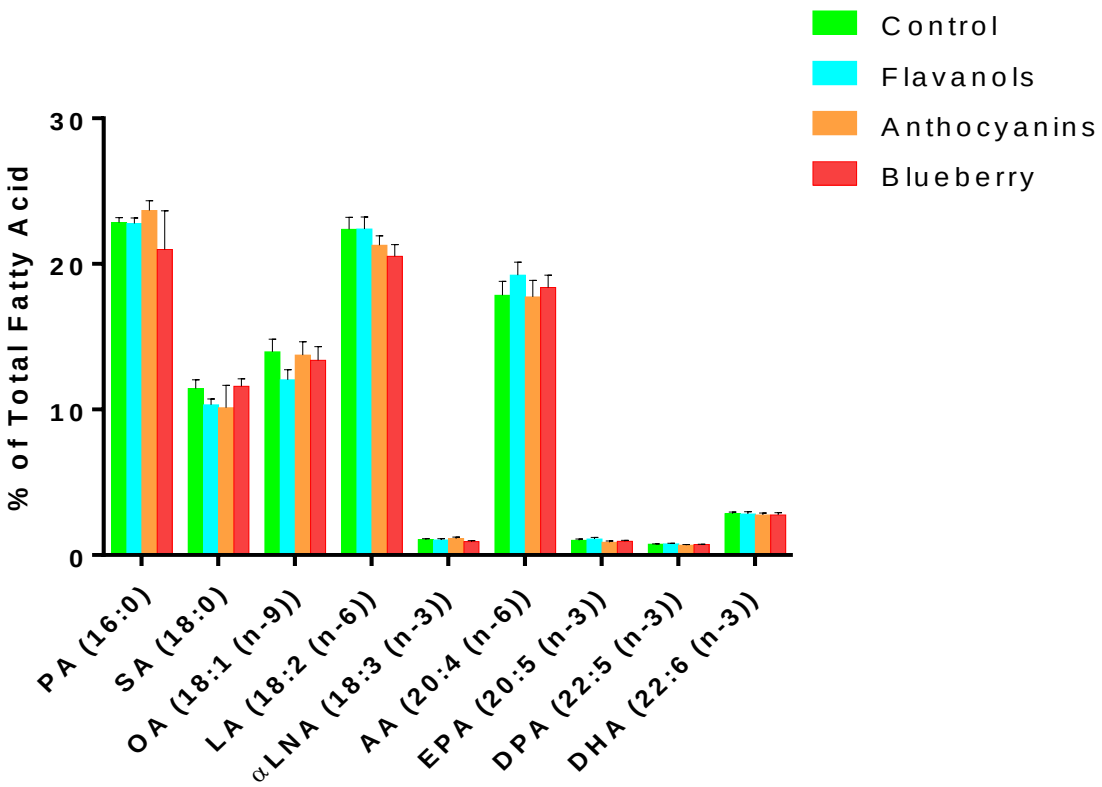
2.3.2 Animal flavonoid and blueberry intervention (Study 2)

The fatty acid profiles were analysed in serum and tissue samples obtained from a study in which 18 month old rats were fed one of four diets. Diet groups were as follows; control, flavan-3-ols, ACNs, or blueberries. There were no significant differences between any of the treatment groups for any fatty acid in the serum (figure 2.4).The brains from these animals were homogenised to allow for extraction of fatty acids. The impact of treatment on the whole brain fatty acid profile is given in in figure 2.5, with no significant differences between any of the treatment groups for any of the fatty acids observed ($P>0.05$). Figure 2.6 shows the average levels of a range of fatty acids for each treatment group for muscle samples. The group of rats that were fed ACNs were shown to have a significant increase in % of OA compared with the control treatment group ($P<0.001$). There were no significant differences for any other fatty acid in the muscle of these rats ($P>0.05$). The distribution of fatty acids in

the heart of the rats can be seen in figure 2.7. The groups of animals fed flavan-3-ols, ACNs or blueberries were found to have significantly higher ($p<0.001$) % of AA in their hearts than those in the control group. The hearts of animals in the control group contained 13.0 ± 2.2 % AA, while the hearts of animals in the flavanol, ACN and blueberry groups contained 16.3 ± 0.9, 17.4 ± 0.5 and 16.3 ± 0.7 respectively. There were no other significant differences between treatment groups for any of the other fatty acids.

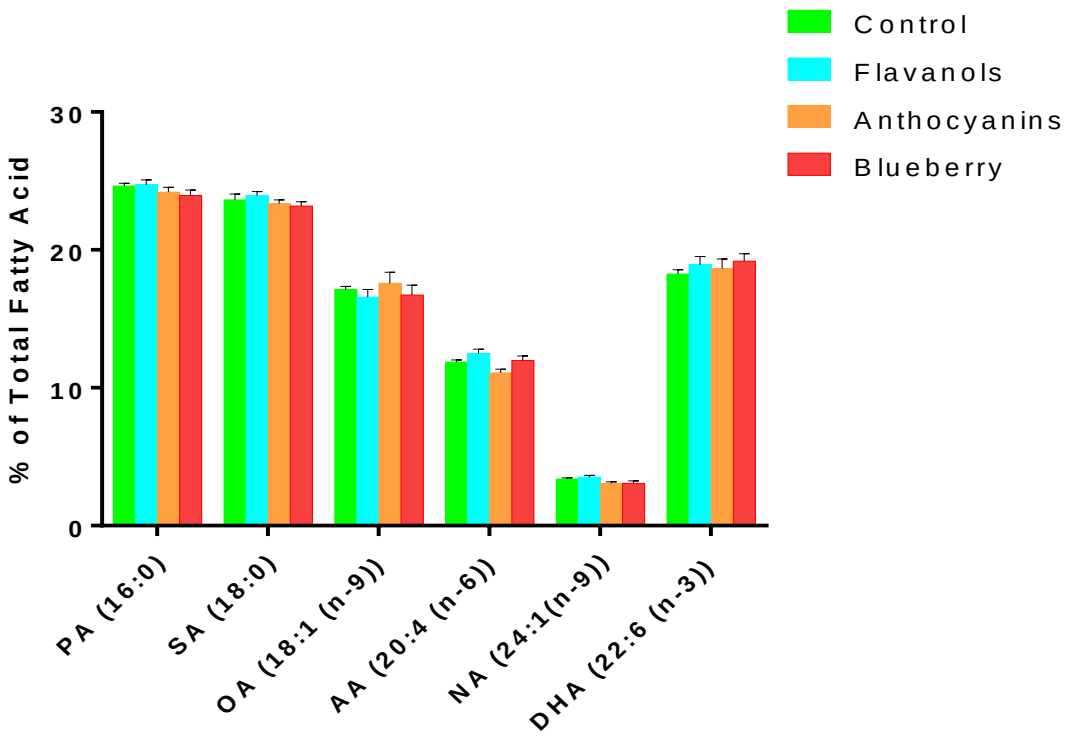
The mean fatty acid % for each tissue across all diet groups is shown in figure 2.8 for comparison. Tissue distribution of AA varied from 5.1 ± 1.0 % in the muscle to 18.3 ± 0.7 % in the serum. The % of EPA were considerably lower; negligible levels were detected in the cortex. The heart contained the highest % of EPA at 1.0 ± 0.2 %, while the serum had a similar % at 1.0 ± 0.1 % of total fatty acids. DHA % in tissues varied widely; DHA made up 2.8 ± 0.1 % of the total fatty acids in the serum and contributed 18.8 % of fatty acids in the cortex.

Figure 2.4. Fatty acids in serum of rats given a placebo, flavan-3-ols, anthocyanins or blueberry



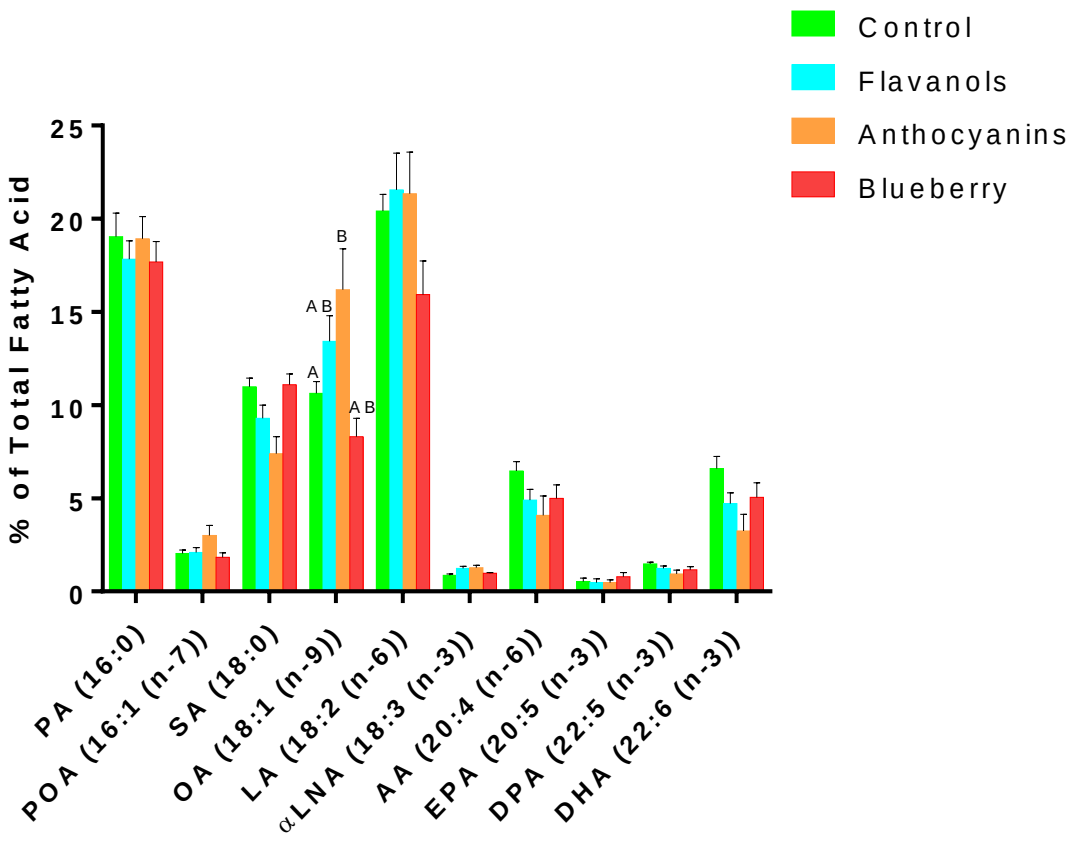
Control group; n=8, Flavanol group; n=8, Anthocyanin group; n=8, Blueberry group; n=9. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid.

Figure 2.5. Fatty acids in cortex of rats given a placebo, flavan-3-ols, anthocyanins or blueberry



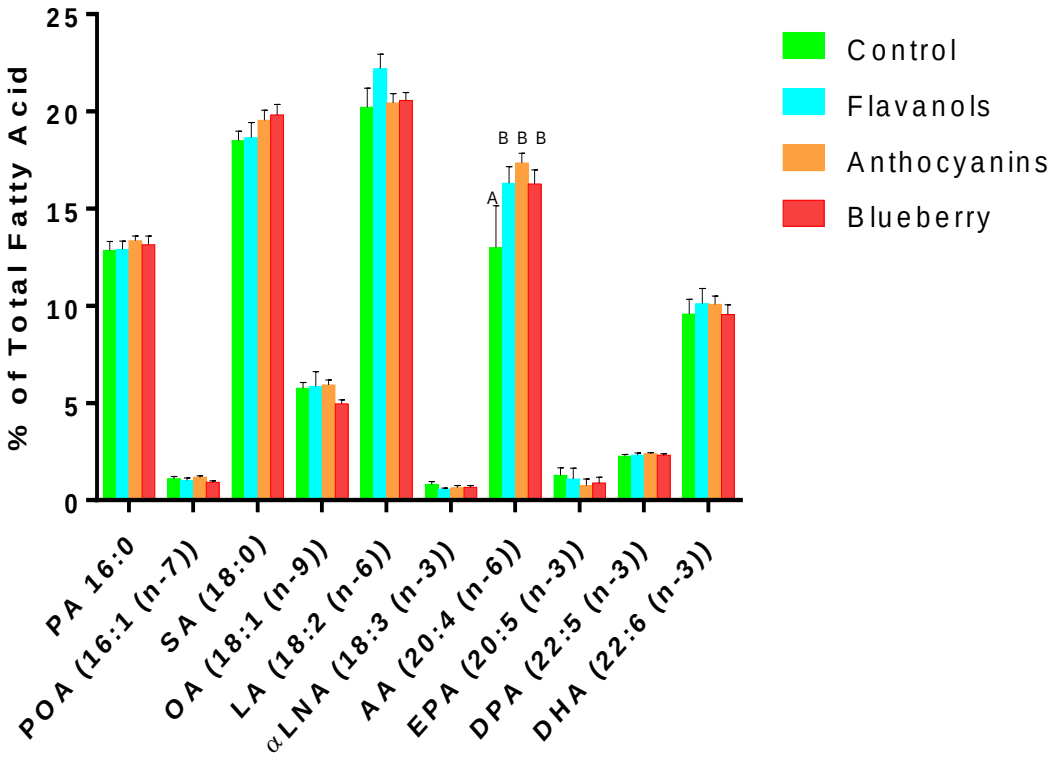
Control group; n=8, Flavanol group; n=8, Anthocyanin group; n=9, Blueberry group; n=9. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid.

Figure 2.6. Fatty acids in muscle of rats given a placebo, flavan-3-ols, anthocyanins or blueberry



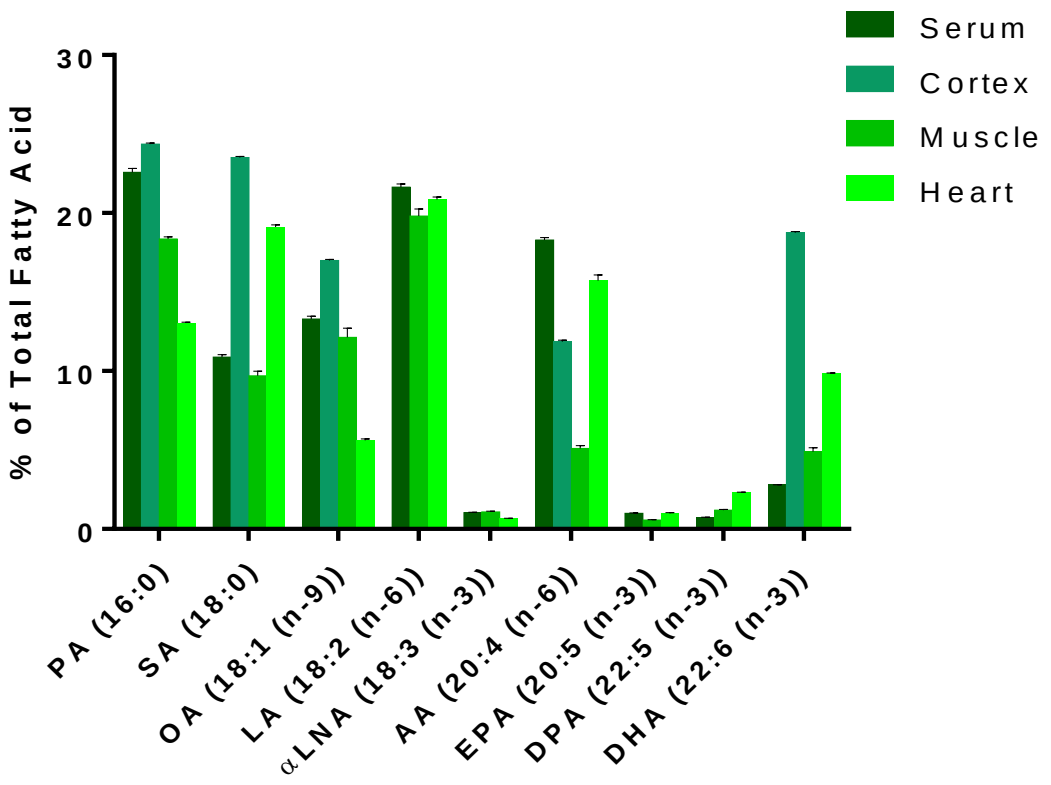
Control group; n=8, Flavanol group; n=8, Anthocyanin group; n=7, Blueberry group; n=8. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. Superscripts not sharing a common letter were significantly different ($P < 0.05$).

Figure 2.7. Fatty acids in heart of rats given a placebo, flavan-3-ols, anthocyanins or blueberry



Control group; n=8, Flavanol group; n=8, Anthocyanin group; n=8, Blueberry group; n=9. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. Superscripts not sharing a common letter were significantly different ($P < 0.05$).

Figure 2.8. Mean fatty acids levels in serum, cortex, muscle and heart of rats

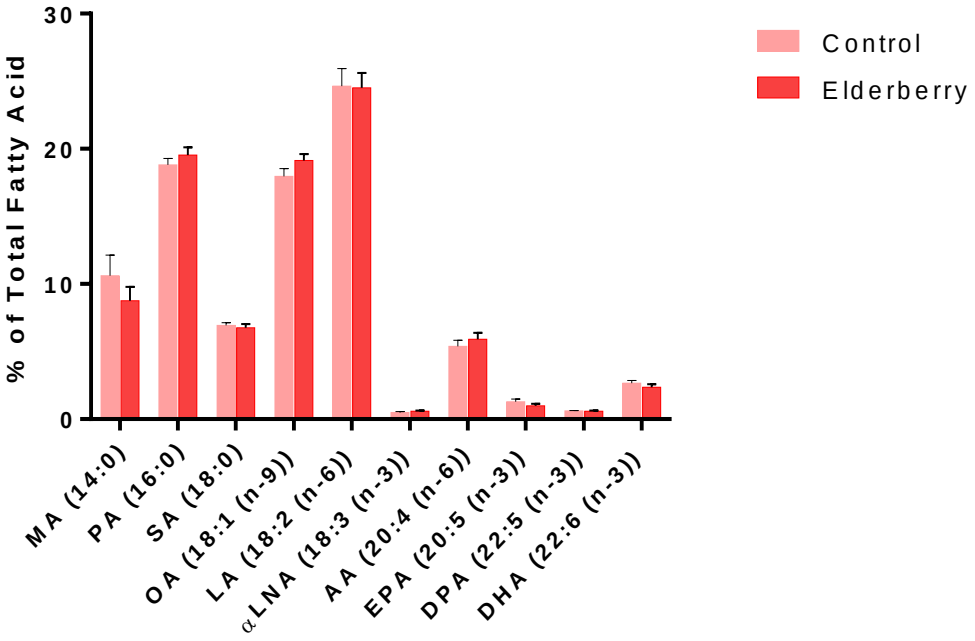


Serum; n=31, Cortex; n=34, Muscle; n=31, Heart; n=33. Data presented as mean ± SEM.

2.3.3 Human anthocyanin intervention (Study 3)

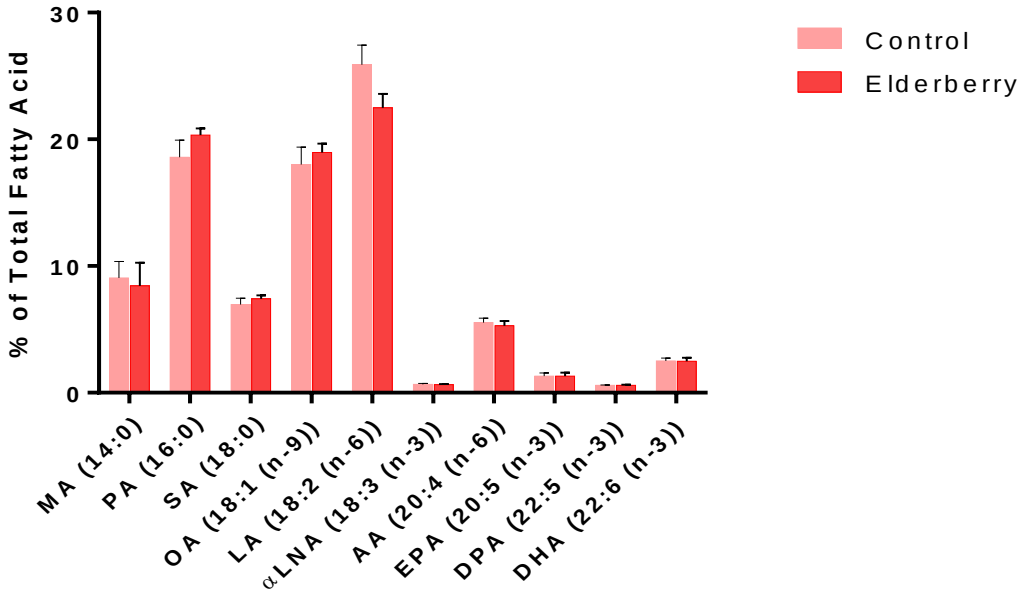
Levels of fatty acids including LA, αLNA, EPA, DPA and DHA were successfully measured in plasma obtained from post-menopausal women. Figure 2.9 shows the control (n=13) and intervention (n=13) groups at baseline. Mean levels of αLNA, EPA and DHA in the control group were 0.5 ± 0.2 %, 1.2 ± 0.6 % and 2.5 ± 0.6 % respectively, and as expected there were no significant differences for any of the fatty acids between both groups. Figures 2.9a and 2.9b shows the comparison between women that were given the treatment of 500mg/d of ACNs and those given a placebo after 12 weeks. 2-way repeated measures ANOVA indicated no significant treatment effect for any of the fatty acids measured ($P>0.05$).

Figure 2.9a. Plasma fatty acids in control and treatment groups at baseline



Control group; n=13, Treatment group; n=13. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid.

Figure 2.9b. Plasma fatty acids in control and treatment groups at follow-up (12 weeks)



Control group; n=13, Treatment group; n=13. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid.

2.4 Discussion

It has recently been suggested that the reduced risk of CVD associated with higher intakes of fruits and vegetables [230] may be in large part attributed to intakes of specific flavonoids [47]. In observational studies, red wine (rich in flavonoids, especially ACNs) consumption has been associated with higher plasma and tissue EPA and DHA levels [156, 231]. Previously, two rodent studies have aimed to examine fatty acid status following consumption of ACN-rich diets but the findings were inconsistent [127, 216]. This project overcame the limitations of these previous studies by investigating the potential effects of not only ACNs but also other flavonoids, champagne polyphenols and alcohol on fatty acid status. We assessed the impact on tissue EPA and DHA status and also response in humans, for the first time. Our results, involving both animal and human studies, demonstrated no impact of ACNs and flavonoids on EPA and DHA profiles and suggest that the positive impact of ACNs and flavonoids on a range of health outcomes is unlikely to be the result of an impact on n-3 PUFA status.

2.4.1 Animal champagne and alcohol intervention (Study 1)

The objective of this study in which aged rats were fed either a control diet or a diet supplemented with alcohol or champagne was to determine if polyphenols present in champagne could have an effect on fatty acid composition of serum and liver samples. A diet supplemented with alcohol was included in this study to account for any effects that the alcohol present in champagne might have. As shown in Figure 2.2, there was no effect of the dietary intervention on the serum fatty acid profile. The serum levels were expressed as the percentage of total fatty acid and the levels detected in this study were within ranges found in other studies [232-235]. There was a significant effect of both alcohol and champagne on levels of PA in the liver. The rats that were fed either alcohol or champagne had significantly lower levels of PA compared with rats on the control diet. The lowering effect of ethanol on levels of PA in the liver has previously been reported [236-239]. A reduction in PA, which may be indicative of reduced hepatic lipogenesis, may be beneficial as increased levels of PA have been shown to increase reactive oxygen species (ROS) products in hepatic mitochondrial cells, reduce insulin sensitivity and increase inflammation and therefore promote injury [240].

There was also a significant decrease in levels of LA in the livers of the animals on the diet supplemented with alcohol compared with those on the control diet. A decrease in LA as a result of alcohol consumption could point to the possibility of increased conversion of

essential fatty acids to longer chained fatty acids, however there was no corresponding increase in AA. There was no significant difference in the fatty acid profile detected between the animals fed the control diet and those fed the diet supplemented with champagne. Ethanol feeding has previously been shown to reduce LA levels in rodents and it has been speculated that this could be the result of enhanced conversion of di-homo linoleic acid, a LA metabolite, to PGE_1 [241-243]. Another possible explanation for a reduction in LA levels in the presence of alcohol consumption could be the result of its requirement in the development of alcoholic liver disease (induced in a rat model) [244]. Furthermore, it has also been shown that reduced LA as a result of alcohol consumption could be related to the formation of cytotoxic fatty acid epoxides as a result of alcohol linked depletion of mitochondrial glutathione [245]. The association of enhanced conversion to PGE_1 and the formation of fatty acid epoxides with ethanol consumption could explain our findings of lower levels of LA without a corresponding increase in LA metabolites such as AA, however this would need to be examined with further research. The fact that there was a significant decrease in LA in ethanol fed rodents, but not in the champagne fed rodents suggests that some component present in champagne, possibly polyphenols, could somehow be ameliorating the effects of alcohol alone.

2.4.2 Animal flavonoid and blueberry intervention (Study 2)

Serum, cortex, heart and muscle samples were analysed to determine fatty acid levels in rats that were fed either a control diet, or a diet supplemented with flavan-3-ols, ACNs or blueberries. We hypothesised that the bioconversion of EFAs would be stimulated and therefore levels of LC-PUFAs would increase following consumption of these interventions. The serum fatty acid profiles from the four dietary groups are shown in figure 2.4, with comparable basal levels detected relative to other studies [234, 235, 240, 246]. No significant impact of intervention on fatty acid status was evident.

DHA levels were higher in the cortex than other tissues analysed, reflecting its important in the brain. DHA is particularly important in the brain, playing a major role in the structure (enhanced membrane fluidity), function (improved plasticity) and perfusion (enhanced cerebral blood flow) of the brain [247]. Levels of fatty acids in the cortex were similar to levels published in other studies in male rats, although levels of DHA were higher than in studies involving aged animals [248-251]. For example, Roy et al. conducted a study on 19 month old rats fed an ad libitum diet and the mean proportion of DHA was 9.7 ± 3.1 % of

total fatty acid [251]. The 20-22 month old rats on the control diet in the study conducted by Little et al. had a mean level of DHA of 7.4 ± 0.5 % of total fatty acids [252]. The mean level of DHA in the rats of our control group, aged 18 months, was 18.3 ± 0.9 % of total fatty acid which is comparable to levels (16.7 ± 1.7 % to 18.5 ± 0.5 %) reported for previous studies in younger rats (aged 2-3 months) [248, 250]. We observed no significant impact of treatment on the fatty acid profile of the brain tissue.

Additionally, muscle samples were analysed to determine if levels of LC-PUFAs had increased as a result of flavonoid consumption. The potential benefits of increased levels of LC-PUFAs in muscle include the stimulation of muscle protein anabolism in healthy individuls, as well as those who experience muscle loss as a result of ageing [253]. However there were no significant differences in EPA, DHA or DPA in any of the dietary groups. The only fatty acid for which there were significant differences was OA. Compared with the control group, there was a significant increase in OA in the rodents that were fed a diet supplemented with ACN. To our knowledge, this finding has not previously been reported. An increase in OA may be beneficial as it has been shown to improve the adaptive response of muscle tissue in conditions of oxidative stress caused by physical activity [254]. Fatty acid levels in the rat muscle were within ranges described in previous studies [248, 255].

Finally, heart samples were analysed to determine if ACN supplementation could have an effect on n-3 fatty acid levels in tissues. Levels of fatty acids found in the hearts of these animals were similar to levels published in other studies [256-258]. AA levels significantly increased in hearts of rats fed ACN, flavanol and blueberry diet groups compared with the control group. This could indicate an increase in bioconversion of LA to AA. However, levels of EPA and DHA did not change between control and other diet groups which suggests that the increase in AA was not a result of increased bioconversion but some other mechanism such as changes in tissue distribution or metabolism of AA. AA is important in cardiac physiology as it is a precursor for eicosanoid signalling molecules and also acts directly to modulate voltage gated ion channel activity and cellular excitability [259]. Lee et al. have previously shown that concentrations of AA tend to be higher in cardiolipin of aged rats compared with younger rats and suggest this may be to help maintain a high unsaturation index [260]. Consumption of a variety of flavonoids has previously been shown to be associated with increased levels of AA and it has been suggested that this is a result of flavonoids inhibiting the conversion of AA into various pro-inflammatory agents, such as leukotriene, by inhibiting lipoxygenase [261-264].

The analysis of these samples to test our hypothesis had a number of limitations. The original study was not designed to test our hypothesis; therefore aspects such as power calculations, intervention treatments and durations were not designed with our hypothesis in mind. Another limitation was that the liver samples were not available for analysis. The liver is the organ where most of the fatty acid bioconversion occurs and therefore would be expected to be most sensitive to any impact of intervention on LC-PUFA synthesis and status. Our results were not consistent with results published in 2011 by Toufektsian et al. [127] in which EPA and DHA levels increased significantly following ACN supplementation. However there were a number of differences between our work and this previous study (see table 2.1), for example the age of the animals at baseline, as well as the amount and composition of ACNs given. These differences could explain the contrasting findings between the studies.

Table 2.1. Comparison of two rodent studies investigating the effects of flavonoid supplementation on fatty acid status

	Toufektsian et al.	**Animal Flavonoid and Blueberry Intervention**
Animal Age at baseline	1 month	18 months
ACN amount (µg/g feed)	240	320
ACN composition	Cyanidin-glucoside Cyanidin-malonylglucoside Pelargonidin-malonylglucoside Cyanidin-dimalonylglucoside	delphinidin-3-galactoside, delphinidin-3-glucoside, cyanidin-3-glycoside, delphinidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidine-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside, malvidin-3-arabinoside

2.4.3 Human anthocyanin intervention (Study 3)

This is the first time that the impact of ACN consumption on plasma fatty acid status has been examined in humans. Plasma levels of various fatty acids were within the range of levels reported previously [265-268]. For example, Rhee et al. report n-3 fatty acid levels in healthy

post-menopausal women at a level of 1.0 ± 1.8, 0.9 ± 1.1 and 2.2 ± 1.5 % of total fatty acid for EPA, DPA and DHA respectively [269]. These figures were similar to the mean baseline levels reported in this chapter; 1.1 ± 0.6, 0.6 ± 0.1 and 2.5 ± 0.6 % of total fatty acids for EPA, DPA and DHA respectively. However, in contrast to the previous single rodent study and in agreement with our studies 1 and 2, no significant effect of ACN supplementation on LC n-3 PUFA status was observed [127]. Our human study had some limitations, the major one being that this study was not specifically designed to test our hypothesis. Power calculations, duration of the study and dosage of ACNs had not been decided with our hypothesis in mind, although we are relatively confident that a 12 week intervention period, with a physiologically relevant dose of ACNs (500 mg/d, equivalent to the ACN levels found in 25 g elderberries, 100 g blueberries or 140 g blackberries) would be sufficient to detect any impact of treatment on EPA and DHA status. Another important consideration in the examination of these results is that our participants were postmenopausal women, who are known to have substantially lower levels of EPA and DHA biosynthesis in comparison to women of reproductive age [270]. Therefore any modest impact of ACN on EPA and DHA formation may be more evident in individuals with a more up-regulated biosynthetic capacity, namely premenopausal women, a point which may be of interest in future studies.

2.5 Additional Investigations at UEA and Conclusion

Serum, plasma and various tissues from humans and animals that were fed defined levels of ACNs were analysed to determine whether consumption could stimulate increased bioconversion of α-LNA to EPA and DHA. Our results showed that ACN consumption had no significant effect on EPA and DHA levels. The major advantages of these investigations over previous studies were the inclusion of a human study, as well as the investigations into a broader range of compounds including not only ACNs but also flavan-3-ols, blueberry extract, alcohol and champagne in blood and a range of tissue samples. A recognised limitation of our rodent study 2 was the lack of liver tissue. The liver is the major site of EPA and DHA synthesis and therefore would be most sensitive to any impact of polyphenols/flavonoids/ACNs on EPA and DHA status. Furthermore the two previously published rodent studies were limited by the lack of purity of the tested compounds; the ACN-rich foods contained other flavonoids subclasses and phenolic compounds in addition to ACNs [127, 157]. In addition, the diets consumed in the previous rodent studies had relatively low levels of αLNA. To overcome these limitations, a specifically designed animal

study was carried out at UEA throughout the summer of 2012 (not specifically a component of my PhD studies) [271].

40 male rats (aged 8 weeks at baseline) were divided into groups of 10 rats and were fed a specific diet for 8 weeks as follows;

- Group 1; control group fed a diet supplemented with palm oil,
- Group 2; fed a diet supplemented with palm oil and purified ACN (240mg/kg feed),
- Group 3; fed a diet supplemented with rapeseed oil (contains ~10% ALNA),
- Group 4; fed a diet supplemented with rapeseed oil and purified ACN (240mg/kg feed) in addition to rapeseed oil.

Palm oil was chosen as the control oil as it contains only trace amounts of αLNA and has a high SFA content typical of a Westernized-type diet. Rapeseed oil is a commonly consumed oil that is a rich source of αLNA (approximately 10% of its total fatty acids). Serum, heart, lungs, kidney, liver, brain, urine, thymus, muscle, gut were all collected and stored in a -80°C freezer. Serum fatty acid levels were analysed and although there were significantly higher levels of serum αLNA, EPA, DPA and DHA following rapeseed oil consumption compared with palm oil consumption, there was no impact of ACN consumption in any of the groups. In addition there was no impact of ACN consumption on liver fatty acid levels. Consistent with fatty acid status work in animals and humans, work in HepG2 cells which were co-cultured with αLNA and various flavonoids for 24h showed no impact of supplementation on *FADS* 2 gene expression [271].

Overall results from this project found no evidence that consumption of ACNs (in addition to other flavonoids and champagne polyphenols) stimulate fatty acid pathways to produce increased plasma or tissue levels of EPA and DHA. It may be speculated that some other plant bioactive component, such as resveratrol, may be responsible for the improved EPA and DHA status in red wine consumers.

The results presented in this chapter have contributed to the following publication; Vauzour D,, Tejera N, **O'Neill C**, Booz V, Jude B, Wolf IM, Rigby N, Silvanc JM, Curtis PJ, Cassidy A, de Pascual-Teresa S, Rimbach G, Minihane AM. Anthocyanins do not influence long-chain n-3 fatty acid status: studies in cells, rodents and humans. *Journal of Nutritional Biochemistry* 2015.

Chapter 3

NU-AGE Study Design;

Investigating the impact of a year-long

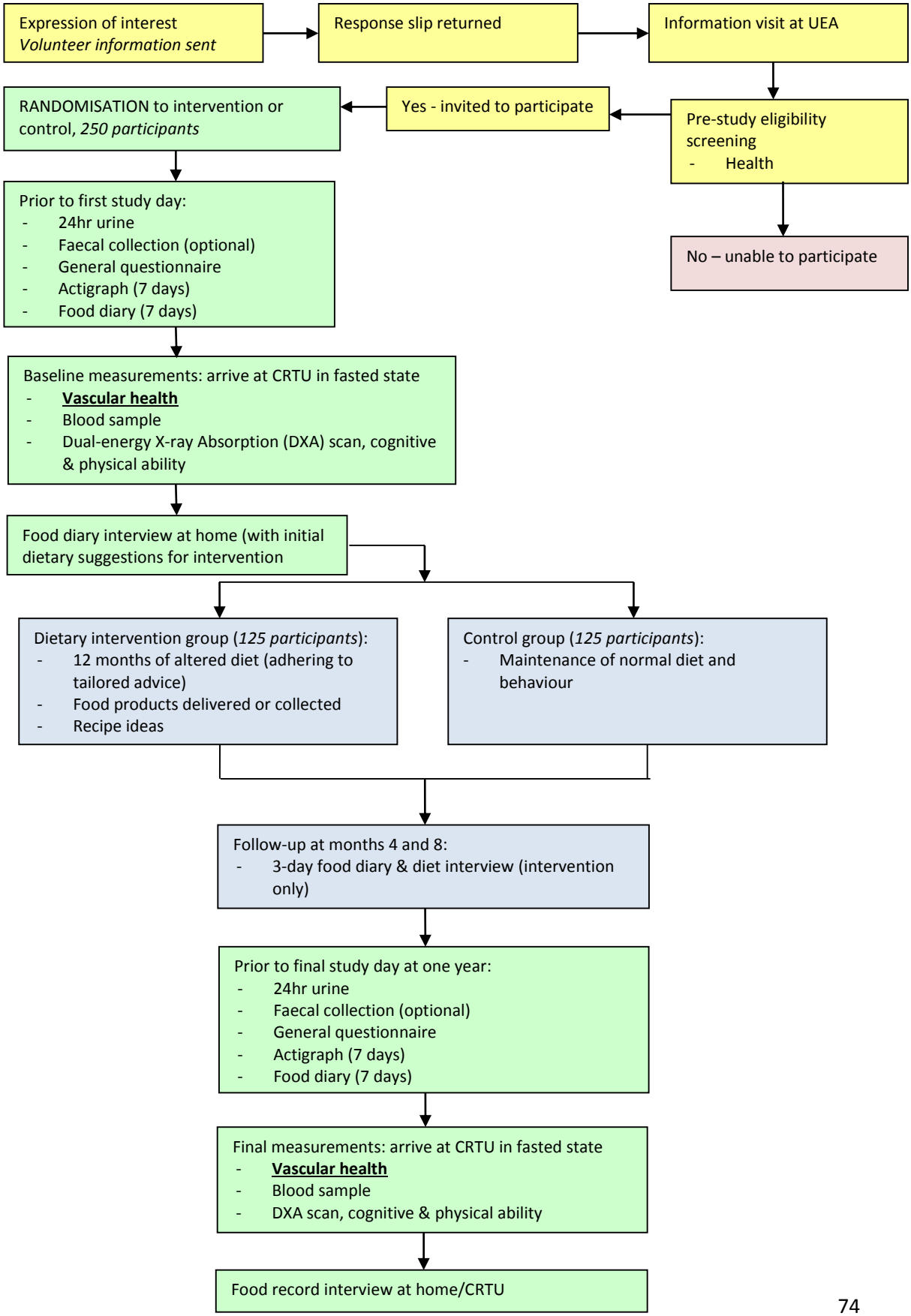
whole-diet intervention on vascular function

3 NU-AGE Study Design; Investigating the impact of a year-long whole-diet intervention on vascular function

3.1 Introduction

The study design for the NU-AGE study has previously been published [182]. NU-AGE (EU FP7) is a multi-centre trial involving 1,250 older adults, aged between 65 and 79 years, in five different centres across Europe, including the UK (UEA), the Netherlands, Italy, France and Poland. UEA was responsible for recruiting 250 volunteers, 125 in the control group and 125 in the intervention group. The aim of NU-AGE was to investigate the impact of a whole-diet intervention for one year on a range of health outcomes, with inflammatory status representing the primary outcome. The whole-diet was based on recommendations specifically designed for older adults (described in table 3.2). The use of the whole-diet intervention allows us to investigate the impact of synergistic and additive effects of a range of foods and dietary components consumed in accordance with the dietary advice provided. This PhD includes data from the Norwich study centre only (with measurements taken at the Clinical Research and Trials Unit (CRTU), UEA) and focused on the impact of intervention on vascular function underlying mechanisms. A flow chart outlining the recruitment plan and completion of the study can be found in figure 3.1.

Figure 3.1. Flow chart of NU-AGE study design (Adapted from NU-AGE UEA protocol [272])



3.2 Study Population

Participants were largely recruited through local advertisements and publicity, targeted at relevant demographics, or through General Practitioner (GP) surgeries. Volunteers were apparently healthy for their age; they were free from current or recent (within last 2 years) chronic diseases. Participants were free-living and responsible for their own shopping/cooking/meal choice and preparation (i.e. not in a nursing or care home). Inclusion and exclusion criteria are listed below [182];

Inclusion criteria

- Men or women aged 65 to 79 years
- Free of chronic disease for the last 2 years (e.g., cancer, severe organ disease)
- Free and independent living, responsible for own shopping/meal choices or preparation
- Willing to participate in a dietary intervention (make changes to their habitual diet) for one year

Exclusion criteria

- Current overt disease such as aggressive cancer or dementia
- Unstable organ failure or organ failure necessitating a special diet
- History of severe heart disease, chronic kidney disease, respiratory insufficiency, liver cirrhosis
- Diabetes Mellitus type 1
- Chronic corticosteroids use
- Use of nonsteroidal anti-inflammatory drug
- Recent (previous 2 months) use of antibiotics
- Recent (previous 3 months) change in habitual medication use (e.g statins, thyroxin)
- Parallel participation in another study involving dietary interventions, or sampling/donation of blood that may increase volume taken above 500mL in a 4 month period
- Malnutrition, as diagnosed by a BMI lower than 18.5 kg/m^2
- Body weight loss of more than 10% within 6 months

- Presence of frailty according to the criteria of Fried et al. [273]
- Individual unable to give informed consent.

3.3 Clinical measures of vascular function

3.3.1 Introduction

A battery of vascular measures that lasted for approximately one hour was performed at baseline and again after one year. These measures were all chosen based on the selection criteria described below;

- must have a sound physiological and mechanistic basis that links it to atherosclerotic risk.
- must be reproducible and easily standardised.
- an improvement in the test result should predict an improvement of subsequent cardiovascular risk, likewise, a worsening in the test result should correlate to increased cardiovascular risk.
- must be non-invasive.
- should be observer independent.

The tests administered included the following measures, which are further described in the subsequent sections;

- Blood pressure
- Pulse Wave Velocity (PWV)
- Cardio-Ankle Vascular Index (CAVI)
- Ankle-Brachial Index (ABI)
- Reactive Hyperaemic Index (RHI) via EndoPAT.

3.3.2 Resting period and blood pressure measurements

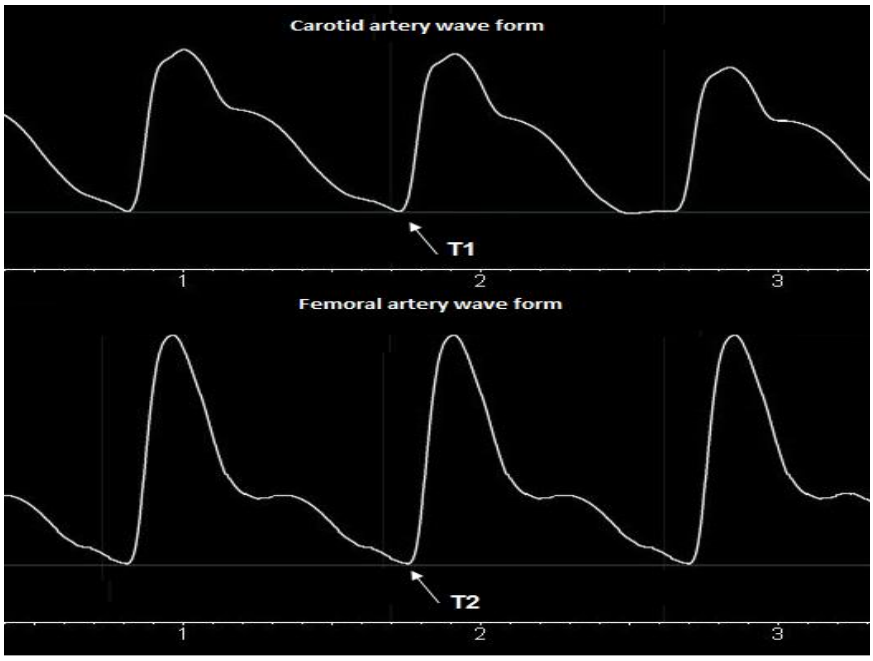
Participants were rested for 15 minutes in a dimly lit, quiet room with a room temperature between 21°C and 24°C. The need for control is due to the known impact of temperature on vascular tone and in particular on the peripheral blood vessels. Following 15 minutes of rest, heart rate, SBP and DBP were measured on the non-dominant arm with an automatic blood pressure measurement device (OMRON M2, Milton Keynes, UK). Measurements were taken

three times, with approximately 1 minute rest between each measurement. The average of the 2nd and 3rd recordings was calculated to give the final blood pressure reading.

3.3.3 Pulse Wave Velocity (PWV)

The Vicorder was used to measure PWV (Skidmore Medical, Bristol, UK). PWV is the velocity of the forward (from the heart to peripheries) propagated arterial pressure wave and is measured by recording the pulse wave at a proximal artery and a distal artery. The gold standard is to measure at the common carotid and the femoral artery, as the distance is comparable to the length of the aorta [274]. According to the manufacturers' instructions, participants were instructed to lie in the semi-prone position (at approximately 30°) to prevent venous contamination of the arterial signal. To ensure adequate recording of the pressure wave form at the carotid artery, the neckpad was carefully placed at the approximate position of the carotid. The femoral artery cuff was placed around the thigh (as close to the hip as possible). Using a measuring tape, the distance between the carotid to sternal notch was measured, as was the distance from the sternal notch to the femoral measurement site. The length (cm) was then calculated by subtracting the "carotid measurement site to sternal notch distance" from the "sternal notch to femoral measurement site distance" in order to approximate the difference in length by which the pulse wave would have to travel, which was then incorporated into the PWV calculation. The participant was instructed to breathe gently and avoid talking or making any major movements. Pulse waves were measured at both sites as the cuffs were inflated. Measurements were taken 3 times and an average of the 3 readings was used as the final result to ensure accuracy. The calculation of PWV is depicted below in Figure 3.3.

Figure 3.2. Diagram representing the carotid artery and femoral artery wave form and calculation of PWV



PWV= L / (T1-T2)

PWV: Pulse Wave Velocity, T: Time, L: Length

3.3.4 Cardio-Ankle Vascular Index (CAVI) and Ankle-Brachial Index (ABI)

CAVI (Vasera™VS-1500, Fukuda Denshi Co, Japan) is another measure of arterial stiffness; it is similar to PWV but it is considered to be independent of pulse pressure. Four blood pressure cuffs were wrapped around the four extremities; the left and right brachial and the left and right ankle. An electrocardiogram was placed on each wrist to monitor pulse and a phonocardiogram microphone was placed on the chest to monitor heart rate. The upper arm and ankle pulse waves, as well as blood pressure, were then monitored as all four cuffs were inflated together. CAVI was determined using PWV from the aortic valve origin to the ankle region and blood pressure measured at the upper arm. The principle underlying CAVI has been previously described [275], but in summary the CAVI result is automatically calculated by the Vasera using the following formula;

$$CAVI = 2\rho \times \ln(Ps/Pd) \times PWV2/\Delta P,$$

where ρ is the blood density, Ps is the systolic pressure, Pd is the diastolic pressure, PWV is the measured pulse wave velocity from the aortic valve to the ankle, and ΔP is the difference between the systolic pressure and diastolic pressure.

The calculations for CAVI are diagrammatically explained in figure 3.4. Measurements were taken three times in a supine position with the average of the 3 readings used as the final

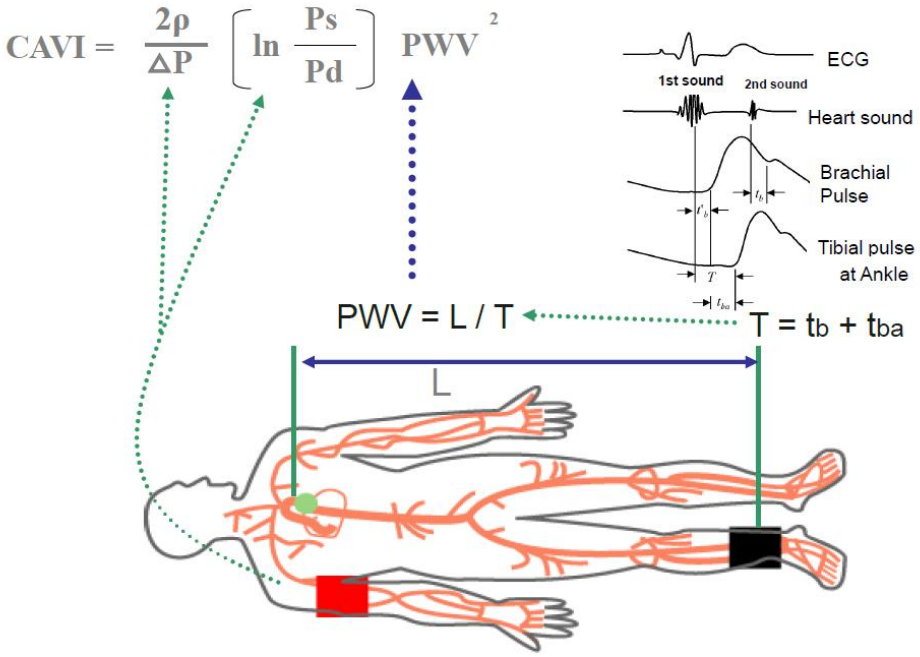
result to ensure accuracy. The Vasera also measured ABI which is used to analyse stenosis and occlusion of the crural arteries and is useful for the early detection of PAD. It is calculated by measuring blood pressures at the brachials and ankles;

ABI= (Systolic Pressure at ankle) / (Systolic pressure at upper arm).

ABI results can be interpreted as follows [276];

- ≤ 0.40; severe occlusion or stenosis suspected
- ≥ 0.41 and ≤ 0.90; slight or mild occlusion or stenosis suspected
- ≥ 0.91 and ≤ 0.99; borderline normal
- ≥ 1.00 and ≤ 1.29; normal
- ≥ 1.30; ankle blood pressure is somewhat high.

Figure 3.3. The measuring method of Cardio-Ankle Vascular Index [275]



Ps; systolic blood pressure, Pd; diastolic blood pressure, PWV; pulse wave velocity, ΔP; Ps - Pd, p; blood density, ΔP; pulse pressure, L; length from the origin of the aorta to the ankle, T; time taken for the pulse wave to propagate from the aortic valve to the ankle, tba; time between the rise of brachial pulse wave and the rise of ankle pulse wave, tb; time between aortic valve closing sound and the notch of brachial pulse wave.

3.3.5 EndoPAT

The EndoPAT2000 (Itamar Medical Ltd, Caesarea, Israel) was used to measure endothelial function. This is a non-invasive technique which measures digital finger pulse volume using PAT. The pulse wave amplitude (PWA) on both index fingers was recorded by pneumatic sensors for a baseline period of 5 minutes. One arm was then subjected to complete occlusion of the brachial artery for another 5 minutes which induced RH. Occlusion of the brachial artery was achieved by placing a blood pressure cuff around the upper arm and rapidly inflating the cuff to supersystolic pressure (60mmHg above the resting SBP or 200mmHg; whichever is higher). Following this, the PWA was recorded for another 5 minutes (post-occlusion). The EndoPAT2000 calculated the RHI by measuring the increase in blood flow in response to the stimulus of shear stress caused by the 5 minute brachial occlusion, and subsequent releasing of the occlusion, causing a sudden increase in blood flow to the finger tips. This RHI measurement is indicative of the capacity of the endothelium to produce vasodilatory substances, in particular NO. The threshold for endothelial dysfunction is defined as an RHI of <1.67 [277].

Figure 3.4. Final result of the EndoPAT test

The final concluded result of the EndoPAT test is a ratio of the post-to-pre occlusion peripheral arterial tonometry (PAT) amplitude of the tested arm, divided by the post –to-pre occlusion ratio of the control arm. A - Mean PAT amplitude between 90s-150s post occlusion of the occluded arm. B - Mean PAT amplitude from the baseline period of the occluded arm. C - Mean PAT amplitude between 90s-150s post occlusion of the control arm. D - Mean PAT amplitude from the baseline period of the control arm

3.4 Sample Size – vascular function measurements

Power calculations were carried out to determine the number of participants required to give sufficient power to address the research questions. Calculations estimated that a sample size of n=150 would have a power of 0.9 to detect clinically relevant changes in the primary outcome measures; EndoPAT and PWV. The calculations assumed no correlation between baseline and end of the intervention. Calculations were two-sided with 5% significance level. For EndoPAT, the calculations were based on RHI and an assumed standard deviation (within patient) of 0.260 [278], a clinically relevant difference of 0.2 RHI units would be detectable with a sample size of 74 subjects. For PWV, assuming a standard deviation of 2.0 m/sec [279], a clinically relevant difference of 1.2 m/sec would be detectable with a sample size of 120

participants. Assuming a dropout of approximately 20% of volunteers, 150 volunteers were analysed so that a total of 120 volunteers completed the study.

3.5 Recruitment

Advertisements and features in the local press or radio were used to raise awareness of the study. The advertisements and flyers encouraged members of the public to contact the study team by telephone or email for further information. Volunteers contacting the study team were sent an information pack that included a cover letter, the Volunteer Information Sheet and response slip. The response slip provided the study team with contact details for the volunteer, and also asked basic information relating to the inclusion/exclusion criteria. Volunteers were also recruited using GP surgeries, which were used both as a place to display posters and as well as an information source; patient databases were used to target potential volunteers. Once in receipt of a response slip, a member of the study team called the volunteer to arrange a face-to-face discussion about the study, or to discuss the eligibility criteria further if the information provided by the volunteer on the response slip indicated they may not be suitable for the study. Alternatively, volunteers contacted the study team directly by telephone to express their interest and discuss their eligibility, and where appropriate a face-to-face discussion appointment was subsequently arranged.

During the face-to-face discussions, volunteers were given the opportunity to ask the study team questions and were given a full explanation of the study requirements. Following the discussion the volunteers were given at least 72 hours to decide on their participation, during which they were not contacted by the study team. If volunteers subsequently decided to participate in the study they were asked to contact a member of the study team to arrange a pre-study health screening appointment. Alternatively, if volunteers were particularly keen they arranged this appointment when visiting the study centre for their discussion appointment, providing it occurred at least 72 hours after their initial visit.

3.6 Pre-Study Health Screening

Prior to being accepted onto the study, all volunteers participated in a pre-study screening. A member of the study team went through the consent form and medical declaration form with the volunteer, who was encouraged to ask any outstanding questions. A copy of each of these two forms was given to the volunteer.

The scientist went through two ‘admission questionnaires’ with the participant. The first questionnaire (Annex 1 and 2) was designed to record information on the volunteer’s health history and adherence to the inclusion and exclusion criteria. Participants were asked to

bring a record of all of their current medications (or the medications themselves) to the appointment. If the outcome of the first questionnaire was favourable then the second admission questionnaire, which assessed frailty, was administered. Only participants scored as non- or pre-frail (defined using a combination of measures, including height, weight, BMI, hand grip strength, gait speed, depression and physical activity) on this questionnaire were recruited to the study, as assessed using a frailty test described by Fried et al. [280]. GPs were informed about their patient's participation in the study and were sent information from the screening session or other study assessments that was deemed of relevance by the study's medical advisor (e.g. full blood count results, frailty assessment etc.). Volunteers agreed to this information being sent to their GP during the consenting process. A minimum of a week was allowed between the screening and starting the study to allow for any intervention by GPs.

Participants received a 7-day food record (described below, section 3.8.3) and an ActiGraph device (as described below, section 3.10), as well as instructions for use. Both the food diary and Actigraph data were collected for the same seven days and completed prior to baseline measurements and randomisation.

3.7 Randomisation

The volunteers were randomised to the intervention or control group. Randomisation was carried out using computer software and stratified by gender, age (65-72 or 73-79 years), frailty status (pre-frail or non-frail) and BMI (<25 or ≥25 kg/m^2) [182]. Couples or others cohabiting were randomised together. This was carried out to ensure that participants in the control group would not alter the dietary habits if in contact with someone in the intervention group and therefore affect the outcome of the study. Due to the nature of the dietary intervention it was impossible to blind the subjects to their allocation and they were explicitly told which group they were assigned to. Although it would be have been preferable to blind the scientists administering the tests and questionnaires to the volunteer groups, this was not possible as all study staff were required to help with follow-up of the two groups, and in particular to assist the intervention group with compliance (e.g. distributing foods, answering queries, providing recipes etc).

3.8 Dietary Intervention

3.8.1 NU-AGE nutrient guidelines

The intervention group received individual, tailored dietary advice that was provided based on the 7-day food diaries (see section 3.8.3) completed at the start of the study. The

personalised dietary advice given aimed to meet the NU-AGE quantitative daily nutrient guidelines (Table 3.1) [182]. The NU-AGE nutrient guidelines were based on recommended daily allowances (RDAs) from each of the countries participating in the study; France [281], Italy [282], Netherlands [283], Poland [284], and United Kingdom [285] and other recommendations bodies including the modified MyPyramid for Older Adults [286, 287], European Commission [288], and Institute of Medicine [289].

Table 3.1. NU-AGE quantitative daily nutrient guidelines [182]

Nutrient	Quantitative requirements*
Energy (MJ)	Individual requirement +/- 0.5 MJ
Protein (EN%)	20-25
Carbohydrates (EN%)	45-55
Fat total (EN%)	25-30
Saturated fat (EN%)	<10
Trans fatty acids (EN%)	<1
MUFA (monounsaturated fatty acids) (EN%)	<26
PUFA (EN%)	<12
Fibre (g)	30-40
Alcohol (g)	<10-20 (1 serving/day for women, 2 servings/day for men, or abstain)
Water (ml)	1500
Sodium (mg)	1300
Calcium (mg)	1200-1300
Iron (mg)	10
Vitamin D (µg)	15
Folate (µg)	400
Vitamin B12 (µg)	5

** Average requirement per day, EN%; percentage of total energy*

3.8.2 NU-AGE food based dietary goals

FBDGs are generally created to make advice on nutrient intake easier to follow for the general population. There were 14 dietary targets in the NU-AGE FBDGs (Table 3.2) that were created based on the nutrient guidelines discussed above (Table 3.1). Achieving the NU-AGE requirement for vitamin D through dietary sources alone would be extremely difficult and exposure to sunlight (and therefore vitamin D status) varies greatly with season and between individuals, particularly across the different European centres [182]. Therefore an additional

target for participants in the intervention group was to take a daily vitamin D supplement (10µg/day). The dietary intervention lasted for one year, thereby reducing seasonal effects by comparing initial (baseline) with final measurements at the same time of the year. A year-long intervention has been shown to be a sufficient time period for alteration of dietary habits to impact on various health outcome measurements, such as blood pressure [290-292]. For example, Vincent-Baudry et al. demonstrated that a time period of just 3 months was sufficient time to observe significant improvements in cardiovascular risk following a whole-diet intervention based on the Mediterranean diet [292].

Participants in both the intervention and the control group were allowed to use nutritional supplements during the study period, which were recorded during the dietary interview at the start of the study. Any changes to supplement use were reported to the study team during the intervention. If participants were taking vitamin D supplements prior to the study they were asked to replace these with the study supplements. All volunteers were asked to continue their normal physical activity and smoking habits [182].

Table 3.2. NU-AGE food based dietary goals [182]

Food group	Amount	Specification/Preference
Whole grains	4-6 servings* per day	Slices of wholegrain bread, breakfast cereals, crackers, etc.
Fruits	At least 2 servings* per day	Fresh, frozen, dried, juice
Vegetables and legumes	At least 300 gram per day and 200 gram legumes per week	Dark-green vegetables (broccoli, salad greens, cooked greens). Orange vegetables (carrots, sweet potatoes). Legumes like lentils, black eyed peas, peas
Dairy and cheese	500 ml dairy per day, including 30 gram cheese	Calcium enriched dairy. Lean, reduced salt cheese.
Fish and other seafood	125 gram of fish twice per week	Preferably fatty fish. Canned tuna, salmon, sardines, herring, frozen fish, smoked salmon, mackerel.
Meat and poultry	125 gram of meat or poultry 4 times per week	Lean meat or poultry. Not fried.
Nuts	20 gram twice per week	Unsalted mixed nuts, almonds
Potatoes and pasta/rice	150 gram per day	Whole grain rice or pasta
Eggs	2-4 eggs per week	
Oil/fat	20 gram of oil per day, 30 gram of margarine per day; maximum 50 gram fat per day	Olive oil rich in N-3/N-6 fatty acids. Low fat margarine rich in MUFA and PUFA
Alcohol	Maximum 1-2 glasses per day for men and 1 glass per day for women	If alcohol consumed, preferably red wine.
Fluid	1.5 litre per day	Including milk
Salt	Maximum 5 grams per day	Reduce adding salt, ready-made meals, soups, gravy, sauce
Sugar	Limit the use of sweets and sweet drinks	Replace sweets with fruits, vegetables or dairy, no sugar in tea or coffee

*1 serving of whole grains equals 1 slice of bread (1.5 cm thick) or 25 g of bread, ½ cup of breakfast cereals (50g), ½ cup of rice (better choose brown), cooked (30g raw), 2/3 cup of pasta, cooked (30g raw), 1/3 cup of muesli, ½ cup of porridge. Examples of fruit serving sizes; 1 apple, 1 banana, 1 orange, 1.2 grapefruit, 8 plums (small), approximately 1 glass of fruit juice

3.8.3 Dietary intake assessment and advice

Dietary intakes of all participants were assessed by means of food diaries that were kept for 7 consecutive days before baseline measurements and randomisation, as well as before follow-up measurements after one year. Relative to other dietary assessment methods, such as FFQs, the advantages of food diaries include their ability to assess portion sizes, take dietary patterns into account, and assess intakes of infrequently consumed foods that can be important sources of specific nutrients for some [182]. A pre-formatted diary was used (see annex 3) and included 8 meal occasions (before breakfast, breakfast, morning snacks, lunch, afternoon snacks, evening meal, evening snacks, night snacks) as well as space for meal recipes for each day. Participants were asked to make diary entries as detailed as possible, including the brand name and weight/portion of each food when possible. Once the food diary was complete and study day had been undertaken, a NU-AGE team member was responsible for reviewing the food diary and reviewing details with the participant. In the UK centre of NU-AGE, the food diaries were coded and translated into nutrients using the nutritional analysis software package WISP (Tinuviel Software, UK).

Participants in the intervention group received **individually-tailored dietary guidance** and advice through regular visits, telephone contact and e-mails. Dietary advice and counselling, which was based on the NU-AGE FBDGs and baseline food diaries, was delivered by trained members of the study team (led by Rachel Gillings, Associate Nutritionist). The dietary goals were split into three blocks of five, and the three blocks were introduced gradually into the diet. The volunteer decided, in collaboration with the study team, which order the goals were introduced. The timeline for the introduction and evaluation of the food goals are shown in table 3.3 which was adapted from the published NU-AGE study design [182]. Subjects in the control group were given a standard healthy living advice leaflet and asked to maintain their normal dietary habits over the course of the year. In the UK, this leaflet was the British Dietetic Association information sheet (annex 4).

Table 3.3. Dietary Counselling Schedule (adapted from Berendsen et al [182])

Month	Occasion	Topic
0	CRTU visit/ Home visit	Dietary assessment intake (7 day diary completed and assessed) Determined the first 5 dietary goals (Vitamin D supplements should be included) Given information for the first set of 5 dietary goals Given vitamin D supplements (enough for 4 months) and food products related to first set of 5 dietary goals
1	Telephone E-mail/post	Evaluated the first 5 dietary goals Determined the second set of 5 dietary goals for the next month Information provision on tips/ recipes for the second set of 5 dietary goals.
2	E-mail/post	Sent out additional information of the first two sets of five dietary goals and on fish consumption
3	Telephone E-mail/post	Evaluated the second set of five dietary goals and determined the third set of five dietary goals Instructions for completing the 3 day food diary before CRTU visit in month 4 Made an appointment for the CRTU visit in month 4 Sent out information regarding the third set of dietary goals Sent out the dietary records for three days plus explanation Sent out confirmation of appointment
4	CRTU visit E-mail/post	Evaluated the dietary goals by going through the food records and ask participant for obtained goals, experience, difficulties. Determined which goals need extra attention. Given vitamin D supplements (enough for 4 months) and food products Given appropriate info sheet for goals that needed extra attention/ were not obtained. Sent out additional information regarding food labelling, recipes, variation
5	Telephone E-mail/post	Evaluated the goals that needed more attention. Determined which goals need extra information/attention Sent out appropriate info sheet belonging to goals that needed extra attention/ were not obtained.
6	E-mail/post	Sent out additional information regarding fruit and vegetable consumption

7	Telephone	Evaluated the goals that needed more attention Asked about sent information; was it clear, any questions Determined which goals need extra information/attention Made an appointment for the CRTU visit in month 8
	E-mail/post	Sent out appropriate info sheet belonging to goals that needed extra attention/ were not obtained. Sent out the dietary records for three days plus explanation Sent out confirmation of appointment
8	CRTU visit	Evaluated the dietary goals by going through the food diaries and asked participant for obtained goals, experience, difficulties. (Dietary goals questionnaire also used to assess compliance) Determined which goals needed extra attention. Given vitamin D supplements (enough for 4 months) and food products
	E-mail/post	Sent out appropriate info sheet for goals that needed extra attention/ were not obtained.
9	Telephone	Evaluated the goals that needed additional attention based on the food records Determined which goals needed extra attention during the last months
	E-mail/post	Sent out appropriate info sheet for goals that needed extra attention/ were not obtained.
10	E-mail/post	Sent out additional information regarding protein intake/hydration
11	Telephone	Evaluated the goals that needed more attention Asked about sent information; was it clear, any questions Made an appointment for the CRTU visit in month 12
	E-mail/post	Sent out confirmation of appointment, delivered pre-appointment equipment
12	Research Unit visit/ Home visit	7-day food record and interview

3.8.4 Mechanisms used to maximise compliance to dietary intervention

Compliance was aided by the distribution of food products, information sheets and recipes to the intervention group. Food products supplied to the intervention group to improve compliance included extra virgin olive oil (high monounsaturated fat content), margarine spread (45% vegetable fat spread with 26% sunflower oil, containing n-3 and n-6 fatty acids), whole-wheat pasta (high in fibre), and lean low-salt cheese (provided by NU-AGE industry partners).

An information sheet for each of the NU-AGE FBDGs was given to the participant which gave details on the NU-AGE goal, the importance and reasons for consuming the food, the amount and preferable type of the food that the participant should consume, as well as ideas on how to reach the goal. For example in the case of wholegrain consumption, the information sheet contained the following information;

- A statement detailing that the NU-AGE goal is 4-6 servings per day,
- A detailed paragraph on the definition of a whole grain (for example; wholegrain refers to the entire grain—this includes the bran, germ and endosperm),
- Information on why wholegrain consumption is important (for example; whole grains are high in fibre, a nutrient which is important for good bowel function and preventing obesity. There is also the combined effect of the variety of other nutrients that whole grains contain, such as B vitamins and folic acid),
- What makes a portion of wholegrain (the weights and food types, for example 65-70 g of cooked brown rice), and
- Tips on how to ensure adequate wholegrain consumption (for example, looking out for wholegrain symbols on food packaging).

Recipes designed to help participants in the intervention group achieve the NU-AGE FBDGs were given throughout the intervention period to assist with compliance. Recipe ideas included hand-outs that were focused on fish, legumes, soups and wholegrain consumption. Fish recipe ideas included cod curry, a healthy version of fish and chips, mackerel fish cakes, fish pie with sweet potato topping, citrus ginger tuna steak. As well as helping participants achieve the goals in relation to fish, these recipes also incorporated other foods, such as olive oil, vegetables, egg and wholemeal breadcrumbs, to help participants achieve other goals simultaneously.

Participants did not receive any financial incentive to participate in the trial, but their travel expenses were reimbursed.

3.9 Assessment of compliance

Following study completion, compliance to the study in both control and diet group was analysed using the 7-day food records at baseline and at follow-up after 1 year. For participants in the intervention group, follow-up 3-day food diaries at month 4 and 8 was used to determine both adherence to the NU-AGE diet, as well as the use of foods supplied. In addition, participants in the diet group were asked to return their remaining vitamin D tablets in order to use this number as measure of compliance to the vitamin D supplement.

However, a limitation of this PhD was that at the time of thesis submission not all of these data were available for the assessment of compliance. Therefore for this PhD thesis, a questionnaire that was designed to both assess and assist compliance was used, in addition to counting leftover vitamin D supplements. This questionnaire (annex 5) was completed at the 8 month follow up visit and was based on the PREDIMED compliance questionnaire [293] but tailored specifically to the NU-AGE diet. The participant was asked how many portions of each of the food goals they consumed per week/day. The answers to these questions were then scored as shown in table 3.4. The majority of the food groups were scored as follows; 1 given when NU-AGE goal was achieved, 0.5 given when NU-AGE goal was not achieved but had been attempted, 0 given for inadequate intake. In some cases, for example when excessive consumption may be detrimental rather than beneficial, a reverse scoring was used. For example, a recommendation of 2 servings of nuts per week was given to participants on the NU-AGE diet. However, nuts are calorie dense and therefore excessive consumption is not recommended. Therefore a score of 0 was given for both inadequate and excessive consumption. In the case of food goals that emphasise restriction rather than consumption the score of zero to one was divided by seven; for example one question examining salt consumption enquired as to how often the participants added salt to their food. This was scored so that if the participant adds salt to their food less than once per week they score a 1, twice per week they score 0.83 and so forth. If they add salt to their food every day they score a zero. The score for each goal was added to give a total percentage of compliance. This was then used as a covariate when investigating the effect of the NU-AGE dietary intervention on various outcomes.

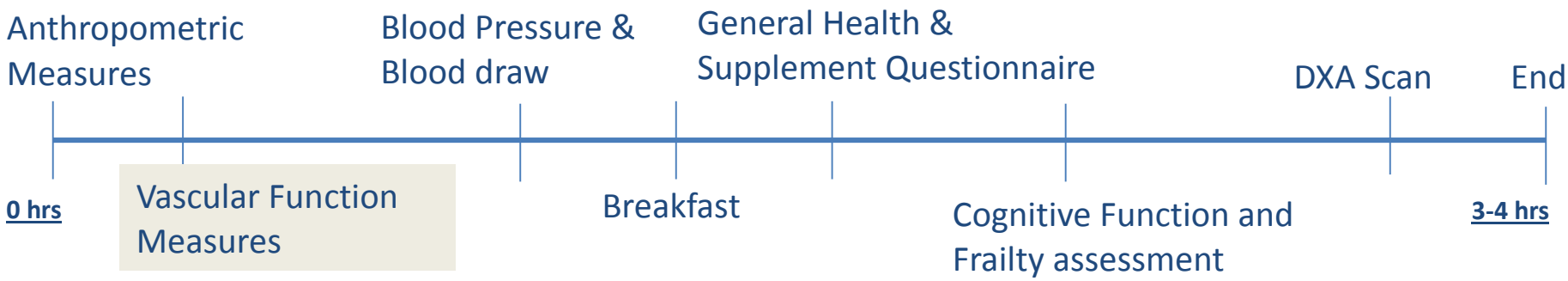
Table 3.4. NU-AGE Compliance Scoring System

Question	Goal	0	0.5	1		0	0.17	0.33	0.50	0.67	0.83	1
1. How many servings wholegrain per day?	4-6 servings	≤2	3	≥4								
2. How many portions of fruit per day?	>2 servings	0	1	≥2								
3. How many portions of vegetables per day?	>3 servings	0	1-2	≥3	% by 2							
4. How many portions of legumes per week?	2-3 servings	0	1-2	≥3								
5. How much milk/yoghurt per day?	500ml	0-250	251-499	≥500								
6. How many servings of cheese per day?	1 serving	>1		≤1								
7. How many servings of fish per week?	2 servings	0	Consuming ≥2 white fish or 1 portion of oily fish	Consuming ≥2 oily fish								
8. How many servings of meat/poultry per week?	4 servings	0-1 or ≥6	2-3 or 5	4								
9. How many servings of nuts per week?	2 servings	0 or ≥ 4	1	≥2								
10. How many eggs per week?	2-4	0 or >6	1 or 5	2-4								
11. How many tbpns of olive oil per day	1.5 tbsp	0 or >3	0.5 or 3	1-2								
12. How many glasses of wine per week?	7 glasses (if alcohol consumer)					≥13	12	11	10	9	8	≤7
13. How many glasses of fluid per day? (subtract no. of caffeinated drinks)	7-8 glasses	0-3	3-6.99	≥7								
14. How often per week do you add salt to food?	Limit					≥7	6	5	4	3	2	<1
15. A) How often per week do you add sugar to food or drink?	Limit				% by 2	≥7	6	5	4	3	2	<1
B) How many times per week do you consume cakes, biscuits or desserts?						≥7	6	5	4	3	2	<1
16. How many vitamin D supplements remaining after one year intervention?	<15% (85% compliant)	≥15%	5-15	<5%								

3.10 Study Day

Prior to the study day at baseline and at the one year follow-up, volunteers were asked to refrain from intensive exercise and alcohol consumption for the 24 hours prior to their appointment. Volunteers were required to wear an ActiGraph device for 7 days between their screening and study day appointments to measure their physical activity. The ActiGraph is capable of measuring a number of parameters related to physical activity, including energy expenditure, the number of steps, intensity of activity and body position. The device is lightweight and fits around the waist on an elastic belt. Volunteers were asked to bring the Actigraph, as well as their urine samples, food diary and questionnaires in to the CRTU with them on the morning of the first study day. The volunteer's weight and height were measured and BMI was calculated. Measurements of waist and hip circumference were also taken. Figure 3.2 provides an overview of the study day attended by participants at baseline and after one year. Participants attended each assessment visit in a fasted state (>8 hours), at either 8am or 9am having fasted for 8 hours overnight (volunteers were encouraged to drink water in the morning), with blood samples and measurements of endothelial and vascular function taken before consumption of breakfast. A variety of other measures were taken following breakfast resulting in each study visit taking 3-4 hours.

Figure 3.5. NU-AGE study day (At baseline and end of 1 year intervention)



3.11 Biochemical measures of vascular function, inflammatory and fatty acid status

3.11.1 Introduction

A range of biochemical markers of vascular function, inflammatory and fatty acid status were measured from the blood samples of participants before and after the NU-AGE intervention. Whole blood was collected directly into heparin vacutainers (for DNA extraction). Blood collected in heparin (for NO, CRP and lipid profile analysis) and EDTA (for ET-1 and fatty acid analysis) was centrifuged at 3300g for 10 minutes to isolate plasma which was stored at -80°C. The methods used to quantify the major analytes included in this thesis are described below. An overview of the all biochemical outcomes to be measured in NU-AGE is given in annex 6.

3.11.2 Plasma Nitrite analysis

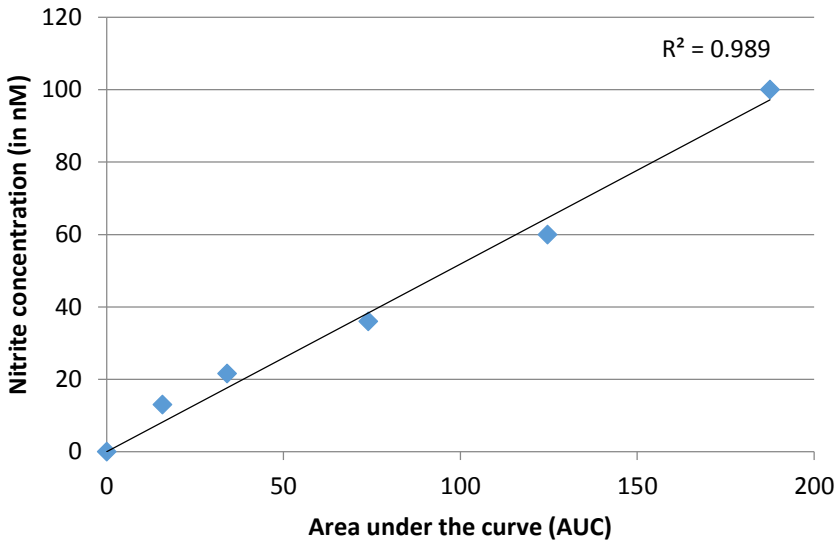
Plasma nitrite levels were measured using a chemiluminescence detector (CLD88), a device that is used to measure levels of NO in an inert carrier gas. At the time of analysis, plasma samples were stored on ice and were injected into a purge vessel that contained reductive solutions. The function of these reductive solutions was to reduce nitrite (NO_2^-), nitrate (NO_3^-) and other oxidative by-products of NO, back to NO for measurement. NO can be produced *in vivo* from both nitrite and nitrate, therefore the measurement of nitrite, nitrate and other oxidation products can be used as an index measure of *in vivo* NO production.

A triiodide solution, made up of a 45mM solution of I- and 10mM solution of I_2 was dissolved in a 93% glacial acetic acid [294], was used to detect NO. NO was carried through to a scrubbing bottle that contained 1M sodium hydroxide (0°C) by passing N gas through the reductive solution. The function of the scrubbing bottle was to trap any traces of acid and iodine before the NO was then carried into the detector via a sampling probe.

A series of serial dilutions of Sodium Nitrite, ranging from 0 to 100nM, were made up and injected in duplicate to establish a standard curve. Samples were run in duplicate once the coefficient of variation for standards and plasma quality controls were confirmed to be within 10%. The triiodide solution was replaced every 40 injections in order to yield optimal reproducibility for peaks derived from both standards and samples. Plasma nitrite concentrations in samples were generated via peak integration and utilisation of standard curves. A representative standard curve can be seen in figure 3.6. The AUC is calculated from

a plot of the concentration of nitrite in blood plasma against time. Materials were obtained from Sigma (Dorset, UK) unless otherwise noted.

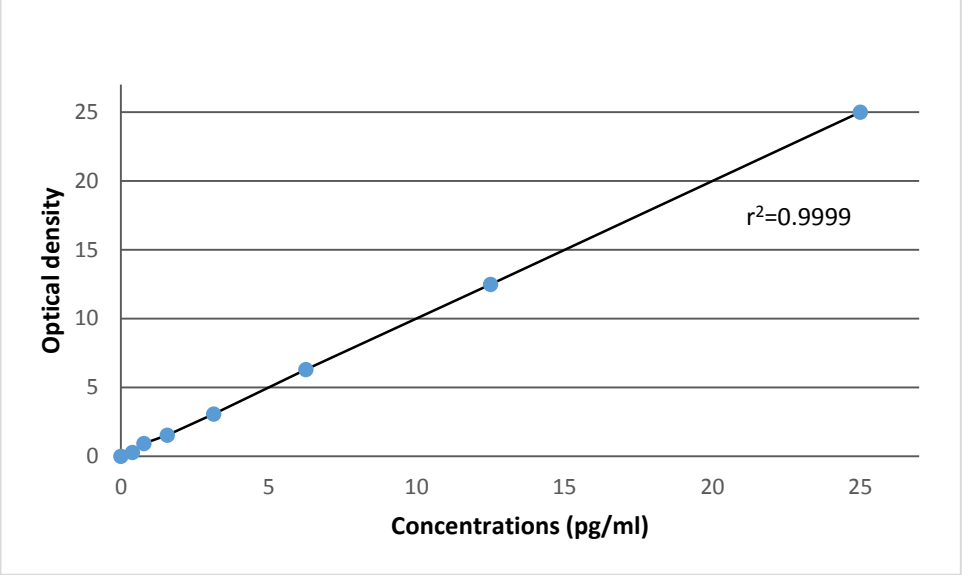
Figure 3.6. Representative calibration curve for nitrite analysis



3.11.3 Plasma Endothelin-1 analysis

ET-1 was determined using a Quantikine ET-1 immunoassay (R&D Systems, Abingdon UK), a solid phase ELISA containing synthetic ET-1 and antibodies raised against synthetic ET-1. The immunoassay utilises the quantitative sandwich enzyme immunoassay technique. To summarise, the microplate was pre-coated with a monoclonal antibody specific for ET-1. The immobilised antibody bound to any ET-1 present in the standards and samples pipetted into the wells. Unbound substances were washed away and an enzyme-linked monoclonal antibody (specific for ET-1) was added to the wells. The plate was washed again to remove any unbound antibody-enzyme reagent. A substrate solution was pipetted into the wells and colour development occurred in proportion to the amount of ET-1 bound in the initial step. A stop solution was used to stop colour development and the intensity of the colour was measured. The optical density of each well was determined within 10 minutes using a microplate reader set to 450 nm and 570 nm. The standard curve was constructed for each set of samples assayed, using a four parameter logistic (4-PL) curve fit. A representative standard curve is shown in figure 3.7. Materials were obtained from Sigma (Dorset, UK) unless otherwise noted.

Figure 3.7. Representative standard curve for Endothelin-1 analysis.



3.11.4 Plasma C-reactive protein analysis

CRP was analysed using a Randox Full Range CRP (fs-CRP) assay (Randox, Antrim, UK). The samples were analysed via spectrophotometric analysis on the ILAB 600 analyser (Instrumentation Laboratory UK Ltd, Warrington, UK), using the latex-enhanced immunoturbidimetric assay. The ILAB 600 was calibrated using the CRP calibrator series, also provided by Randox. The calibrator contained human CRP in a stabilised protein matrix. Randox Specific Protein Controls (Levels 1 and 3) were utilised as daily quality controls to monitor the accuracy and precision of the analysis and to ensure that the concentrations of samples analysed were within the range of linearity for this analysis. Once calibration and quality controls were complete, and the coefficient of variation was observed to be <2.5%, the plasma samples were added to cuvettes for quantification of frCRP. The samples reacted with the buffer and anti-CRP coated latex. The formation of the antibody-antigen complex during the reaction resulted in an increase in turbidity, the extent of which was measured as the amount of light absorbed at 570 nm. The concentration of the CRP in each sample can be determined by correlating this against the standard curve previously established during calibration.

3.11.5 Lipid profile analysis

Plasma TG and cholesterol (total and HDL-C) were also measured via spectrophotometric analysis in the ILAB 600. The measurement of plasma TGs and cholesterol is based on the production of quinoneimine from Hydrogen Peroxide whose formation is coupled in these assays to the presence of TGs. Quinoneimine was measured in this assay at 510nm and

700nm and the absorbance at these wavelengths was proportional to the amount of Hydrogen Peroxide produced, which in turn was dependent on the amount of TGs present in each sample.

HDL-C was determined in plasma samples in a similar manner. HDL-C quantification was based on the principal of anti-human β-lipoprotein binding to all lipoproteins other than HDL. The antigen-antibody complexes that form can block enzyme reactions with these lipoproteins. Cholesterol esterase and cholesterol oxidase were added and there is a selective reaction with HDL-C which allows for HDL-C quantification. The ILAB 600 was calibrated for TGs and TC using a Referr IL G Calibrator (Instrumentation Laboratory UK Ltd). The Referr Il G Calibrator is a multicomponent calibrator for which TG values had been pre-established by the supplier. Calibration for HDL-C measurement was undertaken by using a Referr IL HDL-C Calibrator (Instrumentation Laboratory UK Ltd). Serachem Control Level 1 and Serachem Control Level 2 (Instrumentation Laboratory UK Ltd) were utilised as quality controls in order to monitor the accuracy and precision of the analysis. Serachem controls were constituted from lyophilized bovine serum with TG concentrations lower (Serachem Control Level 1) and higher (Serachem Control Level 2) than observed concentrations in plasma isolated at screening. Once calibration and quality controls were complete, and the coefficient of variation was observed to be <2.5%, the plasma samples were added to cuvettes for quantification of TG. TGs and TC were quantified by measuring absorption at 510nm and 700nm and correlating this against the standard curve previously established during calibration. HDL-C levels were quantified by measuring absorption at 600nm and 700nm. LDL-C was not analysed via the ILAB but was calculated based on the Friedewald formula [295]. In addition, the “TC: HDL-C ratio” was calculated. Materials were obtained from Sigma (Dorset, UK) unless otherwise noted.

3.11.6 Fatty acid analysis

Although a method for fatty acid analysis has previously been described in Chapter 2, our lab group subsequently made some improvements to the method. For the NU-AGE participant samples, total lipids were extracted from 500µl of plasma with chloroform/methanol (2:1 v/v) containing 0.01% BHT as antioxidant [296]. The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically. The total lipid fraction was subjected to acid-catalyzed transmethylation for 16 h at 50 ºC, using 1 ml of toluene and 2 ml of 1% sulphuric acid (v/v) in methanol. The resultant FAMEs were purified by thin layer chromatography and visualised under spraying with 1% iodine in

chloroform [297]. After elution, FAMEs were separated and quantified by gas-liquid chromatography using a Hewlett Packard 5890 GC and a SGE BPX70 capillary GC column (30 m x 0.22 mm I.D.; SGE UK Ltd) with helium as carrier gas and using on-column injection. The temperature gradient started at 115 °C for 3 min, then went to 200 °C at 2 °C/min, 2 minutes at 200 °C, and then to 240 °C at 60 °C/min. After 5 minutes at 240°C, it cooled down to 115 °C and equilibrated for 3 minutes before the next injection. Individual methyl esters were identified by reference to authentic standards and to well-characterized fish oil (PUFA-3 from menhaden oil, SUPELCO, Supelco Park, Bellefonte, USA). Data were collected and processed (peak integration) using GC Chemstation (version B04-02). Materials were obtained from Sigma (Dorset, UK) unless otherwise noted.

Chapter 4

NU-AGE Diet Score; design, validation and results

4 NU-AGE Diet Score; design, validation and results

4.1 Introduction

The majority of nutrition research to date has focused on the effects of single nutrients on health. However we consume foods rather than nutrients, and the majority of the meals we consume consist of a number of different foods. Nutrients within foods may have synergistic and additive effects [183, 184, 298, 299]. While focusing on the effects of single nutrients has led to many important discoveries, it is important that more emphasis is placed on the effects of the whole-diet on health outcomes [177, 179, 180, 300]. As mentioned in section 1.9, foods and nutrients can have additive, synergistic or antagonistic effects when consumed in combination. Identifying various dietary patterns is one method that could be, and has recently been used, for making associations with health outcomes. For example, the Mediterranean diet has been shown to be associated with reduced risk of CVD and certain types of cancer [301].

Older age can be a period of poor nutrition due to a wide range of factors such as reduced income, poor dentition, sensory loss and therefore loss of appetite [302-304]. Older adults are also susceptible to reduced gastrointestinal function, which may be a result of disease and increased medication, and may result in impaired digestion and absorption of nutrients [304, 305]. Other physiological changes associated with senescence, which can affect nutritional requirements due to changes in body composition, include reduction in lean body tissue and potentiating sarcopenia [304, 306]. Therefore nutrient intake and utilisation in the elderly can often be inadequate [307-309], and general adult recommendations for nutrients that are particularly important in old age, such as vitamin D, vitamin B12, folate, iron and calcium, are often not met [310].

Following on from general adult populations, it is important that there are specific dietary guidelines for the elderly which are designed to consider the specific nutritional requirements of this large subpopulation. Table 3.2 (chapter 3) outlines the NU-AGE dietary guidelines. These guidelines have been designed with the primary aim of reducing inflammation in older adults. Berendsen et al. give some insight into how these NU-AGE guidelines were created [182]. For example, oily fish and olive oil were included in the NU-AGE recommendations as previous studies have shown that both oily fish, olive oil, monounsaturated fatty acids (MUFAs) and PUFAs have positive effects on a range of outcomes associated with ageing, including blood pressure [311], inflammation [118],

cognitive function and depression [312]. Guidelines that focus on protein rich foods, such as meat and legumes, are important as protein has been shown to reduce sarcopenia development in old age [313]. Dietary guidelines focusing on dairy products and the provision of vitamin D supplements are important as higher calcium and vitamin D intakes are associated with higher Bone Mass Density (BMD) and reduced morbidity associated with falls in the elderly [314]. In addition, hypovitaminosis D has been reported as being widespread and is said to be re-emerging as a global health issue and in particular in older adults [315, 316]. Wannamethee et al. have previously shown inverse associations between both vitamin C and fruit intake with CRP concentrations [317], while wholegrain foods have been shown to be important in several aspects of healthy ageing including CVD [318] and BMI [319]. A diet combining such foods and nutrients could be more effective than simply targeting single nutrients as foods may have synergistic effects when consumed together.

A number of diet indices/scores have previously been created in order to examine dietary patterns and their potential association with health outcomes. The development of such a score, specific for the older population, based on the NU-AGE diet recommendations is a major aim of this PhD work programme. Such a diet score does not currently exist. Firstly, the developed diet score was used on an existing cohort (TWIN UK data) in order to validate and optimise it. This diet score could be used to assess whether overall shifts in dietary pattern and nutrient intake have an effect on inflammation (CRP) but also on various measures of cardiovascular health in the NU-AGE cohort. However, such analysis is not part of this thesis as follow-up data was not available at the time of submission.

As discussed in section 1.9, there are currently a number of diet scores which are widely used to assess the overall quality of adult diets. For example, the Mediterranean Diet Score is a score which measures adherence to the Mediterranean diet by assigning a score of either 1 or 0 to 9 different dietary components that were considered either beneficial (fruit and nuts, vegetables, legumes, cereal and fish) or detrimental (meat, poultry and dairy products) [181]. A diet score has also been created to assess effects of the DASH diet (a diet designed to reduce blood pressure) on health [208]. The HEI is another major diet score and is based on the FBDGs given by the USDA and consists of 12 food groups. Research has shown that men and women who get the highest HEI score have a 23% lower risk of suffering from CHD, and a 16% lower risk of major chronic disease [209]. Although a diet score designed specifically for the elderly (EDI) has already been published, the NU-AGE diet score is based on different diet goals and also aimed to overcome some of the limitations, as previously discussed. This

could subsequently be used to assess habitual diet quality and adherence to intervention in future observational studies and RCTs in older adult populations.

4.2 Design and validation of the NU-AGE diet score

Before the NU-AGE Diet Score was used on the NU-AGE cohort, it was validated in a different population in order to confirm its functionality and to determine its ability to discriminate individuals for health outcomes, such as blood pressure. This was conducted using data from the TWIN UK cohort, a study which has previously been described [320, 321]. To summarise, these data were collected from 3262 healthy female twin participants residing in the UK. The twins were aged between 18 and 79 years old and completed FFQs, lifestyle questionnaires and attended clinical assessments over the last 20 years. This dataset contains a variety of outcome measures including CRP; the primary outcome of the NU-AGE intervention. A range of other useful health outcomes related to cardiovascular health were also available including PWV, cholesterol (TC, LDL-C and HDL-C), TGs and blood pressure. Although the NU-AGE diet is designed for older adults, all age groups were used in the validation process to increase sample size.

4.2.1 Methods

Food groups

Using the NU-AGE diet information (Tables 3.1 and 3.2 (Chapter 3)), each food group and the recommended guideline intake for each group were examined. 13 food categories were created for use in this diet score (table 4.1). Although recommendations were given to the NU-AGE participants to limit the use of sugar and salt, these were not included as categories of the diet score due to the wide variety of foods in which they are found. The TWIN UK FFQ (annex 7) was used to assign each food to a food category as shown in table 4.1. There were several limitations to the use of this FFQ. For example, there were no data on the amount of oil consumed. It was also sometimes hard to distinguish between a healthy and unhealthy food product; for example in the case of vegetable soup one cannot determine if it is a homemade more healthful version or a high salt processed product. Such products were initially all included, with the exception of fish products. Two fish food categories were created; one included all fish products and the other included non-processed fish only. It was decided that the non-processed fish category would be used as it was thought to represent a healthier food group.

Table 4.1. NU-AGE diet score food groups with each food assigned from TWIN UK FFQ

Food Group	Individual Foods
Fruit (servings/day)	Apples, Bananas, Dried Fruit, Grapefruit, Grapes, Melon, Oranges, Peaches, Pears, Pure Fruit Juice, Strawberries, Tinned Fruit
Wholegrain (servings/day)	All Bran, Branflakes, Brown Bread, Brown Rice, Fruit n Fibre, Muesli, Oat Based Cereal, Wholemeal Bread, Wholemeal Pasta, Porridge
Vegetables (grams/day)	Avocado, Baked Beans, Beansprouts, Beetroot, Broccoli, Brussels Sprouts, Cabbage, Carrots, Cauliflower, Dried Lentils, Garlic, Green Beans, Green Salad, Leeks, Mushrooms, Onions, Parsnips, Peas, Spinach, Sweet Peppers, Sweetcorn, Tofu, Tomatoes, Veg Soup, Watercress, Marrow, Coleslaw
Dairy (grams/day)	Butter, Dairy Cottage Cheese, Dairy Full Fat Yog, Dairy Low Fat Yog, Dairy cheese, Dairy double, Dairy single, Dried Milk, Evaporated Milk, Full Milk, Goats Milk, Semi Skimmed Milk, Skimmed Milk
Cheese (grams/day)	Dairy Cottage Cheese , Dairy cheese
Potatoes, Pasta and Rice (grams/day)	Boiled potato, Brown Rice, Roast Potatoes, White Rice, White pasta, Wholemeal Pasta
Oil and Fat* (grams/day)	Block Marg, Butter, Low Fat Spread, PUFA Marg, Very Low Fat Spread
Non-Processed Seafood (grams/week)	Fish roe, Oily fish, Shellfish, White fish
Meat (grams/week)	Bacon, Beef, Corned Beef, Ham, Lamb, Liver, Pies, Pork, Poultry, Sausages, Burgers, Meat Soup
Nuts (grams/week)	Nuts
Eggs (grams/week)	Eggs
Alcohol (glass/day)	Beer, Liqueurs, Spirits, Wine
Fluid (ml/day)	Channel Island Milk, Coffee, Cocoa, Coffee Decaffeinated, Diet fizzy, Fizzy Soft Drinks, Fruit Squash, Full Milk, Goats Milk, Horlicks, Pure Fruit Juice, Rice Milk, Semi Skimmed Milk, Skimmed Milk, Soya Milk, Tea

* In the TWIN UK FFQ there is no data on amount of oil consumed, therefore for validation this score only contains butter, margarine and spreads

Scoring system

In order to facilitate a sensitive method to distinguish between groups, a scoring system of 0-10 was used to indicate the score for each dietary component, where 0 described inadequate, or sometimes excessive, intake and 10 described the optimum intake. For each category, the sum of all foods was calculated and the total was divided by 7 as the data were given in either servings or grams per week. Grams were used for all categories except fruit and whole grain. Serving data were used for both of these as the grams changed depending on the food. For example, 1 serving of fruit equals 1 apple, 1 banana or 1 orange regardless of the weight. A reverse scoring system was applied to food groups that were recommended to NU-AGE participants but in limited amounts. For example, cheese was given a 0 score for both excess intake and no intake, as were oil/fat and meat/poultry categories (Table 4.2). The alcohol score was based on the NU-AGE recommendations for females of a maximum of 1 glass/day. Table 4.2 lists each food group with the range of scores given from 0-10 and grams/servings to be consumed to attain that score. For example, the recommendation for vegetables is to consume 300g or more each day. If a person reaches this recommendation they were given a score of 10. They were given a score of 0 if they consumed less than 29.99g per day, a score of 1 if they consumed an amount between 30g and 59.99g per day etc. Therefore, intakes of each food within a particular food group determine the score for that food group.

A vegetarian category was created so that vegetarians did not have a reduced score due to the lack of meat/fish consumption. Another category was created so that only those who actually consumed alcohol were scored for the alcohol category. The final score for each participant was the sum of each food group score divided by the total number of food groups (13). However if a person was vegetarian and didn't consume alcohol, their score was averaged out of 11 rather than 13.

To summarise, data from the TWIN UK FFQ were used, foods were divided into their relevant categories (Table 4.1) and servings or grams per day or week were calculated. The final score is the sum of each food score divided by the number of food groups.

Table 4.2. Food groups, recommendations and score assigned to each level of consumption

SCORE	0	1	2	3	4	5	6	7	8	9	10
Whole grains[1]; 4-6 servings/d recommended	0-0.59	0.6-1.19	1.2-1.79	1.8-2.39	2.4-2.99	3.0-3.59	3.6-4.19	4.2-4.79	4.8-5.39	5.4-5.99	6+ servings/d
Fruits[2]; 2+ servings/d	0-0.19	0.2-.39	0.4-.59	0.6-.79	0.8-.99	1-1.19	1.2-1.39	1.4-1.59	1.6-1.79	1.8-1.99	2+ servings/d
Vegetables; 300+ g/d	0-29.99	30-59.99	60-89.99	90-119.99	120-149.99	150-179.99	180-209.99	210-239.99	240-269.99	270-299.99	300g+/d
Dairy; 500 ml/d (including cheese)	0-49.99	50-99.99	100-149.99	150-199.99	200-249.99	250-299.99	300-349.99	350-399.99	400-449.99	450-499.99	500+g/d
Cheese; 30 g/d	0-2.99/ >50	3-5.99	6-8.99	9-11.99	12-14.99	15-17.99 /40-49.99	18-20.99	21-23.99	24-26.99	27-29.99	30-39.99g/d
Potatoes and Pasta/Rice; 150 g/d	0-14.99	15-29.99	30-44.99	45-59.99	60-74.99	75-89.99	90-104.99	105-119.99	120-134.99	135-149.99	150+g/d
Oil/Fat; Oil 20 g/d, margarine 30g/d; maximum 50 gram fat per day	0-2.99/ >50	3-5.99	6-8.99	9-11.99	12-14.99	15-17.99 35-49.99	18-20.99	21-23.99	24-26.99	27-29.99	30-34.99g/d
Fluid; 1.5 l/d	0-149.99	150-299.99	300-449.99	450-599.99	600-749.99	750-899.99	900-1049.99	1050-1199.99	1200-1349.99	1350-1499.99	1500+ ml/d
Fish and other seafood; 250 g per week (2 servings)	0-24.99	25-49.99	50-74.99	75-99.99	100-124.99	125-149.99	150-174.99	175-199.99	200-224.99	225-249.99	250+ g/wk
Meat and poultry; 500 g per week (4 times per week 125 gram meat or poultry)	0-49.99 / >750	50-99.99	100-149.99	150-199.99	200-249.99	250-299.99/ 625-749.99	300-349.99	350-399.99	400-449.99	450-499.99	500-624.99+g/d
Nuts; 40 g per week (2 times per week 20 g)	0-3.99	4-7.99	8-11.99	12-15.99	16-19.99	20-23.99	24-27.99	28-31.99	32-35.99	36-39.99	40g/wk
Eggs; 150 g per week (3 times 50g serving per week)	0-14.99g	15-29.99	30-44.99	45-59.99	60-74.99	75-89.99	90-104.99	105-119.99	120-134.99	135-149.99	150g/wk
Alcohol; Women; Max 1 glass per day	>1	0.1-0.19	0.2-0.29	0.3-0.39	0.4-0.49	0.5-.059	0.6-.069	0.7-0.79	0.8-0.89	0.9-.99	1 glass/d

1; Whole grains

- 4-6 servings/d recommended
- 1 serving of whole grains equals 1 slice of bread (1.5 cm thick) or 25 g of bread, ½ cup of breakfast cereals (50g), ½ cup of cooked brown rice (30g raw), 2/3 cup of wholegrain pasta, cooked (30g raw), 1/3 cup of muesli, ½ cup of porridge.

2; Fruit

- At least 2 servings/d recommended
- 1 serving of fruit equals 1 apple, 1 banana, 1 orange, 1.2 grapefruit, 8 plums (small), approximately 1 glass of fruit juice, ¼ cup of dried fruits.

4.2.2 Statistical analysis

Quartiles of the final diet score were calculated by creating a binned variable of the final diet score and making three cut points within this variable. A 2 way ANOVA was performed to calculate adjusted means and to determine the associations between the diet score quartiles and various CVD outcomes including TC, SBP, DBP, pulse pressure and CRP. If the number of participants was different for each outcome, quartiles of the diet score were made specific to each outcome to avoid skewed results. Results are displayed as means and SEM. Covariates included in the analysis were gender, age, BMI, physical activity, total energy intake and smoking status. 1 way ANOVA was used to determine the associations between the diet score quartiles and characteristics such as nutrient intakes. Chi-square tests were used to examine associations between the diet score quartiles and characteristics that were reported as frequencies or percentages of the total cohort rather than means. All statistical calculations were performed using SPSS statistics (PASW) 18.

4.2.3 First round results and amendments made

The analysis using this initial design of the NU-AGE diet score resulted in no significant associations between increasing diet score on any of CVD health outcomes investigated. The results for this analysis can be viewed in table 4.3.

Table 4.3. First attempt analysis showing associations between diet score quartiles and CVD related outcomes

	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile	p-value
Cholesterol (mmol/l)					
N	624	645	696	626	
Mean (SEM)	5.6 (0.1)	5.6 (0.0)	5.6 (0.0)	5.6 (0.1)	**.701**
Systolic Blood Pressure (mmHg)					
N	792	789	843	727	
Mean (SEM)	123 (1)	121 (1)	121 (1)	121 (1)	**.117**
Diastolic Blood Pressure (mmHg)					
N	792	789	844	727	
Mean (SEM)	77 (0)	76 (0)	77 (0)	77 (0)	**.468**
Pulse Pressure (beats per minute (BPM))					
N	485	495	521	460	
Mean (SEM)	44 (0)	44 (0)	43 (0)	43 (0)	**.261**
C-Reactive Protein (mg/l)					
N	549	592	612	541	
Mean (SEM)	2.7 (0.1)	2.6 (0.1)	2.5(0.1)	2.5 (0.1)	**.187**

Number of participants in each group is detailed in the "N" rows. Results are displayed as mean and SEM. P-values displayed in final column, significant when p>0.05.

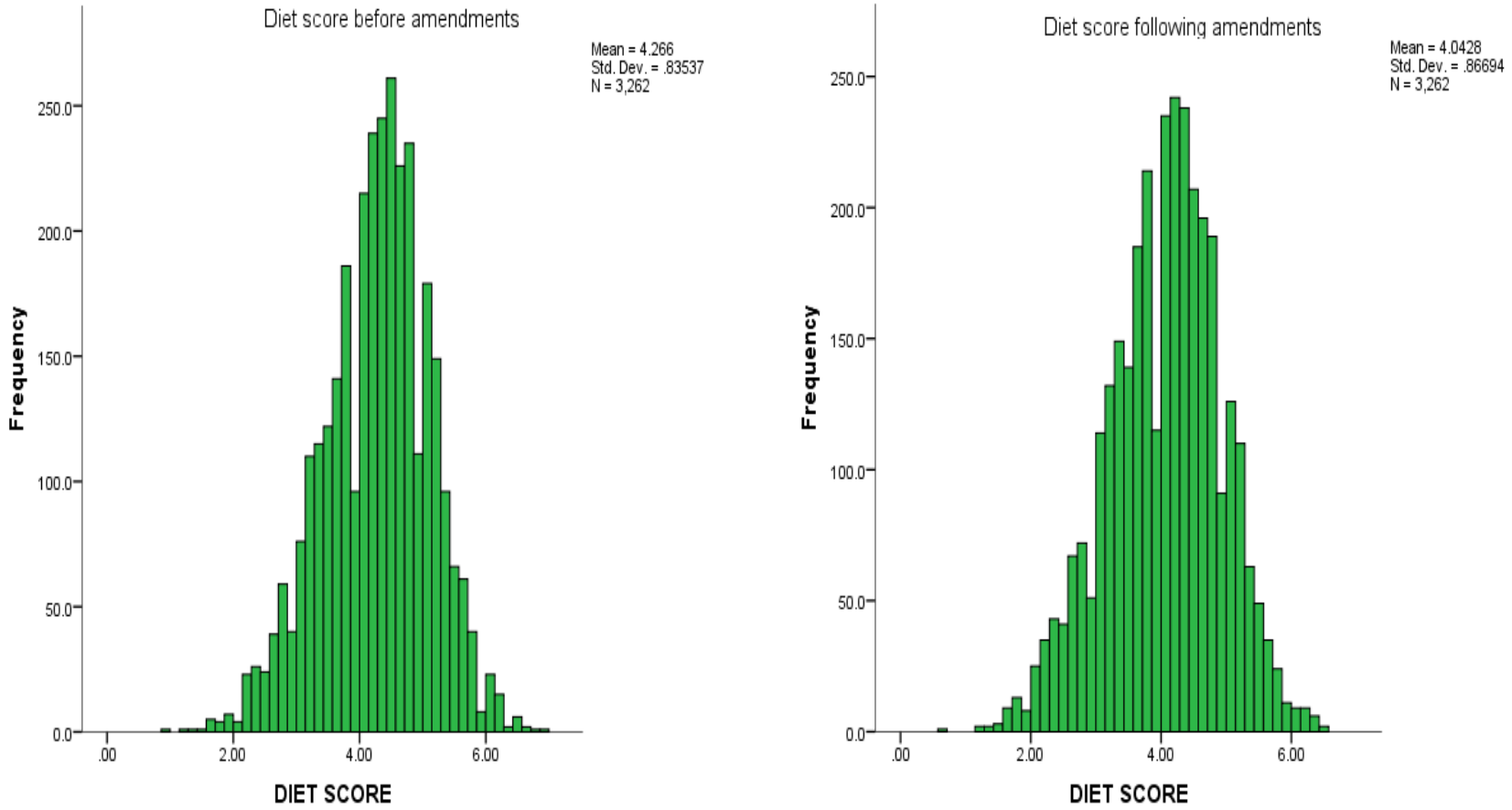
The frequency of scores for each food group was examined and this analysis showed that the data were skewed towards a higher score for most food groups and therefore the diet score did not appear to provide a sensitive measure of the dietary pattern we aimed to define. As a result, a number of changes were made to the diet score in order to try and create a more sensitive measure that resulted in a better distribution of the participants across the diet score. Firstly, several foods that were difficult to classify were removed from a number of the food groups as shown in table 4.4. Most of the foods removed were foods that could not be differentiated into healthy or unhealthy versions in an attempt to make each food group focus on 'healthier' options. In this way, participants potentially consuming unhealthy

alternatives would not be given as high a score. For example, fruit products with the potential to have added sugar were removed from the fruit category. The dairy food category was refined to contain less high fat products by removing products such as butter and cream. The oil and fat categories were refined to remove less healthy options such as butter. All processed meat products were removed. The alcohol category was amended to only include wine. It was also decided that BMI and PWV would be examined as additional outcomes. However the PWV data were collected more recently than other outcomes and therefore were not available for all participants and had its own FFQ data due to the administration of an updated questionnaire. The distribution of the diet score before and after these amendments are graphically depicted in figure 4.1. This figure shows that the amendments made to the diet score resulted in a slightly more evenly distributed diet score among the TWIN UK cohort.

Table 4.4. NU-AGE diet score food groups and food removed from each in an attempt to improve healthfulness

Food group	**Foods removed**
Fruit	Fruit juice, tinned fruit
Wholegrain	None
Vegetables	Baked beans, tofu, veg soup, coleslaw
Dairy	Butter, dairy cottage cheese, dairy cheese, dairy double cream, dairy single cream, evaporated milk
Cheese	None
Potatoes, pasta and rice	None
Oil and Fat	Block margarine, butter
Non-Processed Seafood	None
Meat	Bacon, corned beef, ham, meat pies, burgers, meat soup
Nuts	None
Eggs	None
Alcohol	Beer, liquors, spirits
Fluid	Cocoa, fizzy soft drinks, Horlicks

Figure 4.1. Distribution of diet score results for the TWIN UK cohort before and after amendments



4.2.4 Final results; Analysis of the NU-AGE diet score on TWIN UK population

The validation of the NU-AGE diet score was performed using cross-sectional data from the TWIN UK cohort (n=3262). All participants in this study were female and were aged between 18 and 79 and the mean age (± SEM) was 48 ± 0.

50.7% of the cohort never smoked, while 18.5% and 30.8% were current or former smokers respectively. 23.4% of the cohort were physically active, while 23.0% and 53.6% were inactive and moderately active respectively. In addition, it was shown that 83.1% of the participants drank alcohol. 28.8% of participants were hypercholesterolaemic (defined as TC >6.2mmol/l). 30.2% and 12.5% of the participants were overweight and obese respectively. 20.0% of the cohort had hypertension (defined as having a SBP over 140mmHg or a DBP over 90mmHg [322]).

Table 4.5 shows the associations between nutrient intakes and diet score quartiles in the Twin UK cohort. Total energy intake (kcal) was significantly associated (p= <0.001) with increased NU-AGE diet score; average total energy intakes increased from 1626.2 ± 16.9 (Q1) to 2315.1 ± 16.7 (Q4). In addition to this, nutrient intakes for all other nutrients expressed as grams, with the exception of alcohol, correspondingly increased significantly (p= <0.001) with increased adherence to the NU-AGE diet score. Nutrient intake was therefore calculated as percentage of total kcal (table 4.6). Total fat, saturated fat and MUFA intakes were associated with a significantly lower diet score when expressed as percentage of total energy intakes, while intakes of polyunsaturated fat, carbohydrate, fibre, sugar, protein, sodium and water were associated with a significantly higher score.

Table 4.6 shows the frequencies of covariates according to quartiles. Age was significantly associated with increased score (p= <0.001); from 45 to 51 years between Q1 and Q4 of the NU-AGE diet score. BMI was also associated with significantly increased diet score; (p= <0.001) from 24.7 to 25.3 kg/m^2 between the 1st and 4th quartiles. Higher diet score quartiles were associated with increased physical activity and lower rates of current smokers when compared with the lower quartiles of the diet score.

Table 4.5. Nutrient intake according to NU-AGE diet score quartiles in 3262 participants from the TWIN UK cohort

	1st Quartile (n=768)	2nd Quartile (n=855)	3rd Quartile (n=849)	4th Quartile (n=790)	Total (n=3262)	p-value
Energy (kcal/d) Mean (SEM)	1626.2 (16.9)[a]	1879.0 (15.8)[b]	2089.4 (15.8)[c]	2315.1 (16.7)[d]	1979.9 (9.2)	**0.000***
Fat (% of kcal/d) Mean (SEM)	32. 6 (0.2)[a]	31.5 (0.2)[b]	31.0 (0.2)[bc]	30.3 (0.2)[c]	31.3 (0.1)	**0.000***
Saturated Fat (% of kcal/d) Mean (SEM)	12.5 (0.1)[a]	11.9 (0.1)[b]	11.5 (0.1)[b]	10.8 (0.1)[c]	11.7 (0.1)	**0.000***
Mono-unsaturated Fat (% of kcal/d) Mean (SEM)	10.9 (0.1)[a]	10.4 (0.1)[b]	10.1 (0.1)[bc]	9.9 (0.1)[c]	10.3 (0.1)	**0.000***
Poly-unsaturated Fat (% of kcal/d) Mean (SEM)	6.8 (0.1)[a]	6.8 (0.1)[ab]	6.9 (0.1)[abc]	7.1 (0.1)[c]	6.9 (0.1)	**0.000***
Carbohydrates (% of kcal/d) Mean (SEM)	50.2 (0.3)[a]	51.5 (0.2)[b]	52.7 (0.2)[c]	52.9 (0.2)[d]	51.8 (0.1)	**0.000***
Sugars (% of kcal/d) Mean (SEM)	23.5 (0.2)[a]	25.3 (0.2)[b]	26.5 (0.2)[c]	26.7 (0.2)[d]	25.5 (0.1)	**0.000***
Protein (% of kcal/d) Mean (SEM)	16.1 (0.1)[a]	16.7 (0.1)[b]	16.8 (0.1)[b]	16.9 (0.1)[b]	16.6 (0.1)	**0.000***
Alcohol (g/d) Mean (SEM)	10.0 (0.6)	9.6 (0.5)	8.9 (0.4)	10.5 (0.4)	9.7 (0.2)	**0.082**
Fibre (g/d) Mean (SEM)	13.8 (0.2)[a]	18.3 (0.2)[b]	22.4 (0.2)[c]	26.4 (0.3)[d]	20.3 (0.1)	**0.000***
Sodium (mg/d) Mean (SEM)	1797.4 (0.9)[a]	2092.5 (0.8)[b]	2370.5 (0.8)[c]	2764.6 (0.9)[d]	2258.1 (0.4)	**0.000***
Water (g/d) Mean (SEM)	2182.1 (25.5)[a]	2639.8 (20.4)[b]	2960.4 (23.3)[c]	3276.9 (23.7)[d]	2769.8 (13.5)	**0.000***

Results are displayed as mean and SEM. P-values displayed in final column, significant when p>0.05. Asterix marks significance. Superscripts not sharing a common letter were significantly different (P < 0.05).

Table 4.6. Covariate frequencies according to diet score quartiles in the TWIN UK cohort

	1st Quartile (n=768)	2nd Quartile (n=855)	3rd Quartile (n=849)	4th Quartile (n=790)	Total (n=3262)	p-value
Age (years)						
Mean (SEM)	45 (0)^{a}	48 (0)^{b}	49 (0)^{bc}	51 (0)^{bc}	48 (0)	0.000*
BMI (kg/m^2)						
Mean (SEM)	24.9 (0.2)^{a}	25.3 (0.2)^{b}	25.2 (0.2)^{abc}	25.2 (0.2)^{bc}	25.2 (0.1)	0.013*
Physical Activity						0.000*
Inactive (%)	29.9^{a}	24.0^{b}	20.5^{bc}	18.0^{c}	23.1	
Moderately Active (%)	52.9^{a}	54.7^{a}	54.2^{a}	52.4^{a}	53.6	
Active (%)	17.2^{a}	21.3^{ab}	25.3^{bc}	29.6^{c}	23.4	
Smoking Status						0.000*
Current smokers (%)	27.5^{a}	20.2^{b}	14.8^{c}	12.0^{c}	18.6	
Former smokers (%)	25.3^{a}	31.6^{b}	33.0^{b}	32.9^{b}	30.7	
Never smoked (%)	47.2^{a}	48.2^{a}	52.2^{ab}	55.1^{b}	50.7	

Results are displayed as percentage of group or mean and SEM. P-values displayed in final column, significant when p>0.05. Asterix marks significance. Superscripts not sharing a common letter were significantly different (P < 0.05).

The mean diet score among all participants was 4.04 ± 0.87. Linear regression revealed that the 4th quartile (i.e. participants with highest diet score) was associated with a significantly lower level of CRP compared with the first quartile; CRP decreased from 2.85 (CI; 2.64-3.06) to 2.44 (CI; 2.25-2.64) mg/l (p=0.028). No significant associations were observed between a high diet score and other cardiovascular related outcomes (see table 4.7), although there was a trend towards a decrease in blood pressures with increased diet score.

Table 4.7. Associations between diet score quartiles and CVD related outcomes

	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile	p-value
Cholesterol (mmol/l)					
N	591	675	682	643	
Mean (SEM)	5.6 (0.0)	5.6 (0.0)	5.6 (0.0)	5.6 (0.0)	**.700**
Systolic Blood Pressure (mmHg)					
N	745	834	809	763	
Mean (SEM)	123 (1)	121 (1)	122 (1)	120 (1)	**.115**
Diastolic Blood Pressure (mmHg)					
N	745	834	809	764	
Mean (SEM)	77 (0)	77 (0)	77 (0)	76 (0)	**.354**
Pulse Pressure (BPM)					
N	453	532	509	467	
Mean (SEM)	44 (1)	44 (0)	43 (0)	43 (0)	**.334**
Pulse Wave Velocity (m/s)					
N	214	209	166	169	
Mean (SEM)	9.2 (0.1)	9.2 (0.1)	9.1 (0.1)	9.2 (0.1)	**.742**
C-Reactive Protein (mg/l)					
N	508	611	604	571	
Mean (SEM)	2.9 (0.1)[a]	2.6 (0.1)[b]	2.4 (0.1)[b]	2.4 (0.1)[b]	**.028***
BMI					
N	767	855	848	789	
Mean (SEM)	24.9 (0.2)	25.3 (0.2)	25.2 (0.2)	25.2 (0.2)	**.417**

Number of participants in each group is detailed in the "N" rows. Results are displayed as mean and SEM. P-values displayed in final column, significant when p<0.05. Asterix marks significance. BMI; Body Mass Index

4.2.5 Discussion; design and validation of the NU-AGE diet score on TWIN UK population

To summarise, the design of the NU-AGE diet score involved forming specific food categories based on the NU-AGE dietary guidelines, assigning foods into one of these groups, and calculating servings or grams consumed per day or per week. A scoring system was devised in which each food category was scored from 0 to 10 based on intakes. The total score for each category was then calculated and divided by the number of food groups relevant to each participant to give the final score for each participant. The NU-AGE diet score was based on the NU-AGE diet goals which were guidelines that are similar to current dietary guidelines but tailored specifically towards an ageing population. The aim of the NU-AGE diet score was to assess quality across the whole-diet and to investigate how adherence to the NU-AGE diet affects various outcomes specifically related to CVD. The NU-AGE diet score was developed as a potential method to determine adherence to the NU-AGE intervention as well as a score that could be used to determine the dietary adequacy in older populations (if validated). To validate the NU-AGE diet score, the diet score was applied to the TWIN UK cohort.

The TWIN UK cohort has previously been shown to be representative of the general population with respect to hypertension and dietary intake [323, 324]. Prevalence of overweight and obesity in this cohort is slightly less than the general UK population [325]. The mean cholesterol and CRP of participants is representative of the UK population [326]. Initial analysis examining the impact of the NU-AGE diet score did not result in any significant associations with CRP or any other measures of cardiovascular health (Table 4.3). The data were re-examined and it was determined that the data for each food group highly skewed with the majority of participants easily reaching the food goals. Therefore several amendments were made to the diet score (Table 4.5). The majority of these amendments involved attempting to exclude food products that were not necessarily healthful products but had previously been allowing participants to attain a high score. For example, in the initial analysis the dairy category had contained a large range of high fat products such as cream and butter. These food products were removed and the impact of increased adherence to the NU-AGE diet on CRP and other cardiovascular health outcomes was once again examined following the amendments.

Following these amendments, a significant association was observed; a 0.4 mg/l decrease in CRP between Q1 and Q4. This is similar to the decrease of 0.5 mg/l reported for those consuming the Mediterranean diet with olive oil in the PREDIMED intervention [83].

Considering that the NU-AGE diet was specifically designed to reduce inflammation and the primary outcome of the NU-AGE diet is CRP, the significant reduction of CRP associated with increased NU-AGE diet among TWIN UK participants (assessed by calculating the NU-AGE diet score designed as described) confirmed the functionality of the diet score. Therefore the NU-AGE diet score was considered validated and suitable for subsequent use on the NU-AGE cohort. Although not reaching significance, trends for a reduction in blood pressure related outcomes could be seen in Q4 when compared with the lower quartiles. It is possible that these figures could reach significance if tested on a larger cohort.

We also examined nutrient intakes according to quartiles of the NU-AGE diet score. An increase in total energy intake (kcal) was significantly associated with an increasing NU-AGE diet score. The increases in total energy intakes associated with the NU-AGE diet score for older adults can be beneficial because the elderly can suffer from involuntary weight loss if energy intakes are inadequate [327]. Total fat, saturated fat and MUFA intakes were significantly associated with a lower diet score when expressed as percentage of total energy intakes, while intakes of polyunsaturated fat, carbohydrate, sugar, fibre, protein, sodium and water were associated with a significantly higher score. Reduced fat (excluding polyunsaturated fat) consumption could be contributing to the significant association in CRP levels across quartiles. The intakes of nutrients that were associated with a significant increase in participants in the 4th quartile were generally favourable nutrients (with the exception of sugar and sodium) and this could also have contributed to the reduction in CRP. The increased sugar could be a result of increased fruit consumption, however as sugar based food products were not included as a food group in the diet score it is difficult to decipher exactly why the 4th quartile was associated with a significantly higher consumption of sugar. The higher level of sodium consumption seen in Q4 could be associated with the food goal that recommends the consumption of 30g of cheese per day. Although the NU-AGE guidelines state that a reduced fat and salt cheese is preferable, in this analysis it was not possible to include only cheeses that were reduced in salt. This could also be a potential explanation for why a significant reduction in blood pressure related outcomes was not seen in this analysis. However, neither sugar nor salt intakes were included as food goals in the diet score, therefore the NU-AGE diet score does not account for them in this analysis.

There were several limitations involved in the use of this cohort for validation purposes. This study contains female participants only and therefore it cannot yet be concluded that the diet score is functional for men. Despite the diet score being tailored for the elderly, all age

groups were included in order to have sufficient numbers for each dependent variable. As previously discussed, the use of a FFQ rather than a food diary tends to make it more difficult to distinguish between certain food groups, for example the difference between a homemade healthy soup and a processed version that could be less healthy due to a high salt content. Despite the limitations, overall the TWIN UK dataset was deemed suitable due to the wide variety of foods included in the FFQ as well as the number of cardiovascular related outcomes available.

4.3 Using the NU-AGE diet score on the NU-AGE cohort

Following validation of the NU-AGE diet score on the TWIN UK data, the diet score was tested on the NU-AGE cohort (baseline data) in order to assess if a higher diet score was associated with improved cardiovascular health in this older aged population. 7-day food diaries were used to assess dietary intakes in the NU-AGE cohort which allowed for a more accurate and detailed analysis in comparison to the use of FFQs [328]. However, this also resulted in the need for a more detailed investigation into the division of foods into corresponding food categories as there were over 2,000 food codes used in the analyses of the NU-AGE dietary data.

4.3.1 Methods

Individual foods were automatically assigned to a food group using the nutritional analysis software WISP. However, in certain cases these food groups were not suitable for the current analysis and so further investigation and re-assignment of the food codes was required manually. For example, a group titled “fish products” needed to be investigated to determine which foods should be included in the fish diet score group. Using the WISP food groups, re-assignment was decided as follows;

a) which groups could have all foods contained within that group assigned to a food category,
b) which food groups would be fully excluded from any food category, and
c) which food groups would have to be individually examined to determine which foods are included in which category.

To summarise, these food groups are shown in table 4.8. For section c, the rules that determined what foods were included and excluded from each category are summarised in table 4.9. To decide which foods belonged in the wholegrain category, a number of food

groups had to be examined to select wholegrain only products. The NU-AGE advice for wholegrain foods was to consume brown rice, wholegrain pasta or other grain, wholegrain bread, Ryvita crispbread, and wholegrain cereal, such as oatmeal. These foods were automatically assigned by WISP to the following food groups; flours, grains and starches, breads, rolls, breakfast cereals, biscuits. To determine which foods in these groups should be defined as a wholegrain, we decided to use a method of identifying wholegrain foods that had recently been proposed in a 2013 study as the method to identify the most healthful wholegrain products [329]. Therefore, in order to be assigned from these food groups to the wholegrain category they needed to have a ≤10:1 ratio of total carbohydrate to fibre content. Brown varieties of pasta and rice were selected from the rice and pasta groups to also be included in the wholegrain category. Once the assignment of foods into the correct categories was complete, dietary intakes of each food category were determined for each participant and a diet score was calculated using the same method used on the TWIN UK data discussed above.

Table 4.8. Division of food groups into assigned food categories

A.	**Food Groups with all foods included (Food Group)**	Rice (Pasta, Potatoes, Rice), Pasta (Pasta, Potatoes, Rice), Potatoes (Pasta, Potatoes, Rice), Early Potatoes (Pasta, Potatoes, Rice), Main Crop Potatoes (Pasta, Potatoes, Rice), Skimmed Milk (Dairy), Semi-skimmed milk (Dairy), Whole Milk (Dairy), Yoghurts (Dairy), Yoghurts (Dairy), Whole Milk Yoghurts (Dairy), Low Fat Yoghurts (Dairy), Cheeses (Cheese), Eggs (Eggs), Vegetables, general (Vegetables), Beans and Lentils (Vegetables), Peas (Vegetables), Fruit, general (Fruit), Beef (Meat), Lamb (Meat), Pork (Meat), Chicken (Meat), Duck (Meat), Pheasant (Meat), Turkey (Meat), Rabbit (Meat), Venison (Meat), Offal (Meat), Juices (Fluid), Squash and Cordials (Fluid),
B.	**Food Groups with all foods excluded**	Pizza, Cakes, Pastry, Buns and Pastries, Puddings, Savouries, All creams, Ice Cream, Puddings and Chilled desserts, Savoury dishes and sauces, Herbs and spices, Sugars, Preserves and Snacks, Soups, sauces and miscellaneous foods, Beers, Cider, Liquers, Spirits
C.	**Food Groups to be manually separated (Food Groups)**	Flours, grains and starches (wholegrain), Breads (Wholegrain), Rolls (Wholegrain), Breakfast cereals (Wholegrain), Biscuits (Wholegrain), Rice (Wholegrain), Pasta (Wholegrain),Processed Milks (Dairy), Milk Based Drinks (Dairy), Other Milks (Dairy), Margarines (Fat/Oil), Egg dishes (eggs), Chipped Potatoes (Pasta, Potatoes, Rice), Potato Products (Pasta, Potatoes, Rice), Vegetable Dishes (Vegetables), Fruit Juice (Fruit), Nuts and Seeds (Nuts), White Fish (Fish), Fatty Fish (Fish), Crustacea (Fish), Molluscs (Fish), Fish Products (Fish), Meat Products (Meat), Meat Dishes (Meat), Bacon (Meat), Fats and Oils, Powdered Drinks and essences (Fluid), Infusions (Fluid), Soft Drinks (Fluid), Carbonated Drinks (Fluid), Wines (Alcohol)

A) The groups in which all foods contained within that group are assigned to a food category (assigned food category in brackets), B) The food groups that are fully excluded from any food category, C) The food groups that have been individually examined to determine which foods are included in which category.

Table 4.9. Summary of rules to determine inclusion/exclusion of foods from each food group

Food Group	Rule
Flours, grains and starches (wholegrain)	Content of total carbohydrate to fibre of ≤10:1 ratio
Rice (Wholegrain)	Brown
Pasta (Wholegrain)	Brown
Breads (Wholegrain)	Content of total carbohydrate to fibre of ≤10:1 ratio
Rolls (Wholegrain)	content of total carbohydrate to fibre of ≤10:1 ratio
Breakfast cereals (Wholegrain)	Content of total carbohydrate to fibre of ≤10:1 ratio
Biscuits (Wholegrain)	Content of total carbohydrate to fibre of ≤10:1 ratio
Vegetable Dishes (Vegetables)	Excluded everything, except vegetable stir fry mixes, salads.
Fruit Juice (Fruit)	Excluded all, except freshly squeezed.
Nuts and Seeds (Nuts)	Include nuts. Seeds, nut butters and pastes excluded.
White Fish (Fish)	Exclude fish in batter.
Fatty Fish (Fish)	Excluded pate.
Crustacea (Fish)	Exclude fish in batter.
Fish Products and Dishes (Fish)	Included fish with sauces, breaded fish. Excluded pies, pate, battered, fish cakes, fish fingers.
Meat Products (Meat)	Included meat slices, meat in breadcrumbs. Excluded sausage, pies, pate, beef burgers.
Meat Dishes (Meat)	Included meat dishes with sauce only. Excluded meat in dishes with others foods such as potato.
Fats and Oils,all (fats and oils)	Included vegetable oils like olive, canola, sunflower and soybean oil. Excluded butter, lard, shortening and hard block margarine.
Powdered Drinks and essences (Fluid)	Included those made up with liquid. Excluded powders not yet made up.
Infusions (Fluid)	Included those made up with liquid. Excluded powders not made up
Soft Drinks (Fluid)	Included sugar free.
Carbonated Drinks (Fluid)	Included sugar free.
Wines (Alcohol)	Included red wines only.

4.3.2 Statistical Analysis

The analysis performed on the NU-AGE cohort was the same as described in section 4.2.2 for the TWIN UK data. However, several additional outcomes were analysed including CAVI, ABI and RHI. As both males and females were included in this analysis, gender was also added as a covariate. All other covariates were the same as the TWIN analysis and included gender, age, BMI, physical activity, total energy intake and smoking status. Cardiovascular and hypertensive medication data was also available and therefore was also added as a covariate. Blood pressure measurements and clinical measurements of vascular function were available for 248 participants at baseline. However at the time of analysis, biochemical data was only available for the subgroup of participants (n=136) included in the dataset described in chapter 5.

4.3.3 Results

248 NU-AGE participants were included in this analysis and results shown are based on baseline data. The cohort used for this analysis was made up of both men and women; 37.1% men and 62.9% women. The age ranged from 65-79 years, the mean age (± SEM) was 70 ± 0 years. 61.9% of the cohort never smoked, while 36.8% were ex-smokers and only 1.2% were current smokers. The average energy expenditure (kcal/d assessed by actigraph) for the cohort was 299.7 ± 11.6 kcal/d. 89.9% of the cohort consumed alcohol. 27.2% of the cohort were hypercholesterolaemic (defined as TC >6.2mmol/l). 47.6% and 18.1% of the cohort were overweight and obese respectively, while 44.8% were hypertensive (defined as having a SBP over 140mmHg or a DBP over 90mmHg [322]).

Table 4.10 shows the nutrient intakes according to diet score quartiles in the NU-AGE cohort and statistical significance. There were no significant differences among the quartiles for total energy intake. Total fat, monounsaturated fat, polyunsaturated fat, protein and fibre intakes were all associated with a significantly ($p<0.05$) increased diet score result. Low carbohydrate intakes were significantly associated with an increased diet score, while there were no significant associations for saturated fat, sugar, alcohol, sodium, and water intakes among the quartiles.

The means or frequencies of the covariates for each quartile are shown in table 4.11. There were no significant associations between the quartiles for age, BMI or physical activity. Participants in Q3 seemed to have a slightly higher percentage of former smokers and less people that never smoked when compared with the other quartiles.

Table 4.10. Nutrient intake according to diet score quartiles in the NU-AGE cohort

	1st Quartile (n=62)	2nd Quartile (n=65)	3rd Quartile (n=59)	4th Quartile (n=62)	Total (n=248)	p-value
Energy (kcal/d) Mean (SEM)	1856.8 (55.8)	1840.3 (49.1)	1894.4 (51.4)	1923.6 (41.3)	1878.1 (24.8)	**0.630**
Fat (% of kcal/d) Mean (SEM)	33.8 (0.7)[a]	35.3 (0.7)[ab]	34.2 (0.8)[ab]	36.6 (0.6)[b]	35.0 (0.4)	**0.021***
Saturated Fat (% of kcal/d) Mean (SEM)	13.1 (0.4)	13.2 (0.4)	12.6 (0.4)	13.2 (0.4)	13.0 (0.2)	**0.655**
Mono-unsaturated Fat (% of kcal/d) Mean (SEM)	11.2 (0.3)[a]	11.8 (0.3)[abc]	11.4 (0.3)[ab]	12.8 (0.3)[c]	11.8 (0.2)	**0.001***
Poly-unsaturated Fat (% of kcal/d) Mean (SEM)	5.2 (0.2)[a]	5.7 (0.2)[ab]	5.5 (0.2)[ab]	6.1 (0.2)[b]	5.7 (0.1)	**0.018***
Carbohydrates (% of kcal/d) Mean (SEM)	48.2 (0.9)[a]	46.2 (0.7)[ab]	47.1 (0.8)[ab]	45.0 (0.7)[b]	46.6 (0.4)	**0.025***
Sugars (% of kcal/d) Mean (SEM)	23.7 (0.7)	23.0 (0.6)	23.9 (0.7)	22.7 (0.7)	23.3 (0.3)	**0.539**
Protein (% of kcal/d) Mean (SEM)	15.7 (0.4)[a]	16.6 (0.3)[ab]	17.0 (0.3)[b]	16.2 (0.3)[ab]	16.4 (0.2)	**0.034***
Alcohol (g/d) Mean (SEM)	10.3 (1.7)	10.0 (1.2)	9.8 (1.2)	10.8 (1.2)	10.2 (0.7)	**0.957**
Fibre (g/d) Mean (SEM)	21.4 (0.9)[a]	22.2 (0.8)[ab]	25.6 (1.2)[bc]	26.4 (1.1)[c]	23.9 (0.5)	**0.000***
Sodium (mg/d) Mean (SEM)	2400.9 (114.3)	2254.1 (81.1)	2402.7 (94.6)	2411.2 (97.0)	2365.4 (48.5)	**0.601**
Water (g/d) Mean (SEM)	2482.2 (95.3)	2614.3 (80.4)	2665.2 (90.2)	2712.5 (82.3)	2617.9 (43.6)	**0.276**

Results are displayed as mean and SEM. P-values displayed in final column, significant when p>0.05. Asterix marks significance. Superscripts not sharing a common letter were significantly different (P < 0.05).

Table 4.11. Covariate frequencies according to diet score quartiles in the NU-AGE UK cohort

	1st Quartile (n=62)	**2nd Quartile** (n=65)	**3rd Quartile** (n=59)	**4th Quartile** (n=62)	**Total** (n=248)	**p-value**
Sex (male)						
N	26	23	18	25	92	0.556
Age (years)						
Mean (SEM)	70 (1)	71 (1)	70 (1)	70 (1)	70 (1)	0.552
BMI						
Mean (SEM)	26.5 (0.6)	27.7 (0.6)	27.7 (0.6)	26.0 (0.4)	26.9 (0.4)	0.061
Physical Activity (kcal/d)						
Mean (SEM)	263.7 (19.0)	309.6 (23.5)	343.4 (24.7)	284.2 (25.0)	300.2 (23.1)	0.095
Smoking Status						
Current Smokers (%)	1.7	0.0	1.7	1.6	1.3	0.599
Former Smokers (%)	31.1	38.5	45.8	32.3	36.9	0.503
Never Smoked (%)	67.2	61.5	52.5	66.1	61.8	0.775
Cardiovascular Disease and Hypertensive Medication Users						
(%)	43.5	43.1	37.3	40.3	41.1	0.890

Results are displayed as mean and SEM, number or percentage of total column. P-values displayed in final column, significant when $p<0.05$.

The NU-AGE diet score ranged from 2.5 to 8.5 in the NU-AGE cohort, with a mean of 5.1 ± 0.1. The results determining the effects of diet score quartiles on cardiovascular related outcomes are shown in table 4.12. There were no significant associations between an increased NU-AGE diet score with any of the health outcomes measured in this analysis. Plasma levels of CRP did decrease in Q4 compared with Q1; from 1.72 ± 0.20 to 1.16 ± 0.19 mg/l, however this result was not significant (p=0.121).

Table 4.12. Associations between diet score quartiles and CVD related outcomes in the NU-AGE UK cohort

	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile	p-value
Systolic Blood Pressure (mmHg)					
N	61	65	58	62	
Mean (SEM)	136 (2)	139 (2)	140 (2)	136 (2)	**.419**
Diastolic Blood Pressure (mmHg)					
N	61	65	58	62	
Mean (SEM)	76 (1)	78 (1)	78 (1)	75 (1)	**.257**
Pulse (BPM)					
N	61	65	58	62	
Mean (SEM)	61 (1)	61 (1)	61 (2)	62 (1)	**.897**
Pulse Wave Velocity (m/s)					
N	61	65	56	62	
Mean (SEM)	9.4 (0.2)	9.1 (0.2)	9.0 (0.2)	9.1 (0.2)	**.581**
Cardio-Ankle Vascular Index					
N	60	65	55	62	
Mean (SEM)	8.7 (0.1)	9.0 (0.1)	8.7 (0.2)	8.9 (0.2)	**.421**
Ankle-Brachial Index					
N	60	65	55	62	
Mean (SEM)	1.10 (0.02)	1.09 (0.01)	1.11 (0.02)	1.13 (0.01)	**.132**
Reactive Hyperemia Index					
N	56	61	56	56	
Mean (SEM)	2.48 (0.10)	2.44 (0.10)	2.57 (0.10)	2.38 (0.10)	**.625**
BMI					
N	61	65	58	62	
Mean (SEM)	26.4 (0.6)	27.6 (0.5)	27.8 (0.6)	26.0 (0.6)	**.058**
C-Reactive Protein (mg/l)					
N	31	37	27	35	
Mean (SEM)	1.72 (0.20)	1.69 (0.18)	1.68 (0.22)	1.16 (0.19)	**.121**
Cholesterol (mmol)					
N	32	39	28	37	
Mean (SEM)	5.36 (0.26)	5.44 (0.24)	5.79 (0.28)	5.47 (0.24)	**.708**
Triglycerides (mmol)					
N	32	39	28	37	
Mean (SEM)	1.07 (0.11)	1.16 (0.10)	1.23 (0.11)	1.11 (0.10)	**.769**

HDL-Cholesterol (mmol)					
N	32	39	28	37	
Mean (SEM)	1.66 (0.09)	1.68 (0.08)	1.65 (0.09)	1.59 (0.08)	**.886**
LDL-Cholesterol (mmol)					
N	32	39	28	37	
Mean (SEM)	3.22 (0.20)	3.23 (0.17)	3.59 (0.20)	3.36 (0.18)	**.507**

Number of participants in each group is detailed in the "N" rows. Results are displayed as mean and SEM. P-values displayed in final column, significant when $p<0.05$.

4.3.4 Discussion

The NU-AGE diet score was first tested on the TWIN UK data and an increased diet score was shown to be significantly associated with improved CRP concentrations in this cohort. The NU-AGE diet score was then tailored for analysis on the UK NU-AGE cohort to account for the difference in dietary assessment methods between the two studies. FFQs were used in the TWIN cohort, whereas 7 day food diaries were used for the NU-AGE trial participants. There were also a number of other differences, for example, the TWIN cohort was made up of 3262 females aged between 18 and 79 years old, whereas the NU-AGE cohort was made up of 249 male and female participants aged between 65 and 79 years old which is a limitation that should be considered in the interpretation of these results. A more detailed investigation into the division of foods into food groups was required in the case of the NU-AGE cohort due to the use of food diaries as there were over 2,000 WISP food codes used in analyses of the NU-AGE dietary data. Once the assignment of foods into the correct food groups was complete, dietary intakes of each food category were determined for each participant and a diet score was calculated using the same method used on the TWIN UK data.

Tables 4.5 and 4.10 show the nutrient intakes according to NU-AGE diet score quartiles for the TWIN UK and NU-AGE cohorts respectively. In both cohorts, it was found that polyunsaturated fat, protein and fibre were all significantly associated with an increased diet score result. However results for carbohydrate, total and monounsaturated fat were contrasting between the TWIN UK and NU-AGE cohorts. Carbohydrate intakes were significantly associated with an increased diet score in the TWIN UK population but associated with a significantly decreased diet score in the NU-AGE cohort. It has previously been reported that carbohydrate intakes may be over-reported and less accurate when assessed by an FFQ, which could explain the slightly higher intakes and differences across quartiles in the TWIN UK cohort compared with the NU-AGE cohort [330, 331]. Furthermore,

the slight decrease in carbohydrate intakes in the NU-AGE cohort from 48.2 to 45.0% of total energy intakes between Q1 and Q4 may be the result of increased intakes of fibre. Increased intakes of dietary fibre have previously been shown to be associated with reduced intakes of simple carbohydrates [332]. Total and monounsaturated fat levels also significantly decreased with increased diet score result in the TWIN UK population but significantly increased in the case of the NU-AGE cohort. As mentioned previously, in the case of the TWIN UK dietary data we did not have access to any information on consumption of oil. Consumption of olive oil is an important goal of the NU-AGE diet and the lack of data in the TWIN UK cohort is a distinct limitation and is most likely the main contributor to the differences observed in total and monounsaturated fat levels in the diet score quartiles between the TWIN UK and NU-AGE cohort. The NU-AGE cohort had a low level of current smokers (1.3%) when compared with national statistics data on similarly aged UK participants which is closer to 11% of the population [326]. Levels of hypertension and obesity in the NU-AGE cohort were also lower compared with the general UK population [325, 326]. However the percentage of participants that were overweight and consumed alcohol were similar to the UK population, as were serum cholesterol concentrations and physical activity levels [326, 333].

When associations between the NU-AGE diet score and various cardiovascular health outcomes were assessed in the TWIN UK data it was observed that an increased NU-AGE diet was significantly associated with improved CRP concentrations. Associations between the NU-AGE diet score and various cardiovascular health outcomes were also assessed in the NU-AGE cohort. There were no significant associations between an increased NU-AGE diet score for any of the outcomes measures, although there was a trend for a decreasing concentration of CRP with an increased NU-AGE diet score (1.72 to 1.16 mg/l between Q1 and Q4). A likely explanation for this significant association observed in the TWIN UK cohort but not in the NU-AGE cohort is the large difference in the sample size of the cohorts as mentioned above. The analysis using the TWIN UK cohort consisted of 3262 participants and the NU-AGE cohort used consisted of 249 trial participants at baseline. The complete NU-AGE cohort will consist of over 1000 participants across Europe and therefore future work could involve using this NU-AGE diet score on the entire NU-AGE dataset, both to assess associations with health outcomes and to examine compliance. Another potential explanation for the discrepancies in our results could be the difference in methods for dietary assessment; as mentioned previously the TWIN UK data used FFQs as a dietary assessment

method whereas NU-AGE dietary data were collected via 7 day food diaries. Interestingly, the diet scores previously mentioned (the Mediterranean Diet Score, the DASH diet score and the HEI) have all involved the use of FFQs rather that food diaries [181, 208, 209]. Food diaries are thought to give more accurate and detailed results in comparison to the use of FFQs [328]. However in the case of using the food diary data for the purpose of a diet score, the introduction of more detailed results could potentially have resulted in increased subjectivity and human error when categorising foods into specific food groups. Therefore, the amendments made to the NU-AGE diet score to cater for food diary data still need to be validated on a larger dataset before the NU-AGE diet score can be considered suitable for use in a study using food diaries rather than FFQs.

Although a diet score designed specifically for the elderly has already been published, the NU-AGE diet score was based on different diet goals and also aimed to overcome some of the limitations. The EDI [210] is made up of 10 dietary components, based on a combination of both the modified MyPyramid for Older Adults and the Mediterranean Diet. A strength of the NU-AGE diet score was that it has more dietary components including nuts, eggs, fluid and cheese. The NU-AGE diet score also had a category for potatoes, pasta and rice which could account for a large contribution to energy intake. The NU-AGE diet score contained a wholegrain category rather than just a bread/cereal group which is advantageous considering the importance of fibre in the diet of older adults. To determine which foods should be defined as a wholegrain, we used a method of identifying wholegrain foods that had recently been proposed in a 2013 study as the method (≤10:1 ratio of total carbohydrate to fibre content) to identify the most healthful wholegrain products [329]. However, it should be noted that since then, a multidisciplinary expert discussion has led to the publication of a paper stating that the standard definition of whole-grain should be foods that provide at least 8 g of whole grains per 30-g serving (27g/100g) [334]. Future work regarding the definition of whole grain foods should take this definition into consideration. In addition, both the fish and meat groups have been refined to only include healthier alternatives (i.e. excluding highly processed fish and meat products) in the NU-AGE diet score. The validation of the EDI was conducted using data from 668 elderly participants of the MEDIS study. The EDI was designed using only FFQ data whereas the NU-AGE diet score has been designed for use with both FFQs and food diaries, although as mentioned previously the NU-AGE diet score needs to be tested on a bigger sample size to determine its functionality with the use of food diaries. The NU-AGE diet score has a wider ranged scoring system (a range of 0-10)

compared with the EDI (0-4) which may result in the NU-AGE diet score being more sensitive and potentially more accurate. Another advantage of the NU-AGE diet score is that the NU-AGE diet has been used as an intervention diet in the NU-AGE study, therefore when data are available for the full NU-AGE cohort the NU-AGE diet score can be tested on both baseline and follow-up dietary data to determine both adherence to the diet and the effect of changes in the diet and associated score on various health outcomes. As the majority of diet scores have only been used cross-sectionally, this would also add novelty and validity to the NU-AGE diet approach.

As previously discussed, there were also several limitations associated with the NU-AGE diet score, such as the lack of data for oil consumption in the TWIN UK cohort. The different trends observed between both cohorts in nutrient intakes according to diet score quartiles, in addition to the lack of a significant association between the NU-AGE diet score and CRP levels in the NU-AGE cohort could suggest that the diet score may need to be further validated. Furthermore, a considerable amount of work still needs to be carried out before this diet score can be considered for widespread use. The NU-AGE diet score needs to be tested on the entire NU-AGE cohort, both on baseline and follow-up dietary data. This workload was not possible to complete within the time constraints of this PhD work as the dietary intake data entry had not yet been completed.

4.4 Conclusion

This work aimed to design a diet score based on the NU-AGE diet that would be suitable for use in elderly populations. The diet score was created, and validated using TWIN UK data. This data included 3262 female participants aged between 18 and 79 years old whose diet was assessed using validated FFQs. There were various stages in the development and refinement of the diet score, including the initial design, various amendments, and analyses to detect for associations between the diet score and various cardiovascular health outcomes. As the primary outcome of the NU-AGE diet is inflammation, our primary outcome for validation of the diet score was CRP. Using the TWIN UK data, we saw a significant association between CRP levels and the NU-AGE diet score. However we did not observe significant associations for any of the other health outcomes analysed. The NU-AGE diet score was then used to determine associations between the diet score and various health outcomes in the NU-AGE cohort. The dietary data in the NU-AGE cohort was assessed via 7 day food diaries and so various changes were made to the diet score in order for it to be used in association with both FFQs and food diaries. No significant associations were observed

between the NU-AGE diet score and CRP (although a strong trend was observed), or any of the other cardiovascular related health outcomes measured in the NU-AGE cohort, which consisted of 242 male and female participants aged 65-79 years. Future work is required to examine associations between the diet score and CRP (as well as other health outcomes mentioned previously) in the whole NU-AGE cohort at baseline and following intervention before the NU-AGE diet score can be used as a widespread tool for diet quality assessment in older adults.

Chapter 5

Impact of the NU-AGE intervention on vascular function and inflammation

5 Impact of the NU-AGE intervention on vascular function and inflammation

5.1 Introduction

The percentage of people aged over 65 years in Europe is expected to increase from 25 to 40% by 2030 [335]. It is important to identify realistic dietary strategies that will contribute to healthy ageing, a compression of morbidity and reduced age-related medical costs. Low grade chronic inflammatory status is thought to be a key feature of the ageing process [4]. However the ageing process can be influenced by environmental factors, such as levels of physical activity and the composition of the habitual diet [4]. The aim of the NU-AGE project was to address the effects of the whole-diet on inflammation and other ageing related health outcomes such as cognition, bone density etc. The design of the NU-AGE dietary intervention has been previously published [182]. The aspects of particular relevance to this PhD project, including the study population, power calculations, dietary intervention, have all been described in detail in chapter 3. To summarise, NU-AGE (EU FP7) is a large multi-centre trial that involved 1,250 older adults, aged between 65 and 79 years. The study was carried out in five different centres across Europe, including the UK, the Netherlands, Italy, France and Poland. NU-AGE investigated the effects of a year-long whole-diet intervention on a wide range of health outcomes. The whole-diet was based on recommendations that have been specifically designed for the elderly (Table 3.2 and section 3.8). The control group were given an information sheet containing generic dietary advice from the British Dietetic Association (annex 4).

The focus of this PhD project was to examine the impact of the NU-AGE intervention on measures of vascular function, as well as on inflammatory and fatty acid status. The primary aim was to investigate if adherence to the NU-AGE diet for one year could influence endothelial dysfunction and arterial stiffness in older adults. This was carried out through the utilisation of a range of clinical measures (including EndoPAT, PWV and CAVI). The importance of vascular and endothelial function as early indicators of CVD has been detailed in chapter 1. The loss of vascular reactivity throughout the ageing process is now recognised as a significant CVD risk factor [336, 337]. As discussed in chapter 1, previous dietary studies have shown vascular function can be modulated by single nutrients (particularly n-3 fats and flavonoids, both of which are found in foods recommended as part of the NU-AGE diet) [338], as well as by certain dietary patterns [339, 340]. There are a number of validated clinical

measures of endothelial dysfunction and vascular stiffness currently in use in research, including EndoPAT, PWV and CAVI (detailed in chapters 1 and 3), which were used in this study to test the hypothesis that following the NU-AGE diet for one year may slow the progression of vascular dysfunction in older adults.

A range of biochemical analyses were also examined to assess the impact of the NU-AGE intervention on vascular function and inflammatory status. Their role in CVD risk has been described in chapter 1. NO is recognised as a potent vasoprotective molecule as a result of its various physiological functions, including its ability to act as a vasodilatory signalling and anti-inflammatory molecule [341]. NO production and/or bioavailability can be insufficient in the presence of endothelial dysfunction [342]. Nitrite in plasma is widely used as an index of NO status [341] and was measured in the NU-AGE participants before and after the one-year intervention period to determine the ability of the NU-AGE diet to modulate endothelial function in older adults and to explain potential mechanisms for said modulation. ET-1, which also plays a role in endothelial dysfunction, was measured in the NU-AGE participants [343]. In contrast to NO, ET-1 is a vasoconstricting and pro-inflammatory protein [343]. CRP was analysed as a biomarker of overall inflammatory status. Elevated concentrations of CRP have previously been shown to be associated with increased risk of CVD [71-73]. As an established traditional cardiovascular risk factor, the lipid profile (TGs, TC and HDL-C etc.) of participants was also assessed.

The plasma fatty acid status of the NU-AGE participants was also characterised with the aim to, firstly, investigate if the NU-AGE diet resulted in an altered the fatty acid profile of individuals and secondly, if these changes (and in particular in EPA and DHA status) were related to changes in vascular related outcomes. The impact of common variants in PUFA biosynthesising genes on fatty acid status at baseline and in response to intervention was also investigated, as discussed in chapter 6. The impact of n-3 fatty acids on CVD risk and risk factors, as well as potential mechanisms of action underlying these benefits were previously discussed in chapter 1 (section 1.6). The NU-AGE diet contains several goals related to fat consumption, for example, participants were advised to consume two portions of fish per week (preferably oily, which are rich in EPA and DHA) and were provided with olive oil and margarine spread. Therefore, adherence to the NU-AGE diet could potentially result in an improved plasma fatty acid profile, with lower levels of SFAs and higher levels of MUFAs and PUFAs.

In addition to examining the impact of the NU-AGE intervention on each of these measures individually, we also aimed to compile a vascular risk score that could be used to combine a number of measures related to cardiovascular health. As cardiovascular risk factors are generally interrelated and are considered to have additive effects on the risk of CVD, the use of a clustered score could determine an effect that was not elucidated by looking at individual outcomes [344]. A number of authors have previously designed similar scores to represent the clustering of components related to CVD, particularly in relation to metabolic syndrome risk factors [344-346]. We chose to incorporate blood pressure, BMI, clinical measures of arterial stiffness and endothelial function, as well as biochemical markers of cardiovascular health including TGs, CRP and TC: HDL-C ratio. We aimed to examine the impact of the NU-AGE diet for one year on this calculated vascular risk score in older adults. The anthropometric measurements and various other health related characteristics, such as smoking status, the mean daily nutrient intakes of both the control and intervention groups of the NU-AGE cohort at baseline were first examined (described in section 5.3.1) to ensure that there were no significant differences between the control and intervention groups at baseline and to establish that the NU-AGE cohort was a representative population of the elderly in the UK.

5.2 Methods

5.2.1 Clinical and biochemical measures

The methods that were used to assess vascular function have been described in chapter 3 (section 3.10). To summarise, the assessment of vascular function was carried out at baseline and after the one year dietary intervention period and involved a battery of vascular measures lasting approximately one hour. These assessments took place at either 8am or 9am and participants were requested to fast for at least 8 hours prior to measurements. The participants were requested to rest in a dimly lit and quiet room for 15 minutes beforehand. SBP, DBP and pulse were then measured using an automatic blood pressure measurement device (OMRON M2, Milton Keynes, UK). PWV was measured using a Vicorder device (Skidmore Medical, Bristol, UK). Both CAVI and ABI were measured using Vasera™VS-1500 (Fukuda Denshi Co, Japan). Lastly, the EndoPAT2000 (Itamar Medical Ltd, Caesarea, Israel) was used to measure endothelial function. The following dataset includes n=142 participants. However, n=5 participants were missing values for EndoPAT results (n=3 due to errors in the EndoPAT software, n=2 due to discomfort), n=6 participants were missing values

for PWV results (n=3 due to errors in the Vicorder software/hardware, n=2 due to bad signal in data output, n=1 due to discomfort), n=3 participants were missing values for CAVI/ABI results (n=2 due to discomfort, n=1 due to having had a mastectomy and therefore could not have a blood pressure cuff inflated on both arms).

The methods that were used to analyse biochemical markers of vascular function, inflammatory and fatty acid status have been described in section 3.11. To summarise, plasma concentrations of nitrite were analysed before and after the intervention via the utilisation of a chemiluminescence detector (CLD88). Plasma concentrations of ET-1 were measured using a Quantikine ET-1 immunoassay. CRP, TG and cholesterol concentrations were analysed in the plasma via spectrophotometric analysis in the ILAB 600. Plasma fatty acid status was measured using gas-liquid chromatography following extraction and methylation. Biochemical data were missing for n=2 participants.

5.2.2 Statistical analysis

The data were assessed for normality by visual inspection of normal Q-Q plots. Outliers were determined by inspection of a box-plot for values greater than 1.5 box-lengths from the edge of the box and by calculating studentised residuals (≥ 3 SDs). Homogeneity of variances was assessed by Levene's test for equality of variances ($p > 0.05$). Homogeneity of covariances were assessed by Box's test of equality of covariance matrices ($p > 0.001$). Independent t-tests were used to determine differences in several measures between control and intervention, as well as between males and females at baseline. Repeated measures ANOVA was used in order to quantify the significance level for the time*treatment interactions for the individual vascular and biochemical data, in addition to the vascular risk score (section 5.2.4). Gender, age (T0), BMI (T0), total energy intake (T0), total energy expenditure (T0), heart disease and blood pressure medications (T0) and smoking status (T0) were included in the repeated measures analysis as covariates. Fish oil supplement usage and non-processed seafood intakes were also included as covariates in the examination of the effects of the NU-AGE diet on fatty acid status. A three-way interaction effect (Gender*Time*Treatment) was used to assess gender-specific responses to treatment. Within gender subgroup analysis was performed if a significant effect of gender was detected. All data are presented as mean ± SEM.

5.2.3 Assessing compliance

As described in section 3.8, a compliance questionnaire was administered to participants in the intervention group at month 8 of the intervention. A subgroup analysis was performed

in which the cohort was divided into groups based on compliance, as demonstrated by Marklund et al. [347]. The 75% of the participants with the highest apparent compliance were defined as ‘‘more compliant,’’ whereas the 50% of the participants with the highest apparent compliance were defined as ‘‘most compliant’’. Comparisons of effects were made within these subgroups with the remainder of the cohort. However, the results from this analysis (same outcomes as listed in table 5.3) did not significantly differ from the main analysis (data not shown).

5.2.4 Calculating a vascular risk score

Vascular risk was assessed on the basis of the following seven clinical and biochemical parameters; BMI, SBP, PWV, RHI, TGs, CRP, TC: HDL-C ratio. These variables were chosen from the outcomes measured as they have a known role in CVD progression, with most being independent determinants of CVD risk. In order to combine data from individual variables into a cluster, pre- and post- data were standardised by the calculation of standardised residuals (z-scores). Z= (value – mean)/SD; it is calculated based on the mean and SD of the whole study population. Z-scores rank individuals according to their place in a normal distribution of values. For example, a subject with a z-score of 0.5 has a total risk that is 0.5 SDs higher than the mean of a normalised distribution. This z-score gives equal weight to all factors. Since RHI is inversely related to vascular dysfunction, the calculated z-score was multiplied by -1. The z-scores for the individual risk factors were then added together to create the vascular risk score. A higher score indicates a less favourable vascular profile.

5.3 Results

The data presented in this chapter are from 150 of the participants that underwent the dietary intervention in the UK centre (Norwich) of the NU-AGE study. n=8 out 150 of the participants did not complete the study for the following reasons; n=2 participants experienced discomfort during vascular measurements, n=1 participant experienced an illness in the family and n=5 participants did not wish to continue following the diet. Therefore the following dataset includes n=142 participants.

5.3.1 The health characteristics and daily nutrient intakes of the NU-AGE cohort at baseline

The baseline characteristics of the NU-AGE cohort at baseline are shown in table 5.1. Comparisons between both the control and intervention groups, as well as between males and females are shown in this table. There was a higher proportion of females (n=81) than males (n=61) in the cohort. The age of the cohort ranged from 65 to 79 years, with both a mean ± SEM and median age of 69 ± 1 years. BMI ranged from 18.9 to 43.3 kg/m^2 with a mean of 26.9 ± 0.4 kg/m^2. 46% of the cohort were overweight and 19% were obese. 40% of the cohort were hypertensive (defined as having a SBP over 140mmHg or a DBP over 90mmHg [322])**.** Mean levels of plasma TGs for the Norwich NU-AGE cohort at baseline were 1.13 ± 0.05 mmol/l, while the calculated TC: HDL-C ratio was 4.12 ± 0.41. Data were normally distributed for control and intervention and there was homogeneity of variances ($p>0.05$). There were no significant differences between the control and intervention group for any of the health characteristics (including BMI and blood pressure) measured at baseline. However, there were significant differences between males and females for a number of characteristics at baseline (Table 5.1). In relation to smoking status, more men than females were former smokers, while more females than males had never smoked. In addition, women had significantly higher TC (18%), LDL-C (20%) and HDL-C (19%) compared with men.

Table 5.1. Baseline characteristics of the Nu-Age cohort (n=142)

	Control (n=72)	Intervention (n=70)	p-value	Male (n=61)	Female (n=81)	p-value	Total (n=142)
Sex (male)							
N	34	27	0.192	61	81	N/A	142
Age (years)	70 (0)	69 (0)	0.070	70 (1)	69 (1)	0.230	69 (0)
BMI (kg/m^2)	27.3 (0.5)	26.6 (0.6)	0.382	27.4 (0.6)	26.6 (0.5)	0.323	26.9 (0.4)
Smoking Status			0.753			**0.002***	
Current smokers (%)	1.4	2.9		3.3	1.2		2.1
Former smokers (%)	47.2	42.9		60.7	33.3		45.1
Never smoked (%)	51.4	54.3		36.1	65.4		52.8
Cardiovascular Disease Medication Users							
(%)	40.3	40.0	0.973	47.5	34.6	0.118	40.1
Systolic Blood Pressure (mmHg)	140 (2)	134 (2)	0.061	136 (2)	138 (2)	0.404	137 (1)
Diastolic Blood Pressure (mmHg)	78 (1)	76 (1)	0.080	77 (1)	76 (1)	0.526	77 (1)
Pulse (BPM)	62 (1)	60 (1)	0.271	61 (10	61 (2)	0.964	61 (1)
PWV (m/s)	9.3 (0.2)	8.7 (0.2)	0.058	9.0 (0.2)	9.0 (0.2)	0.927	9.0 (0.2)
CAVI	8.8 (0.1)	8.7 (0.1)	0.703	8.8 (0.2)	8.7 (0.1)	0.530	8.7 (0.1)
ABI	1.11 (0.01)	1.11 (0.2)	0.951	1.13 (0.02)	1.09 (0.02)	0.113	1.11 (0.01)
RHI	2.47 (0.09)	2.43 (0.08)	0.774	2.34 (0.07)	2.53 (0.09)	0.122	2.45 (0.06)
Triglycerides (mmol/l)	1.15 (0.61)	1.12 (0.55)	0.762	1.12 (0.08)	1.14 (0.06)	0.863	1.13 (0.05)
Total cholesterol (mmol/l)	5.64 (0.2)	5.38 (0.2)	0.333	4.89 (0.18)	5.97 (0.17)	**0.000***	5.51 (0.13)
HDL-Cholesterol (mmol/l)	1.65 (0.06)	1.67 (0.06)	0.813	1.46 (0.06)	1.80 (0.06)	**0.000***	1.66 (0.05)
LDL-Cholesterol (mmol/l)	3.47 (0.14)	3.21 (0.13)	0.176	2.91 (0.13)	3.65 (0.12)	**0.000***	3.34 (0.10)
Total Cholesterol: HDL-Cholesterol ratio	4.38 (0.67)	3.86 (0.48)	0.529	4.25 (0.76)	4.02 (0.44)	0.778	4.12 (0.41)

Results are displayed as mean and SEM. Independent samples t-tests were utilised to determine significant differences between the control and intervention groups, as well as between males and females, for outcomes at baseline. Asterix marks significance ($p<0.05$). BMI; Body Mass Index, PWV; Pulse Wave Velocity, ABI; Ankle Brachial Index, CAVI; Cardio-Ankle Vascular Index, RHI; Reactive Hyperaemic Index.

For the dietary data (Table 5.2), data were normally distributed for control and intervention and there was homogeneity of variances (except for total energy intake, sodium and protein for which homogeneity of variances was violated). There were no significant differences between the control and intervention group for any of the nutrients measured at baseline. Differences in dietary intakes at baseline between males and females were also examined. Males consumed greater than 20% more calories, 27% more alcohol, 22% more fibre, and 22% more sodium compared with women ($P<0.05$). Although a detailed analysis of EPA and DHA dietary intakes was not available, it was calculated that 51% of the NU-AGE cohort were taking fish oil supplements and participants had a mean intake of 209.1 ± 17.1 g of non-processed fish per week at baseline.

It should be noted that follow-up dietary intake data was not complete at the time of thesis submission and therefore could not be included as part of this PhD work.

Table 5.2. Daily nutrient intakes for the NU-AGE cohort at baseline (n=142)

	Control (n=72)	Intervention (n=70)	p-value	Males (n=61)	Females (n=81)	p-value	Total
Energy (kcal/d)	1986 (55)	1910 (40)	0.266	2229.8 (50.1)	1736.3 (29.2)	**0.000***	1948 (34)
Fat (% of kcal/d)	34.4 (0.7)	35.0 (0.7)	0.541	34.0 (0.7)	35.1 (0.7)	0.265	34.7 (0.5)
Saturated Fat (% of energy)	12.9 (0.4)	13.1 (0.4)	0.781	12.9 (0.4)	13.1 (0.4)	0.220	13.0 (0.3)
Monounsatur-ated Fat (% of energy)	11.6 (0.3)	11.7 (0.3)	0.884	11.5 (0.3)	11.8 (0.3)	0.518	11.6 (0.2)
Polyunsaturat-ed Fat (% of energy)	5.4 (0.2)	5.4 (0.2)	0.986	5.5 (0.2)	5.4 (0.2)	0.720	5.4 (0.1)
Carbohydrates (% of energy)	47.1 (0.7)	48.0 (0.7)	0.323	48.4 (0.7)	46.9 (0.6)	0.110	47.5 (0.5)
Sugars (% of energy)	23.2 (0.6)	23.4 (0.6	0.732	23.2 (0.7)	23.4 (0.5)	0.085	23.3 (0.4)
Protein (% of energy)	16.2 (0.3)	16.2 (0.4)	0.945	15.8 (0.3)	16.5 (0.3)	0.970	16.2 (0.2)
Alcohol (g/d)	11.3 (1.1)	8.5 (1.0)	0.065	11.7 (1.3)	8.6 (0.9)	**0.045***	9.9 (0.8)
Fibre (g/d)	26.0 (1.1)	24.6 (0.8)	0.341	29.0 (1.3)	22.6 (0.7)	**0.000***	25.3 (0.7)
Sodium (mg/d)	2531 (118)	2472 (87)	0.691	2859 (122)	2232 (78)	**0.000***	2502 (73)
Water (g/d)	2650 (77)	2653 (79)	0.980	2724.5 (85.7)	2597.0 (71.5)	0.253	2652 (55)

Results are displayed as mean and SEM. Independent samples t-tests were utilised to determine significant differences between the control and intervention groups, as well as between males and females, for outcomes at baseline. Asterix marks significance ($p<0.05$)

5.3.2 Clinical measures of vascular function; response to the NU-AGE intervention

Compliance to intervention was assessed as detailed in section 5.2.3. Mean overall compliance to the NU-AGE dietary goals was calculated to be 74%.

The effects of the NU-AGE intervention on BMI, blood pressure and clinical measures of vascular function are shown in table 5.3 (displayed as unadjusted means and SEM for both the control and intervention group at baseline and follow-up). There were no outliers in the BMI, SBP, DBP, pulse or CAVI data, with one outlier removed for PWV and two for RHI. The data for all these outcome measures were normally distributed for both the control and intervention groups and there was homogeneity of variances ($p>0.05$). There were no significant effects of treatment on BMI ($p=0.576$, $F(1, 134) = 0.315$, partial $\eta2 = 0.002$), SBP ($p=0.552$, $F(1, 133) = 0.356$, partial $\eta2 = 0.003$) or DBP ($p=0.952$, $F(1, 133) = 0.004$, partial $\eta2 = 0.000$). Analysis showed no significant treatment*time interaction ($p=0.563$, $F(1, 133) = 0.335$, partial $\eta2 = 0.003$) or treatment effect ($p=0.799$, $F(1, 133) =0.065$, partial $\eta2 = 0.000$) for pulse. However, the main effect of time showed a statistically significant difference in pulse at the different time points ($p=0.035$, $F(1, 132) =4.548$, partial $\eta2 = 0.033$) effect for pulse.

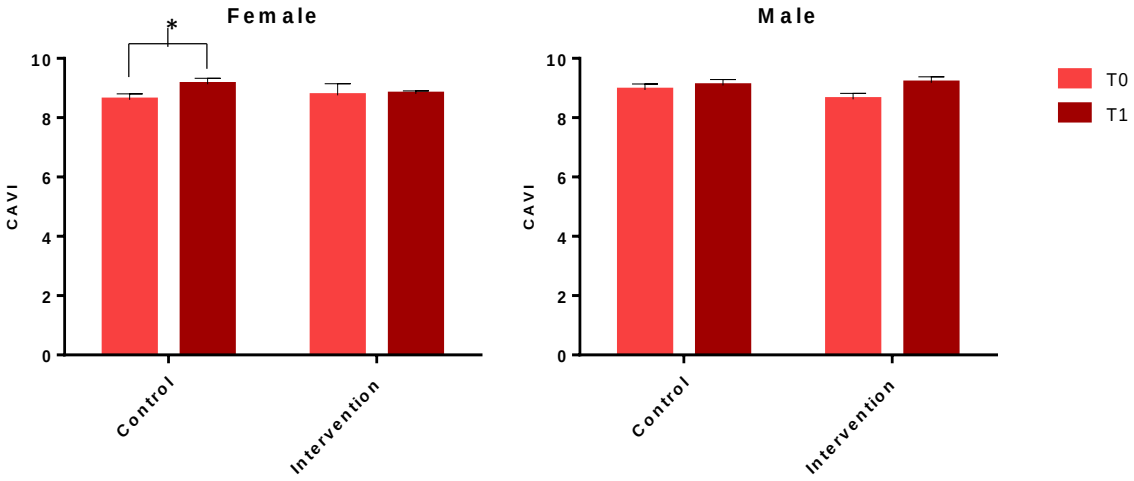
Table 5.3. Anthropometric measurements and clinical measures of vascular function in subjects that were on the Nu-Age whole-diet intervention or a control diet for one year (n=142)

	Control (n=72)		**Intervention (n=70)**		***P value***
	T0	T1	T0	T1	
BMI (kg/m^2)	27.3 (0.9)	26.3 (0.9)	26.6 (1.0)	26.2 (0.9)	*0.567*
Systolic Blood Pressure (mmHg)	140 (3)	136 (3)	134 (3)	134 (3)	*0.552*
Diastolic Blood Pressure (mmHg)	78 (2)	77 (2)	75(2)	76 (2)	*0.952*
Pulse (BPM)	62 (2)	60 (2)	60 (2)	59 (2)	*0.563*
PWV (m/s)	9.2 (0.4)	9.2 (0.4)	8.7 (0.4)	8.8 (0.4)	*0.157*
CAVI	8.8 (0.2)	9.1 (0.2)	8.7 (0.2)	8.9 (0.2)	*0.250*
ABI	1.11 (0.03)	1.19 (0.08)	1.11 (0.03)	1.09 (0.08)	*0.652*
RHI	2.47 (0.15)	2.51 (0.17)	2.42 (0.16)	2.40 (0.18)	*0.469*

Values are unadjusted means ± SEM. BMI; Body Mass Index, PWV; Pulse Wave Velocity, ABI; Ankle Brachial Index, CAVI; Cardio-Ankle Vascular Index, RHI; Reactive Hyperaemic Index. P values determined using repeated measures ANOVA.

Analysis showed no significant treatment*time interaction (p=0.157, *F*(1, 126) = 2.025, partial η2= 0.17) for PWV**,** RHI, as assessed using the EndoPAT (p=0.469, *F*(1, 126) = 0.529, partial η2= 0.004) or CAVI (p=0.250, *F*(1, 130) = 1.337, partial η2= 0.010). However, in the case of CAVI there was a significant effect when gender was included in the analysis (p=0.011, *F*(1, 130) = 6.681). Subgroup analysis showed that CAVI in the females in the control group increased, indicating a significant increase in the stiffness of arteries over the 1 year intervention period compared with those following the NU-AGE diet (p=0.024, *F*(1, 72) = 5.328, partial η2= 0.070)(figure 5.1). Analysis showed no significant treatment*time interaction (p=0.652, *F*(1, 130) = 0.205, partial η2= 0.002) for ABI which is measured simultaneously with CAVI.

Figure 5.1. The differential response of the NU-AGE intervention on Cardio-Ankle Vascular Index (CAVI) in females and males



Female control group; n=36, female intervention group; n=43, male control group; n=33, male intervention group; n=27. Data presented as mean ± SEM. Repeated measures ANOVA was conducted to examine the impact of treatment on CAVI separately in men and women. Asterix indicates significance (P < 0.05).

5.3.3 Biochemical Measures of vascular function and inflammatory status; response to the NU-AGE intervention

The effects of the NU-AGE intervention on biochemical measures of vascular function, inflammatory and lipid status are shown in table 5.4 (displayed as unadjusted means and SEM for both the control and intervention group at baseline and follow-up).

Five outliers were detected in the nitrite analysis and 4 outliers were detected in the ET-1 analysis but these were included in the analysis as they did not significantly affect outcomes, as determined using a sensitivity analysis. In relation to inflammation, a CRP reading of >10 mg/l is indicative of acute rather than chronic infection [74, 348, 349] and therefore participants that had > 10mg/l at T0 or T1 were excluded from the analysis (n=6). There were no outliers for any other analysis. The data were normally distributed for control and intervention and there was homogeneity of variances for all analysis ($p>0.05$). There was no significant impact of the NU-AGE intervention on treatment*time interaction ($p=0.565$, $F(1, 130) = 0.332$, partial $\eta^2 = 0.003$) for nitrite, ET-1 ($p=0.691$, $F(1, 130) = 0.158$, partial $\eta^2 = 0.001$), CRP ($p=0.384$, $F(1, 124) = 0.765$, partial $\eta^2 = 0.006$), TGs ($p=0.670$, $F(1, 130) = 0.183$, partial $\eta^2 = 0.001$), TC ($p=0.642$, $F(1, 130) = 0.218$, partial $\eta^2 = 0.002$), HDL-C ($p=0.827$, $F(1, 130) = 0.048$, partial $\eta^2 = 0.000$), LDL-C ($p=0.407$, $F(1, 130) = 0.692$, partial $\eta^2 = 0.005$), or the calculated TC: HDL-C ratio ($p=0.264$, $F(1, 130) = 1.259$, partial $\eta^2 = 0.010$).

Table 5.4. Range of biochemical analytes measured in plasma in subjects that were on the Nu-Age whole-diet intervention or a control diet for one year

	Control diet (n=70)		Nu-Age diet (n=70)		P-value
	T0	*T1*	*T0*	*T1*	
Nitrite (nmol)	92.1 (13.4)	85.3 (12.9)	92.3 (13.4)	93.1 (12.9)	*0.565*
Endothelin-1 (pg/ml)	2.46 (0.19)	2.50 (0.21)	2.32 (0.19)	2.40 (0.21)	*0.691*
C-Reactive Protein (mg/l)	1.92 (0.30)	1.50 (0.33)	1.48 (0.31)	1.65 (0.35)	*0.384*
Triglycerides (mmol/l)	1.15 (0.12)	1.12 (0.08)	1.12 (0.12)	1.02 (0.08)	*0.670*
Total cholesterol (mmol/l)	5.64 (0.30)	5.69 (0.21)	5.38 (0.30)	5.17 (0.21)	*0.642*
HDL-Cholesterol (mmol/l)	1.65 (0.11)	1.77 (0.08)	1.67 (0.11)	1.73 (0.08)	*0.827*
LDL-Cholesterol (mmol/l)	3.47 (0.22)	3.41 (0.17)	3.21 (0.22)	2.98 (0.17)	*0.407*
Total Cholesterol: HDL-Cholesterol ratio	3.63 (0.22)	3.41 (0.15)	3.41 (0.22)	3.07 (0.15)	*0.264*

Values are unadjusted means ± SEM (n=140). P values determined using repeated measures ANOVA. HDL; High Density Lipoprotein, LDL; Low Density Lipoprotein. P values determined using repeated measures ANOVA.

5.3.4 Plasma fatty acid status; response to the NU-AGE intervention

The effects of the NU-AGE intervention on plasma fatty acid status is shown in table 5.5. There were no significant differences between the control and intervention group for any of the fatty acids, as assessed using repeated measures ANOVA analysis.

There were no outliers in the data, the data were normally distributed for control and intervention and there was homogeneity of variances ($p>0.05$). To summarise the findings, there were no significant effects of time, treatment or time*treatment interactions evident for any of the individual fatty acids, total n-3 PUFA, total n-6 PUFA, n3-n6 ratio or desaturase activity.

Table 5.5. Plasma fatty acids from total lipids (% of total fatty acid) in subjects that were on the Nu-Age whole-diet intervention or a control diet for one year (n=140)

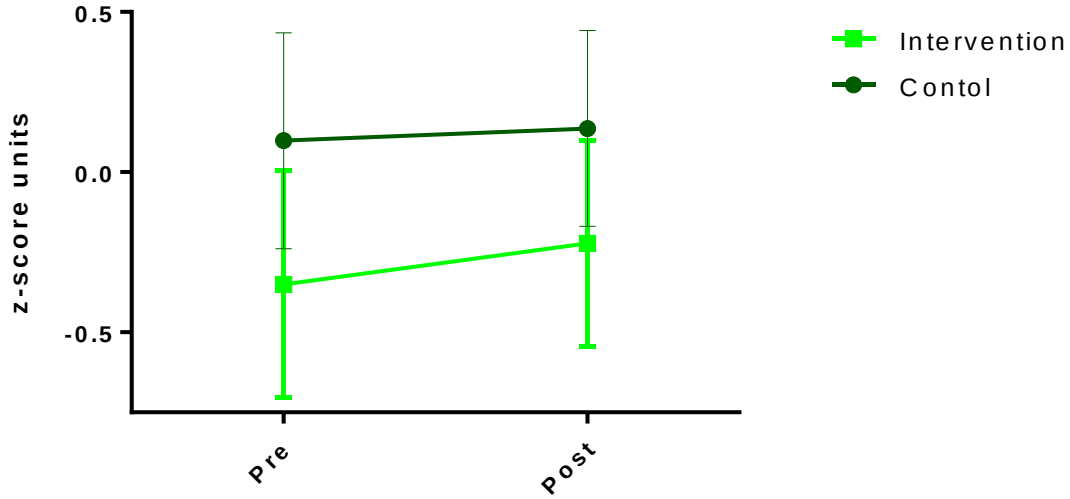
	Control diet		Nu-Age diet		P-value
Fatty acid	*T0*	*T1*	*T0*	*T1*	
Palmitic acid	23.71 (0.41)	23.84 (0.42)	23.27 (0.42)	23.57 (0.42)	*0.701*
Palmitoleic acid	2.44 (0.15)	2.35 (0.14)	2.41 (0.15)	2.28 (0.14)	*0.982*
Stearic acid	8.07 (0.23)	8.24 (0.29)	8.09 (0.23)	8.47 (0.29)	*0.525*
18:1*	23.22 (0.42)	22.71 (0.44)	22.92 (0.44)	22.68 (0.46)	*0.356*
Linoleic acid	26.98 (0.68)	26.98 (0.78)	26.86 (0.68)	26.77 (0.78)	*0.750*
α-linolenic acid	0.67 (0.04)	0.64 (0.04)	0.69 (0.04)	0.71 (0.04)	0.186
Dihomo-γ-linolenic acid	1.45 (0.06)	1.42 (0.07)	1.45 (0.06)	1.46 (0.07)	*0.241*
Arachidonic acid	5.67 (0.36)	5.74 (0.37)	6.12 (0.37)	6.04 (0.37)	*0.519*
EPA	1.45 (0.14)	1.46 (0.13)	1.52 (0.14)	1.70 (0.14)	*0.156*
DPA	0.14 (0.08)	0.18 (0.17)	0.20 (0.09)	0.19 (0.12)	*0.259*
DHA	2.34 (0.12)	2.37 (0.13)	2.42 (0.12)	2.56 (0.13)	*0.210*
Total n-3 PUFA	5.60 (0.35)	5.53 (0.29)	5.72 (0.35)	5.95 (0.29)	*0.373*
Total n-6 PUFA	34.77 (0.75)	34.87 (0.85)	35.03 (0.73)	34.87 (0.83)	*0.531*
n-3/n-6 PUFA ratio	0.17 (0.02)	0.16 (0.01)	0.17 (0.01)	0.17 (0.01)	*0.284*
Desaturase Activity	0.21 (0.02)	0.21 (0.02)	0.23 (0.02)	0.23 (0.02)	*0.706*

Values are adjusted means ± SEM (n=142). P-values calculated using repeated measures ANOVA. Totals include some minor components not shown. PUFA: Polyunsaturated fatty acids. Total n-3 PUFA consists of 18:3n3, 18:4n3, 20:3n3, 20:4n3, 20:5n3, 22:5n3 and 22:6n3. Total n-6 PUFA consists of 18:2n6, 20:2n6, 20:3n6, 20:4n6 and 22:2n6. Desaturase activity was calculated using a product to precursor ratio of AA to LA. *Contains n-9 and n-7 isomers.

5.3.5 The impact of the NU-AGE intervention on a calculated vascular risk score

The z-score sum of all 7 risk factors as a measure of vascular risk burden ranged from -9.60 to 8.93, with higher levels indicating increased burden of risk. Outliers were detected but included in the analysis as they did not significantly affect outcomes, determined using a sensitivity analysis. The data were normally distributed for control and intervention and there was homogeneity of variances ($p>0.05$).There was no significant difference between the z-scores for the control and intervention groups at baseline. The vascular risk score increased from -0.12 ± 2.70 to -0.03 ± 2.44 for the cohort as a whole over the one year period. Analysis showed no significant treatment*time interaction ($p=0.865$, $F(1, 115) = 0.029$, partial $\eta 2= 0.000$) for the vascular risk score. Results are shown diagrammatically in figure 5.2. The mean z-score for the control group changed from 0.10 ± 0.30 to 0.14 ± 0.27, while the mean z-score for the intervention group changed from -0.35 ± 0.31 to –0.22 ± 0.29.

Figure 5.2. The impact of the NU-AGE intervention on change in vascular risk score over the period of one year



5.4 Discussion

5.4.1 Baseline Characteristics of the NU-AGE cohort

There were no significant differences between the control and intervention group for any of the characteristics measured at baseline. The mean BMI of the participants was 26.9 ± 0.4 kg/m^2, specifically 27.4 ± 0.6 kg/m^2 for men and 26.6 ± 0.5 kg/m^2 for women. Although a BMI of >25 kg/m^2 is categorised as overweight, a meta-analysis examining the relationship between BMI and all-cause mortality in older adults found that for adults aged >65 years there was a 4-10% lower mortality risk for participants in the overweight range relative to those with a BMI <25kg/m^2 [350]. Data from the NDNS shows similar, although slightly higher BMI figures for >65 year olds in the UK; the average BMI was 27.9 ± 0.3 kg/m^2 for men and 27.8 ± 0.4 kg/m^2 for women [351].

In relation to smoking status, the percentage of older adults that had never been regular smokers was identical between the NU-AGE participants (52.8%) and the UK population (53%), however the proportion of current smokers was higher in the UK population (9%) compared with the NU-AGE population (2.1%). Furthermore, significantly more male than female NU-AGE participants were former smokers, while more females than males had never smoked. 40.1% of the NU-AGE cohort were taking medication for the treatment of hypertension, hypercholesterolemia or for the treatment/prevention of CVD. In the UK, 14% of the population are prescribed lipid-lowering medications and 15% are prescribed anti-hypertensive medications, with these figures generally increasing with age [352]. For example, 41% of the population aged 65 to 74 years are prescribed lipid-lowering medications and 39% are prescribed anti-hypertensive medications [352].

The mean SBP and DBP for the NU-AGE cohort were within a healthy range; 136 ± 2 mmHg and 77 ± 1 mmHg respectively for males and 138 ± 2 mmHg and 76 ± 1 mmHg respectively for females. The NDNS data show that for >65 year olds the mean SBP were 138 ± 1 mmHg and 137 ± 1 mmHg for men and women respectively [351]. The average DBP were 74 ± 1 mmHg and 73 ± 1 mmHg for men and women respectively [351]. Therefore the NU-AGE cohort is representative of the general UK population in terms of blood pressure.

The UK guidelines state that TGs should ideally be <1.7 mmol/l and the TC: HDL-C ratio should be <5:1 [353]. Mean concentrations of plasma TGs for the NU-AGE cohort at baseline were 1.13 ± 0.05 mmol/l, while the calculated TC: HDL-C ratio was 4.12 ± 0.41. The UK older adult population has a slightly higher mean TG concentration of 1.27 mmol/l and a slightly lower TC: HDL-C ratio of 3.44 [351]. Similarly to patterns in the UK older adult population, females had significantly higher TC (18%), LDL-C (20%) and HDL-C (19%) compared with males [351].

Normal values of PWV for older adults across Europe aged between 60-69 years has been established at 10.3 (5.5-15.0) m/s and for adults aged ≥70 years the mean is higher at 10.9 (5.5-16) m/s [354]. The baseline results for the NU-AGE cohort as a whole were substantially lower at 8.9 ± 0.2 m/s. Mean CAVI scores for men and women were similar to established scores based on age and other CVD-risk related criteria [355]. The NU-AGE cohort was also, on average, within the normal range for ABI levels at baseline. The average RHI of the NU-AGE cohort was 2.45 ± 0.06, which is above the threshold for endothelial dysfunction, defined as an RHI of <1.67 [277]. Therefore overall, the NU-AGE cohort were generally healthy and representative of the UK elderly population in terms of health outcomes, such as BMI, blood pressure and lipid profile.

5.4.2 Daily nutrient intakes of the NU-AGE cohort at baseline

In relation to dietary intakes, there were no significant differences between the control and intervention groups at baseline (Table 5.2). The NDNS data show that the mean energy intake for over 65 year olds in the UK is 1697 kcal per day; this is approximately 13% lower than the energy intake of the NU-AGE cohort [351]. The aim of the NU-AGE study in relation to fat was that total fat consumption should be 25-30% of energy intake. The data in table 5.2 shows that the NU-AGE cohort had a total fat intake of 34.7 ± 0.5% of total energy at baseline. This figure is similar to the mean UK fat intakes, which the NDNS report to be 35.5% of total energy [351]. The figures for saturated fat were also similar between the NU-AGE cohort (13.0 ± 0.3% of energy intake) and UK population (13.8% of energy intake), but again higher than the NU-AGE recommendation (<10% of energy intake). The NU-AGE study recommends that PUFA provides <12% of energy intake and the total of MUFAs and PUFAs should be between 8-28%. The MUFA and PUFA intakes of the NU-AGE cohort fell within these requirements at 11.6 ± 0.2% and 5.4 ± 0.1% of energy intake at baseline respectively. In addition, these levels were closely matched to the mean MUFA and PUFA intakes in the UK of 12.1% and 5.9% of energy [351].

The carbohydrate intakes of the NU-AGE cohort at baseline (47.5 ± 0.5% of energy) were representative of the UK population (47.2%). The NU-AGE carbohydrate recommendation was to consume carbohydrates as 50-60% of total energy intakes. However, the aim of the NU-AGE study was to limit the intakes of sugars and place emphasis on the consumption of fibre (30-40g). The sugar intakes of the NU-AGE cohort at baseline and UK population were similar; 23.3 ± 0.4% and 21.6% of energy respectively [351]. The fibre intakes of the NU-AGE

cohort at baseline were below the NU-AGE recommendations at 25.3 ± 0.7g, but were substantially higher than the NSP intakes of the UK population, reported by the NDNS as 13.9g [351]. The aim of the NU-AGE study in relation to alcohol consumption is for intakes to be <20 g per day; one unit for women and two for men. The UK NU-AGE cohort had intakes within this range (9.9 ± 0.8 g), as did the UK population of over 65 year olds (8.4 g) [351]. The sodium intakes of the NU-AGE cohort (2502 ± 73 mg) were higher than both the NU-AGE recommendation (2000 mg) and the UK older adult population (2058 g) [351].

Although data on intakes of specific fatty acids were not available, 51% of the NU-AGE participants were taking fish oil supplements at baseline. This is a high percentage in comparison to the UK levels for older adults. The NDNS data show that only 24% of adults aged over 65 years are taking fish oil supplements [351]. Further to this, baseline dietary data show that the NU-AGE cohort had a mean intake of 209.1 ± 17.1 g of non-processed fish per week. Although there is not a specific category for non-processed seafood in the NDNS data, total fish intakes for over 65 year olds is reported to be 217 ± 14 g per week, 98 ± 7 g of which is oily fish [351].

Comparisons between male and female participants showed, as expected, that males consumed significantly more calories (20%), alcohol (27%), fibre (22%), and sodium (22%) compared with women.

5.4.3 Anthropometric measurements and clinical measures of vascular function; response to the NU-AGE intervention

Clinical measures of vascular function, including blood pressure, arterial stiffness and endothelial function, were assessed to determine if adherence to the NU-AGE diet for a one year period could slow the progression of vascular dysfunction in older adults.

BMI

Dietary interventions have shown that both calorie intakes and diet quality can have an impact on BMI in older adults [356-361]. For example, Howarth et al. showed that higher total energy intakes and increased eating frequency was associated with a higher BMI in older adults [356]. Furthermore, Pala et al. identified a number of dietary patterns in an elderly cohort and found “pasta and meat” and “prudent” diets to be strongly positively associated with BMI in both men and women [357]. The NU-AGE intervention did not have a significant impact on BMI. This result was not overly surprising given that calorie restriction

was not involved and weight loss was not an aim of the NU-AGE study. Total body composition and body fat distribution are more indicative of cardiovascular risk compared with BMI in the elderly. Although DXA measures of body composition at baseline and follow-up were conducted for the entire NU-AGE cohort these data were not part of my PhD project.

Blood pressure

There are over 970 million people worldwide with hypertension and the WHO rates hypertension as a major contributing cause of premature death and CVD [362]. It is known that diets high in salt, saturated fat and alcohol consumption increase the risk of hypertension in older adults [362]. The NU-AGE diet had a number of goals that could have potentially resulted in a lowering of blood pressure in older adults, for example restriction of salt intake (the provision of a reduced salt cheese) and alcohol consumption. However, no changes from baseline were detected in SBP, DBP or pulse in response to the NU-AGE intervention. Several observation studies have shown various dietary patterns to be associated with lowering blood pressure [185, 186, 363-366]. In terms of whole diet intervention studies, the DASH diet, the PREDIMED diets and a number of other diets have been shown to lower blood pressure (as described in section 1.9.1) [194, 198-201, 367, 368].

It is difficult to determine exactly why there was no impact of the NU-AGE intervention on blood pressure but there are several potential factors. Many of the studies above examined populations that were at risk; many included populations with pre-existing hypertension or the metabolic syndrome. Our inclusion criteria did not require participants to be hypertensive or at higher risk of CVD but were representative of a healthy population of that age group. As such, it may have been more difficult to observe beneficial effects in a population that did not have high blood pressure to begin with. At baseline, 27% of the cohort were prescribed medications to treat hypertension. However subgroup analysis to determine differences in effects of the NU-AGE diet on blood pressure in medicated versus non-medicated participants showed no difference in response between groups (appendix 1). The sample size of 150 subjects was calculated based on PWV and EndoPAT figures and therefore a larger sample size may be required to determine any subtle effects of the NU-AGE diet on blood pressure. This will be further examined when the Norwich data is combined with that from the other four NU-AGE centres (n=1,250 participants across Europe) which will dramatically increase statistical power of the study.

Pulse Wave Velocity

PWV is a measure of arterial stiffness and has been shown to be a strong predictor of cardiovascular risk and mortality [55, 369]. It would be expected that follow-up results for the cohort as a whole would be slightly increased compared with baseline, due to the negative effect of ageing on vascular function. As expected, PWV modestly increased from 8.9 ± 0.2 to 9.1 ± 0.2 m/s for the cohort as a whole after one year.

Various dietary components [65] have been shown to modulate and slow the progression of arterial stiffness and therefore we hypothesised that participants following the NU-AGE diet would have improved arterial stiffness compared with the control group. A systematic review examining the effects of nutrient and non-nutrient dietary interventions on PWV showed potential for intakes of fish oils containing n-3 fatty acids (540 mg EPA combined with 360 mg DHA, Cohen's d = 0.21–0.81) and soy isoflavones (112 mg, Cohen's d = 0.35–0.39) in the treatment of arterial stiffness [65]. There is also limited but consistent evidence to indicate that salt restriction and bioactive peptides from fermented milk products could improve arterial stiffness [65]. ACN and flavone intakes (present in fruit) have also been shown to be inversely associated with arterial stiffness, as measured by PWV [63]. The NU-AGE dietary recommendations support intakes of oily fish (source of n-3 fatty acids) and fruit (ACNs and flavones), as well as salt restriction. However, despite this there was no impact of the NU-AGE intervention on PWV in older adults.

Although many studies have examined the impact of various nutrients on PWV as summarised above, research focusing on the effects of dietary patterns on PWV is limited, particularly in older adults. A limited number of dietary studies have been successful in improving PWV [200, 370-373]. However, to the best of our knowledge this study is the first study in which the effects of a whole-diet intervention for one year in healthy older adults on PWV have been examined. Considering that the NU-AGE cohort had substantially lower PWV measurements at baseline in comparison to the age matched European population, it could be speculated that the NU-AGE population were "too healthy" to see improvements in arterial stiffness. Further to this, although differences between the control and intervention group at baseline were not significantly different, the intervention group did have slightly lower blood pressure and PWV compared with the control group which could have exacerbated such an effect.

Another potential factor to explain the lack of improvement following intervention could be that modest changes in individual dietary components may not have been substantial

enough to result in improvements of PWV. The NU-AGE diet was designed with the primary outcome of improving inflammation rather than arterial stiffness [182]. Further research on the impact of a whole-diet specifically designed to improve arterial stiffness (perhaps with more emphasis on fruits particularly high in ACNs and flavones, n-3 fatty acid supplementation, and further restrictions on salt consumption) in healthy older adults is warranted.

Cardio-Ankle Vascular Index

In addition to PWV, CAVI was used to measure arterial stiffness. CAVI is thought to be a particularly useful indicator of arterial stiffness in elderly cohorts because it is independent of blood pressure and is therefore thought to evaluate arterial stiffness more accurately than PWV in those taking anti-hypertensive medications or those with masked hypertension [58, 59]. We aimed to determine, for the first time, if intervening with a healthy diet for one year could slow the progression of arterial stiffness, as assessed by CAVI, in older adults. Overall results showed there was no impact of the NU-AGE intervention on CAVI results. However, there was a significant interaction between gender and treatment. Subgroup analysis showed that female participants in the control group had a significant increase in CAVI over the period of one year, whereas in females following the NU-AGE diet this increase was significantly ameliorated. This effect was not seen in male participants.

As CAVI is a relatively novel tool, research on the impact of nutrition on CAVI is limited to a few cross sectional and intervention studies with small numbers. However, these interventions have shown the ability of various dietary compounds, including EPA, isoflavones and plant stanol esters, to affect arterial stiffness as assessed by CAVI through mechanisms independent of blood pressure [66, 67]. The only intervention study that has reported a gender specific investigation on the response to CAVI was a study looking at the effects of plant stanol ester consumption for 6 months on arterial stiffness in 92 subjects with a mean age of 51 years [68]. There was no impact of the intervention on CAVI in the whole study group, but in control men CAVI increased by 3.1% ($p=0.06$) and was unchanged in men following the intervention. In contrast to our results, they saw an effect on CAVI in response to diet in men only, however the authors did not speculate on a potential mechanism underlying the gender specific response. It is difficult to make comparisons between these results as this study only looked at the effect of plant stanol ester consumption, whereas our work involved investigating the effects of a whole-diet intervention. Although research has shown gender-related differences in baseline CAVI

levels [355], there is no other research on gender related differences in response to dietary interventions or the potential mechanisms involved. We speculated that perhaps women were more likely to be compliant to the NU-AGE diet then men. However, a one-way ANOVA examining potential differences in compliance across gender revealed compliance (as assessed via 8 month questionnaire previously discussed) to be almost identical among men and women. Age-related arterial stiffening has previously been shown to be more pronounced in women and the differences are thought to be the result of the differential effects of male and female hormones on vascular structure and function [374, 375]. It is also important to note that considering we did not see a beneficial effect of the NU-AGE diet on PWV or blood pressure values, the mechanism by which the NU-AGE diet beneficially impacted on CAVI in females is independent of blood pressure related mechanisms. Further research is needed to gain understanding of the greater CAVI responsiveness in females.

Ankle-Brachial Index

ABI is used to evaluate the degree of stenosis and the occlusion of the crural arteries. It is therefore valuable for the early detection of PAD and risk of cardiovascular events. Incidence of PAD increases with age and occurs in about 20% of the population aged over 60 years [376]. Based on the guidelines previously discussed, the NU-AGE cohort was, on average, within the normal range at baseline and at follow-up [276]. Smoking is the most important risk factor for PAD but diet is also known to play an important role [376]. Our result indicated no impact of the NU-AGE diet on ABI and therefore potentially PAD. Research on the impact of the whole-diet /dietary patterns on ABI in the elderly is limited. The PREDIMED assessed ABI and concluded that the Mediterranean diet was associated with reduced risk of PAD [195]. The most probable explanation for the lack of effect of the NU-AGE diet on ABI in this project is that PAD was within the normal range at baseline. In fact, 83.7% of the cohort fell within the normal range at baseline and only 6 participants had an average ABI ≤ 0.90, which could potentially make it difficult to observe improvements. Future work investigating the impact of diet on PAD in the elderly should be conducted in participants with increased risk or already existing PAD.

Reactive Hyperaemic Index (EndoPAT)

EndoPAT is used for the non-invasive quantification of endothelium-mediated changes in vascular tone and has previously been shown to have the ability to predict cardiovascular events beyond the Framingham risk score [26]. There was no significant effect of the NU-AGE diet on RHI results, as assessed by EndoPAT. There have been a number of studies

examining the impact of dietary components on RHI; however this is the first study to look at the impact of a whole-diet intervention on RHI in older adults. Previous research has shown associations between nutrients, such as vitamin D and n-3 fatty acid intakes, with RHI [377, 378]. Dietary interventions have shown that supplementation with dietary components, such as lycopene and n-3 fatty acids, can have a beneficial impact on RHI [41, 278]. It could be hypothesised that, through additive or even synergistic effects of these nutrients, the NU-AGE diet could have the potential to improve RHI in older adults. No such effect was seen. However, it is important to note that for many studies examining the impact of diet on RHI, no change in RHI has been detected [340, 379-381].

Furthermore, a recent study (published following NU-AGE initiation) has suggested that EndoPAT is not useful to detect the effect of robust interventions on endothelial function compared with the conventional techniques including FMD [382]. FMD is considered one of the gold standards and involves the use of ultra-sound imaging and RH, usually performed on the brachial artery [39]. This study showed that EndoPAT cannot detect changes in endothelial function in renally impaired and type-II diabetic subjects, when compared with a healthy population. In addition, EndoPAT could not detect changes in endothelial function in response to various interventions, such as glucose load and smoking, in healthy populations. Another recently published study indicated that FMD, but not EndoPAT, was significantly related to the Framingham risk score in a healthy middle-aged cohort [383]. It could therefore be speculated that the RHI, as measured by the EndoPAT, may be suitable for qualitatively establishing the presence of endothelial dysfunction, but may not be optimal for use in quantitative measurement of endothelial function or in studies examining subtle changes in response to intervention. As such, although these data have shown that the NU-AGE diet had no effect on endothelial function, this result should be interpreted with caution due to differences in various methods used to assess endothelial function and we recommend the use of FMD in future research in this field.

5.4.4 Biochemical measures of cardiovascular health; response to the NU-AGE intervention

A secondary aim of this PhD work was to examine the impact of the NU-AGE intervention on a number of biochemical measures of cardiovascular health, including nitrite, ET-1 and CRP plasma status.

Nitrite was measured in the plasma of NU-AGE participants before and after the intervention as a marker of NO synthase activity and potential cardiovascular related disease [341]. The mean plasma nitrite levels of the NU-AGE cohort fell within the normal range at baseline and after the intervention [341], and were not affected by the NU-AGE intervention. Various dietary compounds, including MUFA, EPA, L-arginine, folic acid and soy, have been shown to modulate levels of nitrite, nitrate and NO_x [43, 384, 385]. Furthermore, an intervention sub-study of 200 participants taking part in the PREDIMED trial showed that total polyphenol excretion in urine samples was positively correlated with plasma NO in Mediterranean diets supplemented with either extra-virgin olive oil or nuts [386]. This study also showed that the statistically significant increases in plasma NO were associated with a reduction in both SBP and DBP levels. However there have also been several dietary studies that showed no effect of intervention on NO levels [43, 387-389].

The plasma ET-1 means for the NU-AGE cohort at baseline and follow-up were similar to other studies in older adults [390, 391]. However levels of plasma ET-1 did not differ between the control and intervention group over the one year intervention period. Animal studies have shown that dietary interventions, including caloric restriction and salt consumption, can affect ET-1 expression and response [36, 392]. Human intervention studies have shown that ET-1 can be influenced by dietary salt, genistein, a vitamin D fortified yoghurt and a low calorie diet in combination with exercise [37, 393-395]. Other dietary interventions that were unsuccessful in modulating ET-1 included supplementation with walnuts and flaxseed [396, 397].

This is the first study to examine the impact of a year-long whole-diet intervention (tailored specifically towards the elderly) on endothelial function in UK older adults, using both clinical and biochemical markers. Overall, these data show that there was no effect of the NU-AGE intervention on endothelial function, as assessed by EndoPAT, plasma nitrite and plasma ET-1.

Elevated concentrations of CRP (defined as > 3 mg/l) are associated with an increased risk of cardiovascular events [398]. The baseline average CRP for the NU-AGE cohort as a whole was

1.86 ± 0.16 ml/l (SEM) which was considerably lower than that of the UK older adult population; a high, but widely varied figure of 5.36 ± 9.73 mg/l (SD) as reported by the NDNS [351]. The NU-AGE intervention did not impact plasma CRP concentrations in this subsection of UK-based older adults. Epidemiological research has shown that overall diet quality and healthy dietary patterns are associated with lower levels of CRP in the general population [186, 399-401], as well as in older adults [402]. Furthermore, increased adherence to the Mediterranean diet has emerged as being particularly associated with lower CRP concentrations across all age groups, including older adults [189-191]. Therefore, studies investigating the impact of the whole-diet on CRP have focused particularly on the Mediterranean diet. Many of these interventions have been successful in lowering CRP [81-84]. The DASH diet has been shown to be successful in lowering CRP concentrations in two small studies, but further research is required to establish benefits [403, 404]. We did not see an impact of the NU-AGE intervention on CRP in this UK sub-sample but this result should be interpreted with caution due to lack of power. The NU-AGE power analyses resulted in a sample size requiring 1000 participants in order to detect a difference of 0.6 (± 0.4 SD) unit change in CRP [182]. Future analysis and publications will report on the impact of the NU-AGE intervention on CRP in the entire cohort.

Mean concentrations of TC, HDL-C, LDL-C and TGs in the NU-AGE cohort at baseline were similar to the average values for UK older adults [351]. Healthy dietary patterns, including the Mediterranean diet, have previously been shown to be associated with an improved lipid profile [186, 192, 193]. Whole-diet interventions have shown that the Mediterranean diet and the Nordic diet can both improve lipid profile [83, 405-408]. Adherence to the NU-AGE diet over a one year period did not significantly improve lipid profile in UK older adults. However, modest improvements in the lipid profile were evident in the intervention group, with for example the TC: HDL-C ratio decreasing from 3.41 ± 0.22 to 3.07 ± 0.15, with such changes in the lipid profile analysis on the entire NU-AGE cohort likely to reach significance with an increased sample size. These results will be presented in future NU-AGE publications.

5.4.5 Plasma fatty acid status

Analysis of plasma samples revealed that there was no effect of the NU-AGE intervention on fatty acid status in older adults. The mean levels of fatty acids in the plasma of the NU-AGE cohort were similar to levels in previously published papers examining levels in older adults in the UK [213, 409]. Epidemiological and intervention based studies have shown that whole

diet interventions, including the Mediterranean diet, the PREDIMED diets and the Nordic diet, can beneficially influence plasma fatty acid status [187, 188, 410, 411] [197] [207]. Considering that participants in the intervention group of the NU-AGE study were advised to consume 2 portions of oily fish and 20 g of nuts per week, and were supplied with olive oil and margarine spread, it was surprising that there were no significant changes in any of the fatty acids following the intervention. Examination of data from the compliance questionnaire completed at the 8 month follow-up visit shows that only 50.7% of the intervention group were eating 2 or more portion of oily fish per week, 37.7% were having 1.5 or more tablespoons of olive oil per day, while 75.4% were consuming 2 or more servings of nuts per week. The low adherence to recommendations involving oily fish and olive oil consumption may explain the lack of change in fatty acid status in older adults following the one year intervention. The lack of dietary intake data at follow-up to assess compliance was a major limitation of this work. Future work involving the utilisation of follow-up dietary data will allow us to examine compliance and intakes in a more detailed manner. In line with the majority of the published prospective epidemiology and RCT data in humans we chose to present our fatty acid data as a percent of total fatty acids rather than as absolute concentrations [412]. There is currently a lack of direct comparison and consensus regarding the optimum method to present fatty acid data with respect to disease risk prediction or response to dietary fatty acid change. In the NU-AGE RCT, as no overall impact of intervention on plasma TG levels was evident, which is the predominant form of fatty acids in the circulation, the presentation as absolute concentration rather than % of total fatty acids, is unlikely to have had an effect on the key findings.

5.4.6 The impact of the NU-AGE intervention on a calculated vascular risk score

Initial analysis of individual outcomes showed no effect of the NU-AGE intervention on any of the individual outcomes measured. We created a cluster of vascular risk factors to determine if the NU-AGE intervention influences the overall risk score, however no effect on this integrative risk measure was evident.

The calculation of z-scores allowed the combination of data of different units. We designed a vascular risk score combining BMI, blood pressure, PWV, RHI, CRP, TGs and TC: HDL-C ratio; all of which have been shown to be associated with cardiovascular health [26, 55, 362, 398, 413]. As expected, there was an increase in vascular risk among the NU-AGE cohort over the one year period. The NU-AGE diet did not prove to be effective in ameliorating the risk.

Several scores have previously been proposed to identify adults at potential high risk for future disease, but as of yet there is no single cluster of risk factors that has been defined as the gold standard [344-346]. Although there were advantages to the utilisation of z-scores, there were also a number of limitations. Our risk score, like others, was not validated with actual cardiovascular outcomes to determine the degree of prediction. This should be addressed in future work and a defined set of outcomes should be determined that can be used across all studies. In addition, such scores are sample specific and the score derived in one study generally cannot be compared with other studies [345].

5.5 Conclusion

The main focus of this PhD project was to examine the impact of the NU-AGE intervention on measures of vascular function, as well as on inflammatory and fatty acid status in older adults. Overall, there was no significant effect of the NU-AGE intervention on any of the clinical or biochemical outcomes measured or the combined risk score. However, a subgroup analysis showed that there was a modest beneficial impact of the NU-AGE diet on cardiovascular health in older females.

This is the first study to assess the impact of a whole-diet intervention for one year on both arterial stiffness and endothelial function in older adults in the UK. Some of the strengths of this project have already been discussed, for example the use of validated clinical measures to assess vascular health, a diet designed specifically to target the nutritional needs of the elderly, the provision of certain food products and vitamin D supplements. Another strength is the long duration of the intervention. One year has previously been shown to be sufficient time to improve dietary intakes and observe changes in health outcomes [414, 415]. Seasonal-dependent changes in outcomes can also be avoided as outcomes were measured at the same time of year. However, there were also a number of limitations, which may explain the lack of beneficial effect of the NU-AGE diet on vascular health. As mentioned previously, the NU-AGE diet was designed with the primary goal to improve inflammatory status rather than vascular function. In addition, a major limitation was that the follow-up dietary data was not yet analysed at the time of thesis submission and so exact changes in diet composition cannot be determined. This could have helped to examine compliance in a more detailed manner. The sample size of 150 subjects was calculated based on PWV and EndoPAT figures and therefore a larger sample size may be require to determine an effect of the NU-AGE diet on other outcomes, such as blood pressure and CRP. A limitation that applies to many health related RCTs is the “healthy volunteer effect”; that volunteers are

more likely to be health aware compared with the general population [416-418]. However this can be particularly relevant to dietary interventions if the recruited cohort already has a healthy diet as they may already be achieving many of the dietary goals. Furthermore, volunteers may have signed up to this study with the intention of becoming more "healthy" and so those assigned to the control group may have been otherwise motivated to make dietary changes. Suggestions for future work examining the impact of a dietary intervention on vascular function in older adults include the use of an "at risk" population rather than a healthy cohort. Such a group is likely to be more responsive and gain more clinical benefit compared with a healthy older adult group. In addition, the use of gold standard methodologies to detect changes in vascular function is recommended, specifically the use of FMD rather than EndoPAT.

Chapter 6

Impact of *fatty acid desaturase* genotype and haplotypes on fatty acid status and response to the NU-AGE intervention in older adults

6 Impact of *fatty acid desaturase* genotype and haplotypes on fatty acid status and response to the NU-AGE intervention in older adults

6.1 Introduction

As previously discussed, plasma and tissue PUFA concentrations are associated with the risk of several diet related chronic diseases, including CVD [101, 104, 121, 419, 420]. Therefore it is important that the determinants of PUFA metabolism, and concentrations in the circulation and in target tissues are fully understood. LC-PUFAs are provided by the diet but can also be synthesised endogenously [421]. FADS and elongases are responsible for the conversion of PUFAs in humans and the synthesis of AA, EPA and DHA from their shorter chain essential fatty acid precursors namely LA and αLNA. These biosynthetic pathways have previously been described in Chapter 1. The D5D and D6D enzymes, which have been mentioned in a number of sections of this thesis, are the key rate-limiting enzymes in this pathway [122]. When examining the effects of the FADS enzymes on fatty acid status and cardiovascular related health outcomes, it is important to consider the impact of *FADS* polymorphisms. It has been reported that there are strong associations between gene variants in *FADS*1 and *FADS*2 and blood levels of LC-PUFAs, the most significant of which were found for AA [422]. Schaeffer et al. reported that the *FADS* genotype can account for up to 28% of AA variation, up to 7% of EPA variation, and up to 3% DHA variation in the blood [422]. Furthermore, there are also several studies which suggest that genetic variation in the *FADS* gene region, specifically SNPs associated with higher desaturase activity, are associated with higher levels of inflammation and CVD risk [131, 132, 142, 143, 423].

*FADS*1 SNPs include rs8448, rs145902, rs174547, rs174546. *FADS*2 polymorphisms include rs174575, rs1535, rs174605. However, it appears there are no key SNPs with respect to phenotype, with the whole region seeming to be important. We therefore aimed to identify tag SNPs to cover the whole *FADS* gene region. A tag SNP is a SNP in a region of the genome that has high linkage disequilibrium (LD) and can represent multiple genetic variants on one chromosome, known as a haplotype. LD refers to correlations among neighbouring alleles, reflecting haplotypes descended from single, ancestral chromosomes [424]. The utilisation of tag SNPs and haplotyping makes it possible to identify genetic variation and phenotype association without the requirement to genotype all SNPs in that chromosomal region or conduct sequencing [425]. Although it is recognised that the tag SNP may not be the

functional SNP, once an association with a tag SNP has been identified, subsequent genotyping of the gene regions for all known SNPs or sequencing, can then be conducted in order to identify the functional SNP. The goal of the international HapMap Project is to develop a haplotype map of the human genome. The HapMap can be used to describe common patterns of human DNA sequence variation, which in turn allows for the identification of tag SNPs that can represent the haplotype block [426]. We aimed to statistically reconstruct haplotypes from our selected *FADS* SNPs to represent the biologically functional genetic unit [129].

Although it is known that age influences the impact of *FADS* genotypes on fatty acid concentrations, research examining the impact of the *FADS* genotype on plasma fatty acid status in the elderly is limited [427]. The studies that have been conducted previously have involved a small number of SNPs that were selected based on previously published data [428-430]. Studies in older adults have shown that, in general, carriers of minor alleles of these SNPS have significantly lower AA and EPA [428-430]. This is the first study to examine the impact of *FADS* tagging SNPs and haplotypes in a healthy elderly UK cohort on plasma fatty acid status.

Research on whether dietary total fat and fatty acid intake influences the relationships between *FADS* gene variants and circulating fatty acid levels is also limited. Considering the future potential to provide personal nutritional advice to individuals based on their genetic make-up, along with other phenotypic characteristics such as health status, gender etc., it is important to examine how habitual fat intake and its manipulation can modify the relationship between *FADS* SNPs and fatty acid status. Although carriers of the major allele have been shown to benefit from increased EPA and DHA status, several studies have shown that the major allele is in fact associated with increased inflammation, cholesterol and CAD risk [131, 142-144]. This could potentially be the result of the high levels of n-6 fatty acids, such as LA, present in the typical Western diet [147]. High levels of LA, in conjunction with the presence of the major allele leads to increased conversion of LA to AA, which is a direct precursor of pro-inflammatory eicosanoids, such as prostaglandins and leukotrienes [147, 148]. The NU-AGE study was designed to target chronic inflammation in older adults [182]. The NU-AGE recommendations for the consumption of oily fish, as well as the provision of an n-3 rich spread, could help improve the ratio of n-6: n-3 intakes of participants following the NU-AGE diet. This could result in a beneficial, rather than detrimental, effect of the major allele on fatty acid status and inflammation. As discussed in chapter 1, a handful of dietary

interventions have previously been shown to modify the relationship between the *FADS* genotype and fatty acid status [154, 155, 431], however none of these interventions examined the impact of a whole-diet (including significant fatty acid manipulation) intervention in the elderly. Therefore, we aimed to examine whether the NU-AGE diet could influence the relationship between the *FADS* genotype and plasma fatty acids status. Specifically, we investigated if the NU-AGE diet could overcome any identified negative impacts of *FADS* minor alleles on EPA and DHA status, as well as the negative effect that the major allele has on AA status.

As discussed in detail in the previous chapter, the NU-AGE intervention significantly influenced arterial stiffness as measured by CAVI in the female group, which is likely to be in part due to an impact of fatty acid intake in the intervention group. Therefore, as an additional outcome, we aimed to examine whether arterial stiffness could also be influenced by *FADS* genotype and whether this influence was also gender specific. Although research has previously shown the *FADS* genotype to be associated with inflammation, CAD and cholesterol concentrations, no studies have been conducted to examine the impact of the *FADS* genotype on arterial stiffness [131, 142, 143]. Our hypothesis was that vascular function, and specifically arterial stiffness, could be influenced by the *FADS* genotype (most likely as a result of its impact on fatty acid status). If a significant effect was observed, we also aimed to determine if the NU-AGE intervention could modulate the relationship between genotype and arterial stiffness.

To summarise, we aimed to address the following;

1. The impact of the *FADS* genotype/haplotype on baseline fatty acid status in older adults
2. The potential of a year-long whole-diet intervention in older adults to influence the relationship between *FADS* gene variants and circulating fatty acid levels
3. The impact of the *FADS* genotype on vascular function, specifically the impact on CAVI

6.2 Methods

The full details of the study design have been described in Chapter 3, including the description of the blood sampling procedure. The method for the analysis of fatty acids in plasma has also been described in Chapter 3. All materials for genotyping were provided by LGC Genomics (Herts, United Kingdom) and Qiagen (Manchester, United Kingdom) unless otherwise noted.

6.2.1 Selection of tag SNPs and characteristics

In this project, tag SNPs were selected from the CEU (northern and western European) population of the HapMap (release 28) through the utilisation of the tagger functionality within haploview, configured to the pairwise tagging algorithm (http://www.broadinstitute.org/mpg/tagger) [425]. The *FADS* gene region of interest was entered as 61,323,679–61391401 on chromosome 11. This SNP genotype data was downloaded and subsequently analysed using haploview software. The minimum minor allele frequency was set to >10% and r^2 was set to >0.8. Following the genotyping of participants for these selected tag SNPs, the allele frequencies were calculated using the Hardy-Weinberg principle. For example for a SNP A/G;

$$p^2 + 2pq + q^2 = 1$$

The genotypic frequencies are as follows; AA is p^2, AG is $2pq$ and GG is q^2. p is allele frequency for A and q is allele frequency for G. In order to measure pairwise LD between each pair of SNP loci, Lewontin's disequilibrium coefficient D' and the squared correlation coefficient r^2 were estimated and plotted with JLIN v1.0 software (http://www.genepi.com.au/jlin).

6.2.2 DNA purification from blood sample

Genomic DNA was isolated from whole blood samples taken from participants at baseline. DNA was isolated using the QIAamp DNA blood mini kit. In brief, 20 µL of a protease solution (QIAGEN Protease) was added to 1.5 ml eppendorf tubes. 200 µL of the whole blood sample was then added to the protease solution. 200 µL of lysis buffer (QIAGEN Buffer AL) was added and eppendorfs were briefly vortexed and then incubated at 56°C for ten minutes. 200 µL of ethanol was added after incubation and samples were once again vortexed. Subsequent to the addition of ethanol, samples were transferred to a QIAamp spin column and were centrifuged twice at 8000rpm for 1 minute with wash buffers added in between

centrifugation steps in order to remove impurities. Genomic DNA was then eluted from the column by incubating the column with 200 µL of elution buffer before centrifugation at 8000rpm for 1 minute. Genomic DNA content was subsequently quantified using a nanodrop spectrophotometer and then stored at -20°C until use.

6.2.3 Polymerase Chain Reaction for genotyping

All DNA samples were genotyped via Kompetitive Allele Specific genotyping (KASP) by LGC Genomics (Herts, United Kingdom). The principle of KASP genotyping is based on the PCR process in which genomic DNA is denatured, primers are annealed, and complement allele specific tails with FAM™ or HEX™ fluorescent tags are elongated dependent on which allelic variants are present.

6.2.4 Statistical analysis

The data was assessed for normality by visual inspection of normal Q-Q plots. Outliers were determined by inspection of a box-plot for values greater than 1.5 box-lengths from the edge of the box and by calculating studentised residuals (≥ 3 SDs). Homogeneity of variances were assessed by Levene's test for equality of variances ($p > 0.05$). Homogeneity of covariances were assessed by Box's test of equality of covariance matrices ($p > 0.001$). A one-way ANOVA was used to determine the impact of each *FADS* SNP on LA, αLNA, AA, EPA and DHA concentrations (expressed as a % of total fatty acids). The impact of genotypes on desaturase activity, calculated using a product to precursor ratio of AA to LA, was also established. Covariates added in the ANOVA included gender, age (T0), BMI (T0), total energy intake (T0), total energy expenditure (T0), heart disease and blood pressure medications (T0), smoking status (T0), fish oil supplement use (T0) and non-processed fish intakes (T0). Subsequent post-hoc analysis was undertaken using Bonferroni adjustment. A one-way ANOVA was also used to determine the impact of each *FADS* SNP on a number of fatty acids at baseline in the group as a whole and also in the intervention group only. This analysis was repeated on follow-up data to determine if the impact of the *FADS* genotype was still significant post-intervention. A one-way ANOVA was used to determine the impact of each *FADS* SNP on baseline CAVI values. Covariates included in the repeated measures analysis were gender, age (T0), BMI (T0), total energy intake (T0), total energy expenditure (T0), heart disease and blood pressure medications (T0), smoking status (T0). All data are presented as mean ± SEM. Relative percentage changes were calculated by dividing the absolute value of the change by the baseline value and multiplying by 100.

6.2.5 Reconstruction of *FADS* haplotypes

The most likely predicted haplotype pairs per subject for all 10 selected SNPs were reconstructed using the haplo.em function (expectation-maximization algorithm) of the R package HaploStats in collaboration with Mr Jason Sinnwell (Mayo Clinic, Rochester, US) [432]. The region was inspected for recombination hotspots with both the D' and r^2 LD measures, and a small recombination region was found between markers 3-5. Sub-haplotypes were selected by inspection of the LD structure of the 10 selected marker SNPs. If recombination cut points (natural recombination hotspots) were observed across the 10 loci, sub-haplotypes of split regions were constructed using haplo.em and ld.pairs functions. In order to limit the number of covariates (therefore maintaining power and avoiding collinearity), we examined correlations of the covariates. Covariates were narrowed down to four key variables; sex, BMI, fish oil supplement use and non-processed fish intakes and we validated that these were not highly correlated in the analysis. Key responses and covariates were determined by using specific haplo.score models and correlation heatmaps [433]. Haplo.score and haplo.score.slide were run on the complete 10 marker haplotype to assess global and max-stat significance of the entire region and sub-haplotypes. The sliding window approach involves a set of adjacent SNPS being analysed progressively across a region to identify the most significant region of association. For the regions with the strongest association between haplotypes and fatty acids, haplo.glm (haplotype-based generalised linear model) was used to estimate frequencies, regression coefficients and p-values for individual haplotype effects, assuming an additive effect of haplotypes on the response The haplo.glm analysis performs a re-weighted least square approach to jointly estimate the regression coefficients and posterior probability of each haplotype pair using the expectation maximization algorithm. The reference haplotype was selected to be the most frequent haplotype as a baseline for linear regression by the software. Rare haplotypes were pooled and included in the model as a group called 'haplo.rare'.

6.3 Results

6.3.1 Selection of tag SNPs and their characteristics

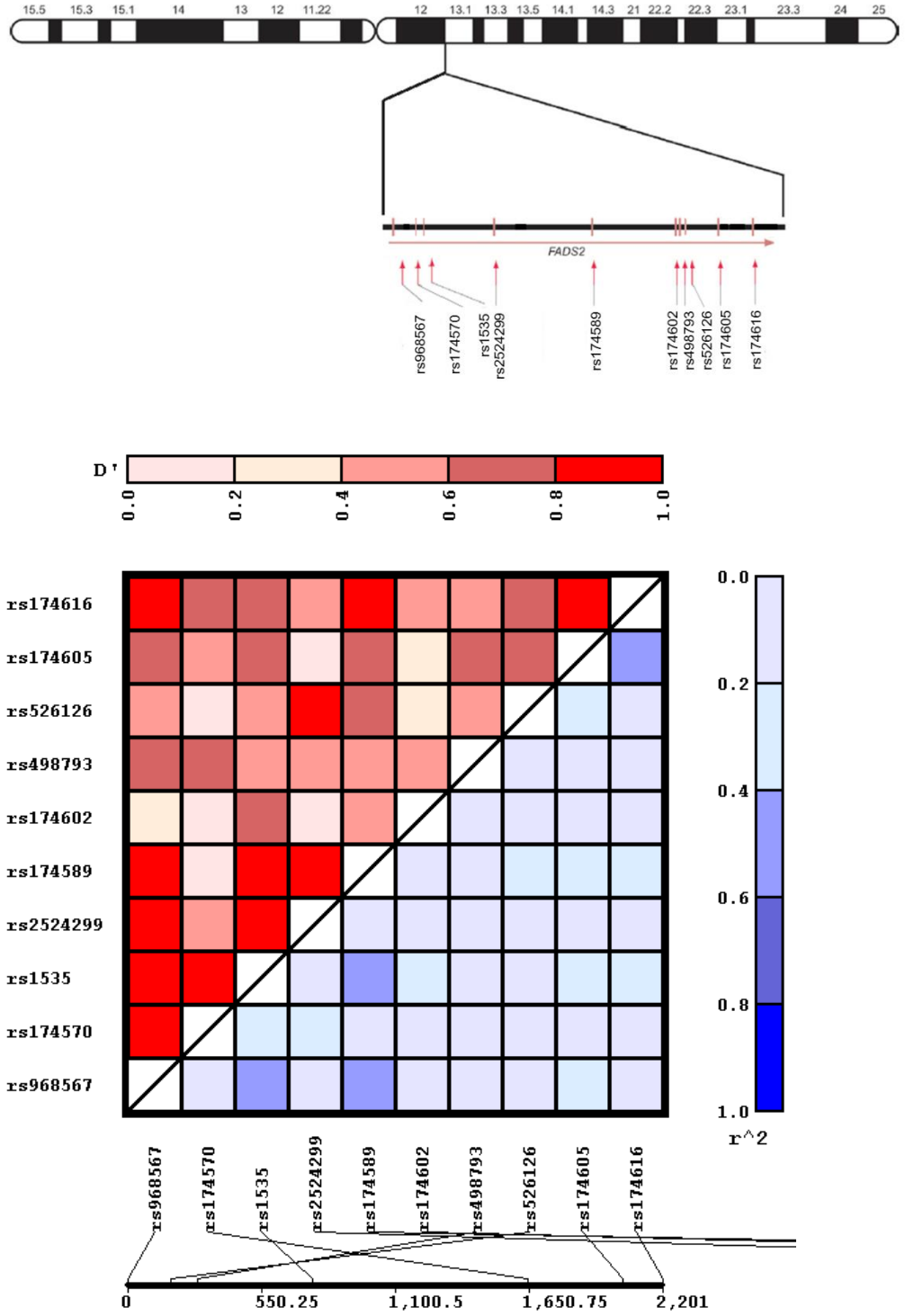
Using the HapMap, 10 tag SNPs were selected and genotyped to cover the *FADS* gene region. These SNPs and their characteristics, including their positions on chromosome 11, their possible functions and their allele frequency and that from the CEU HapMap population are shown in table 6.2. The minor allele frequency ranged from 13 to 51% in the NU-AGE cohort and from 15 to 47% in the CEU population. The distributions of genotypes for all analysed SNPs were consistent with Hardy–Weinberg equilibrium. Figure 6.1 shows the position of each of the selected SNPs on chromosome 11, as well the degree of LD between the analysed genetic polymorphisms. Examination of the LD shows that although several of the tag SNPs were linked, none of them showed a particularly high correlation with another.

Table 6.1. Characteristics of 10 *FADS*2 SNPs analysed

SNP ID	Position (base pair)	Most serious consequence	Alleles (1/2)*	Nu-Age Allele Frequency (%)		European Allele Frequency (%) [434]	
				Major	Minor	Major	Minor
rs968567	**61595564**	5 prime UTR variant	C/T	78	22	84	16
rs174570	**61597212**	Intron Variant	C/T	86	14	84	16
rs1535	**61597972**	Intron Variant	A/G	61	39	64	36
rs2524299	**61604782**	Intron Variant	A/T	87	13	85	15
rs174589	**61615803**	Intron Variant	G/C	75	25	79	21
rs174602	**61624414**	Non coding exon variant	A/G	77	23	79	21
rs498793	**61624705**	Intron Variant	G/A	63	37	58	42
rs526126	**61624885**	Intron Variant	C/G	78	22	81	19
rs174605	**61626921**	Intron Variant	G/T	67	33	72	28
rs174616	**61629122**	Intron Variant	C/T	49	51	53	47

*1/2; 1= Major allele, 2= Minor allele

Figure 6.1. Structure of the FADS2 gene cluster, its location on chromosome 11 and pairwise LD D' and r^2 plots of 10 SNPs across the *FADS*2 gene cluster.

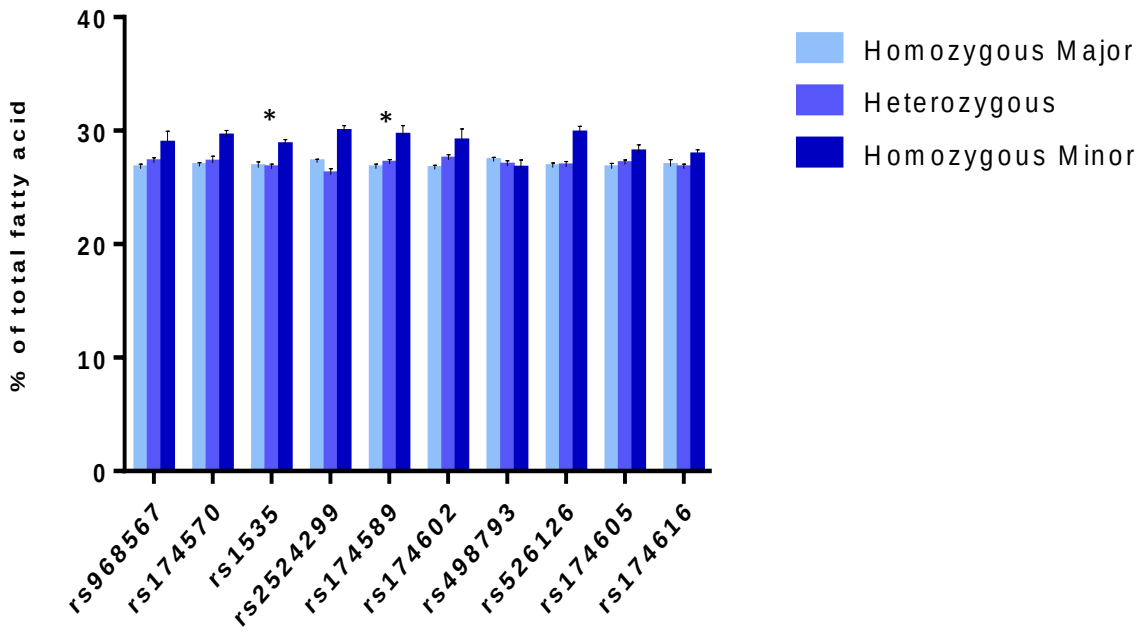


JLIN software was used to calculate the LD values of alleles at adjacent loci and to generate the plot graph.

6.3.2 Impact of *FADS* genotype on fatty acid status in older adults

The impact of each of the 10 selected *FADS* tag SNPs on plasma LA in NU-AGE older adults is depicted in figure 6.2. This graph shows that the majority of *FADS* SNPs had similar patterns, with carriers of the minor allele exhibiting higher concentrations of LA. Both the rs1535 (p=0.033) and the rs174589 (p=0.030) SNPs were significantly associated with plasma LA concentrations. Post-hoc analysis revealed that participants that were homozygous for the minor allele had significantly higher levels of LA compared with those that were homozygous for the major allele, 6.7% and 9.8% concentrations for rs1535 and rs174589 respectively. There were similar trends for rs526126 (p=0.050) and rs2524299 (p=0.078) that approached significance.

Figure 6.2. The impact of 10 *FADS* SNPs on Linoleic Acid (% of total fatty acids) in older adults

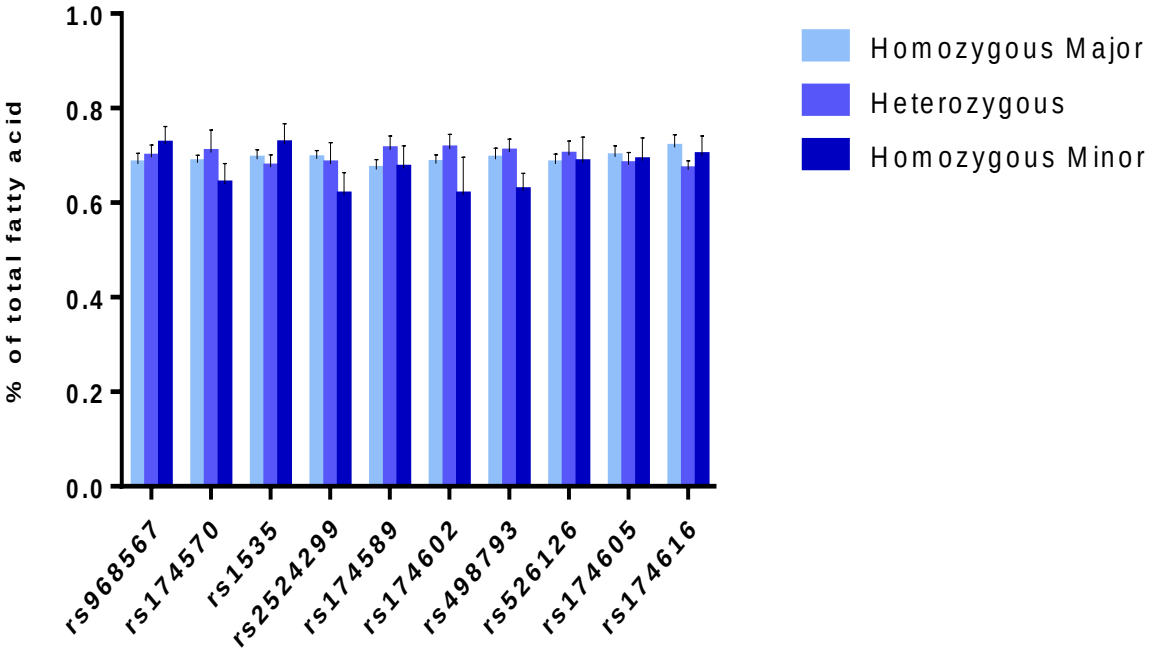


n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

Figure 6.3 displays the influence of the *FADS* genotype on plasma αLNA in older adults, with no significant effects of any of the 10 *FADS* SNPs on αLNA evident. The influence of the *FADS* SNPs on AA plasma levels is shown in figure 6.4. The majority of *FADS* SNPs had similar

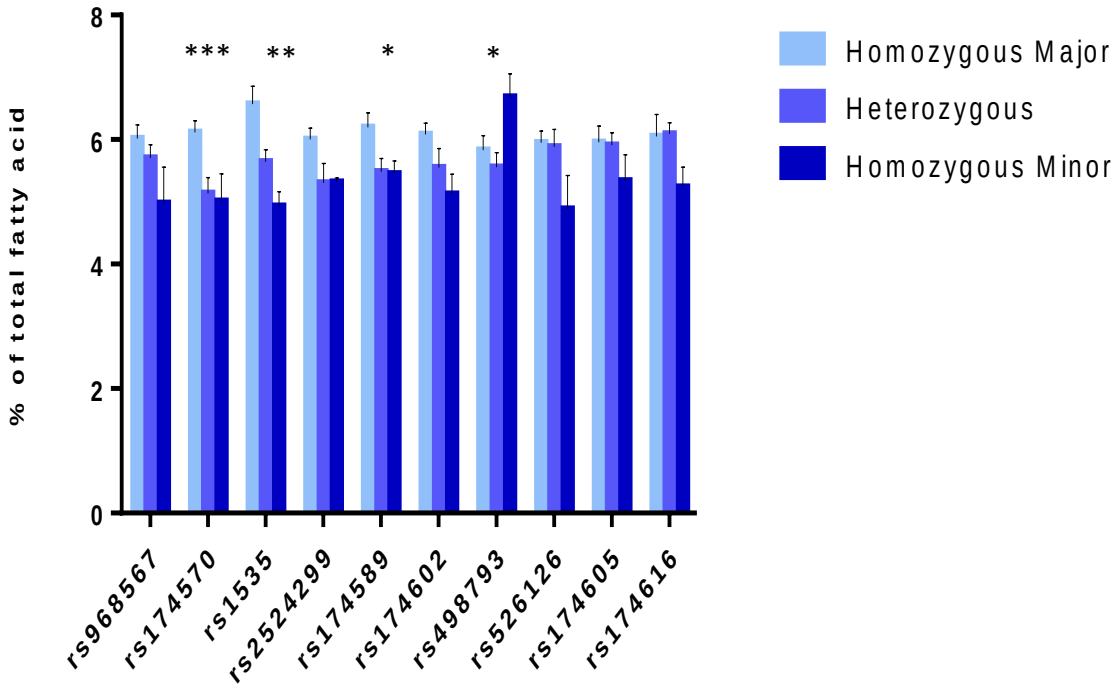
patterns, with carriers of the minor allele having lower levels of AA, with the exception of rs498793 which had the opposite effect. rs174570 (p=0.010), rs1535 (p=0.003), rs174589 (p=0.018) and rs498793 (p=0.021) SNPs were all associated with AA plasma levels. Post-hoc analysis shows that for rs174570 and rs174589 participants that were heterozygous had significantly lower, 15.7 and 12.1% respectively, levels of AA compared with participants that were homozygous for the major allele. Furthermore, participants that carried the minor allele for rs1535 also had significantly lower (9.7%) plasma AA. Conversely, participants that were homozygous for the minor allele in the case of rs498793 had significantly higher levels of AA (16.8%) compared with heterozygous participants.

Figure 6.3. The impact of 10 *FADS* SNPs on α-Linolenic Acid (% of total fatty acids) in older adults



n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid.

Figure 6.4. The impact of 10 *FADS* SNPs on Arachidonic Acid (% of total fatty acids) in older adults

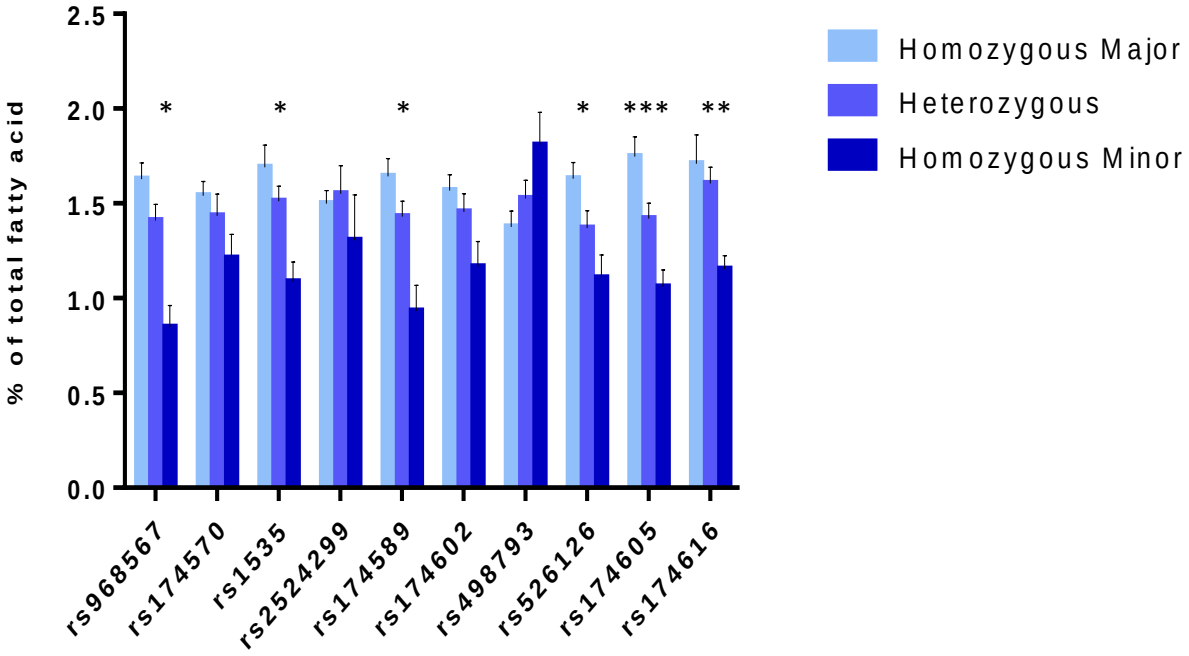


n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

Figure 6.5 shows the impact of the 10 *FADS* SNPs on plasma EPA levels in older adults. It can be observed that the majority of *FADS* SNPs have similar effects on EPA, with carriers of the minor allele of rs968567 (p=0.020), rs1535 (p=0.002), rs174589 (p=0.017), rs526126 (p=0.016), rs174605 (p=0.000) and rs174616 (p=0.001) being associated with lower plasma levels, with rs498793 which, although not significant, having contrasting effects. Post-hoc analysis shows that participants that were homozygous for the minor allele had significantly lower levels of EPA compared with participants that were homozygous for the major allele for rs968567 (47.9%), rs174589 (43.3%) and rs526126 (31.9%), while both participants that were heterozygous or homozygous for the minor allele had significantly lower levels of EPA compared with participants that were homozygous for the major allele for rs174605, 18.9% and 39.4% respectively. Participants that were homozygous for the minor allele had significantly lower levels of EPA compared with participants that were heterozygous or

homozygous for the major allele for rs1535 (10.7% and 35.5% respectively) and rs174616 (5.9% and 32.4% respectively).

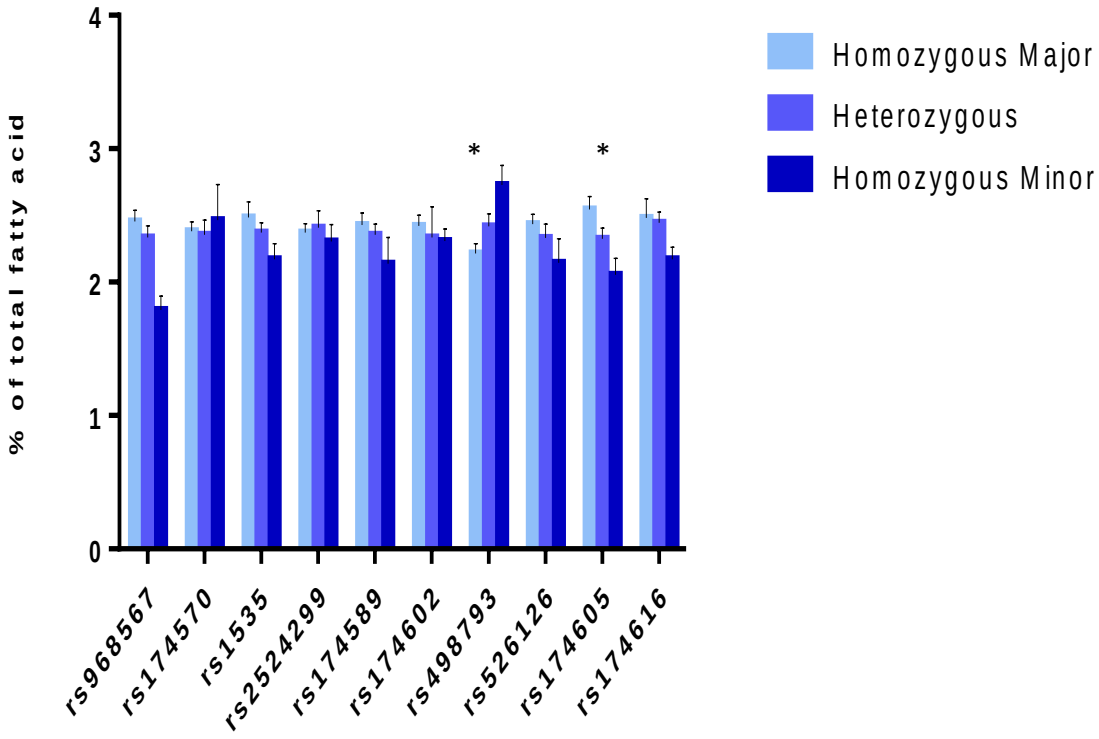
Figure 6.5. The impact of 10 *FADS* SNPs on EPA (% of total fatty acids) in older adults



n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

In terms of the impact of the *FADS* SNPs on DHA, figure 6.6 shows that the majority of *FADS* tag SNPs have a similar impact on DHA, with carriers of the minor allele tending to have lower plasma levels, with the exception of rs498793 which, again, had contrasting effects. Statistical analysis revealed that participants that were homozygous for the minor allele had significantly lower levels of DHA than participants that were homozygous the major allele for rs174605 (p=0.017, 19.3% lower) while opposite effects were seen for rs498793 (p= 0.012, 19.0% higher).

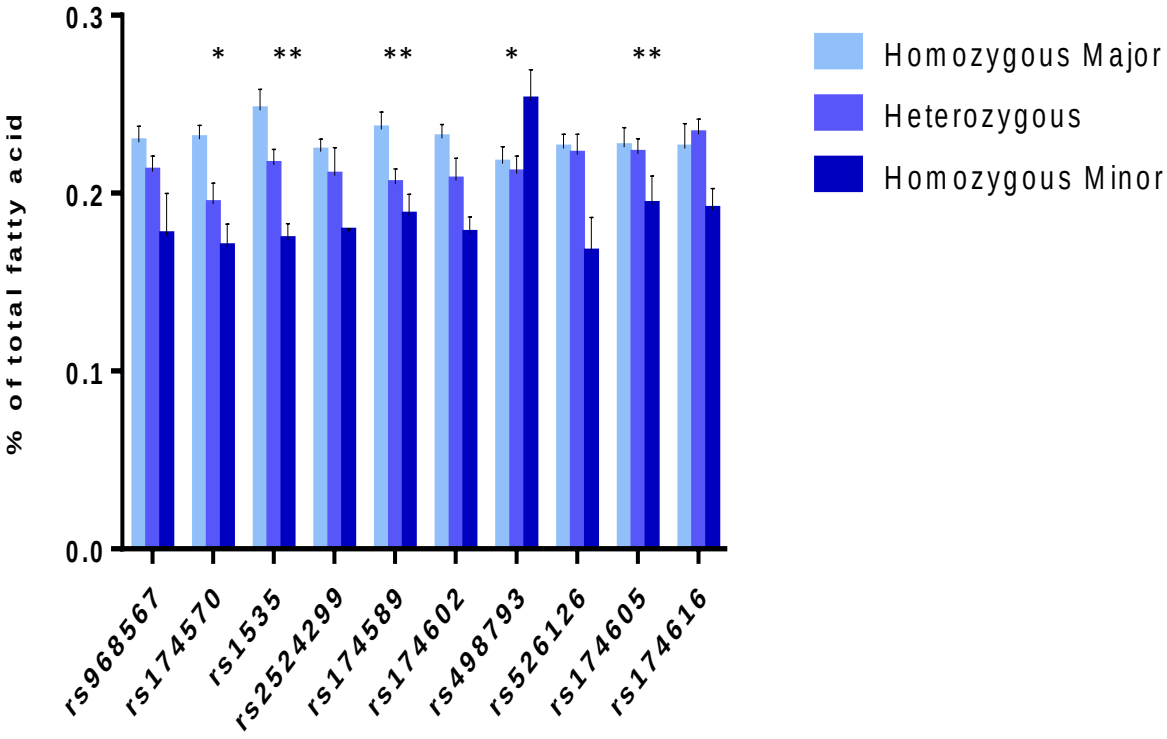
Figure 6.6. The impact of 10 *FADS* SNPs on DHA (% of total fatty acids) in older adults



n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

Lastly, the impact of each of the 10 *FADS* SNPs on a calculated measure of desaturase activity was analysed and can be observed in figure 6.7. There were significant associations of rs174570 (p=0.011, 26.4% size effect), rs1535 (p=0.003, 29.6%), rs174589 (p=0.007, 20.6%), rs5498793 (p=0.033, 16.0%) and rs174605 (p=0.003, 14.3%) with desaturase activity. Post-hoc analysis showed that carriers of the minor allele had significantly lower desaturase activity compared with participants that were homozygous for the major allele for each of these SNPs, with the exception of rs498793.

Figure 6.7. The impact of 10 *FADS* SNPs on desaturase activity in older adults



n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

6.3.3 Reconstruction of *FADS* haplotypes and associations with fatty acids

Haplotypes were statistically reconstructed for three different windows, as shown in table 6.3. The first window contained all 10 SNPs in the analysis, with 9 major haplotypes accounting for over 70% of all 10-locus haplotypes. The most common haplotype occurred in 26.6% of the cohort and contained all major alleles, with the exception of rs498793 which was shown to have generally opposite effects compared with other alleles (as described in section 6.3.2).

Table 6.2. Haplotype characteristics for 10-, 7- and 5- locus haplotypes

Haplotype	Alleles	Frequencies (%)
10-locus haplotypes	(rs968567-rs174616)	
Haplo.common	AAAAAAaAAA	26.6
Haplo.1	AAAAAAAAAA	12.6
Haplo.2	aAaAaAAaaa	6.0
Haplo.3	AAAAAAAAAa	5.8
Haplo.4	aAaAaaAaaa	5.4
Haplo.5	AaaaAAAAaa	4.8
Haplo.6	AAAAAAAAaa	3.7
Haplo.7	aAaAaAAAaa	3.1
Haplo.8	aAaAaaAAaa	2.4
Haplo.9	AaaAaaAaaa	2.3
Haplo.rare	**********	27.1
7-locus haplotype	(rs968567,rs174570, rs1535, rs2524299, rs174589, rs174602, rs498793)	
Haplo.common	AAAAAAa	29.2
Haplo.1	AAAAAAA	24.8
Haplo.2	aAaAaAA	10.3
Haplo.3	AaaaAAA	7.8
Haplo.4	aAaAaaA	7.2
Haplo.5	AaaAaaA	5.4
Haplo.rare	*******	15.4
5-locus haplotype	(rs968567, rs174570, rs1535, rs2524299, rs174589)	
Haplo.common	AAAAA	58.0
Haplo.1	aAaAa	21.3
Haplo.2	AaaaA	8.7
Haplo.3	AaaAa	6.1
Haplo.rare	*****	5.8

A; major allele, a; minor allele. 10-locus haplotype in order of chromosome location as follows; rs968567, rs174570, rs1535, rs2524299, rs174589, rs174602, rs498793, rs526126, rs174605, rs174616

There was one minor split observed in the 10-marker LD-block, which suggested a rationale to investigate sub-haplotype regions of interest across the whole region, rather than split into distinct LD-based blocks. To find highly-associated sub-haplotypes, we used the haplo.score.slide function of haplostats with sliding window sizes of 3 and 5 markers. The haplo.score.slide function is used to identify sub-haplotypes from a group of loci and calculates global and maximum score statistics. Sub-haplotypes of the first 7- and first 5-locus haplotypes were derived due to higher scores in the first 7- and first 5- markers. For example, in the case of AA (20:4n-6), scores above 2 were found in the first 7 at both slide 5 and slide 3 settings with or without covariates. There were 6 major 7-locus haplotypes. The most common haplotype was present in 29.2% of the cohort and contained all major alleles,

again with the exception of rs498793. 4 major 5-locus haplotypes emerged, with the most common haplotype occurring in 58% of the cohort and containing all major alleles.

Haplotype association analysis indicated significant associations and trends between a number of haplotypes and levels of plasma fatty acids, as shown in tables 6.4 and 6.5. For example, haplo.2 of 10-locus haplotypes contained 6 minor alleles, as well as the major allele for rs498793, was present in 6% of the cohort (table 6.3) and was found to be significantly associated with 29.1% lower EPA and 14.2% lower DHA levels (table 6.4). EPA levels were also significantly lower in those with haplo.4 (24.1%) and haplo.7 (38.0%), which contained 7 and 5 minor alleles respectively.

Sub-haplotypes showed similar patterns and haplotypes carrying minor alleles generally tended to be associated with increased levels of LA and decreased levels of AA, EPA and DHA, which was in line with the results of individual SNP analysis (as reported in section 6.3.2). For example, haplo.2 of the 7 locus haplotype was significantly associated with 32.4% lower EPA and 15.0% lower DHA compared with the common haplotype. In relation to the 5 locus haplotype, haplo.1 was significantly associated with 29.6% lower EPA compared with the common haplotype.

Table 6.3. Association of 10-locus *FADS* haplotypes with fatty acids; p-values and coefficients.

Haplotype		Linoleic Acid	α-Linolenic Acid	Arachidonic Acid	EPA	DHA
10-locus haplotypes (rs968567-rs174616), 10-locus haplo.common as reference						
Haplo.1	P-value	0.16	0.27	0.71	0.33	0.81
	Coefficient	-1.06	0.05	0.15	-0.21	-0.04
Haplo.2	P-value	1.00	0.48	0.38	**0.01***	**0.03***
	Coefficient	0.00	0.04	-0.40	-0.46	-0.37
Haplo.3	P-value	0.18	0.65	0.91	0.26	0.31
	Coefficient	1.84	0.05	-0.07	0.17	-0.24
Haplo.4	P-value	0.14	0.46	0.29	**0.05**	**0.08**
	Coefficient	2.04	-0.04	-0.52	-0.38	-0.32
Haplo.5	P-value	0.81	0.61	**0.06**	0.14	0.11
	Coefficient	0.25	-0.03	-1.08	-0.32	-0.34
Haplo.6	P-value	0.96	0.40	**0.05**	0.70	0.62
	Coefficient	0.05	-0.06	1.13	-0.09	-0.11
Haplo.7	P-value	**0.03***	0.61	0.38	**0.01***	**0.09**
	Coefficient	2.60	0.03	-0.50	-0.60	-0.36
Haplo.8	P-value	0.64	0.22	0.75	0.34	0.68
	Coefficient	0.66	0.11	-0.25	-0.27	-0.12
Haplo.9	P-value	0.21	N/A	**0.06**	0.10	0.82
	Coefficient	1.39	N/A	-1.18	-0.40	0.05
Haplo.rare	P-value	0.14	0.33	**0.06**	**0.03***	0.12
	Coefficient	0.93	0.03	-0.58	-0.26	-0.18

* P-values <0.05, p-values in bold <1.0

Table 6.4. Association of 7- and 5- locus *FADS* haplotypes with fatty acids; p-values and coefficients.

Haplotype		Linoleic Acid	α-Linolenic Acid	Arachidonic Acid	EPA	DHA
7-locus haplotypes (rs968567,rs174570, rs1535, rs2524299, rs174589, rs174602, rs498793), 7-locus haplo.common as reference						
Haplo.1	P-value	0.82	0.72	0.28	0.98	0.34
	Coefficient	-0.15	-0.01	0.35	0.00	-0.11
Haplo.2	P-value	0.40	0.50	0.15	**0.00***	**0.00***
	Coefficient	0.59	0.03	-0.51	-0.47	-0.38
Haplo.3	P-value	0.54	1.00	**0.05**	0.51	0.53
	Coefficient	0.53	0.00	-0.84	-0.11	-0.10
Haplo.4	P-value	**0.06**	0.93	0.27	0.17	0.42
	Coefficient	1.83	0.00	-0.56	-0.26	-0.14
Haplo.5	P-value	0.30	0.39	**0.00***	**0.06**	0.58
	Coefficient	1.02	0.05	-1.37	-0.35	-0.09
Haplo.rare	P-value	0.64	0.30	0.75	0.19	0.51
	Coefficient	0.42	-0.05	-0.12	-0.22	-0.10
5-locus haplotypes (rs968567, rs174570, rs1535, rs2524299, rs174589), 5-locus haplo.common as reference						
Haplo.1	P-value	0.10	0.53	**0.02***	**0.00***	**0.06**
	Coefficient	0.89	-0.02	-0.63	-0.37	-0.19
Haplo.2	P-value	0.26	0.88	**0.02***	0.31	0.78
	Coefficient	0.82	0.01	-0.84	-0.15	-0.04
Haplo.3	P-value	0.12	0.41	**0.00***	**0.06**	0.87
	Coefficient	1.30	0.04	-1.42	-0.33	-0.03
Haplo.rare	P-value	0.22	0.53	**0.03***	0.64	0.58
	Coefficient	-1.20	-0.04	-1.10	-0.10	-0.11

* P-values <0.05, p-values in bold <1.

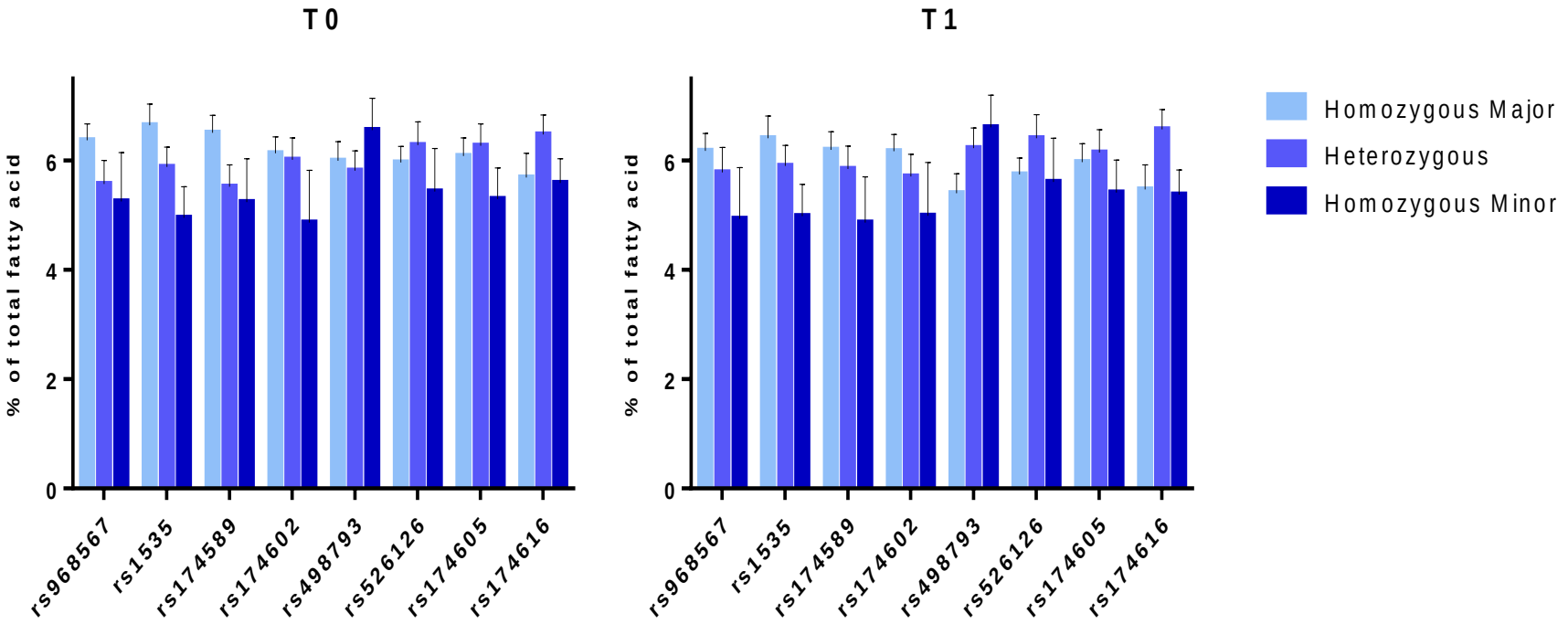
6.3.4 The potential of the NU-AGE intervention to overcome the impact of *FADS* genotype on fatty acid status in older adults

In order to test the ability of the NU-AGE intervention and increased n-3 fatty acid intake to overcome the negative impact of *FADS* genotype on EPA or DHA status, a subgroup analysis was conducted in the NU-AGE intervention group only (n=63). Due to minor allele frequencies of <5%, both rs174570 and rs2524299 were removed from this analysis. In this small subgroup there was no significant impact of any of the *FADS* SNPs on the essential fatty acids, LA and αLNA, at baseline and so the results discussed in the following section are those of the longer chained fatty acids (AA, EPA and DHA) only. The influence of the NU-AGE intervention on AA before (T0) and after (T1) intervention is graphically depicted in figure 6.8. There were no significant associations for any of the eight *FADS* SNPs with AA at T0 or T1.

The influence of the NU-AGE intervention on EPA before and after intervention is graphically depicted in figure 6.9. At T0, rs498793 (p= 0.002), rs968567 (p= 0.003), rs1535 (p= 0.002), rs174589 (p= 0.008), rs526126 (p= 0.009), rs174605 (p= 0.000) and rs174616 (p= 0.001) all had a significant association with EPA status. Post intervention, rs498793 (p= 0.022), rs174605 (p= 0.020) and rs174616 (p= 0.030) had a significant association with EPA plasma levels. For an example of a significant size effect reduction following intervention, the difference between EPA levels of participants that were homozygous for the major allele compared with those homozygous for the minor allele decreased from 43.8% to 34.3% for the rs174616 SNP.

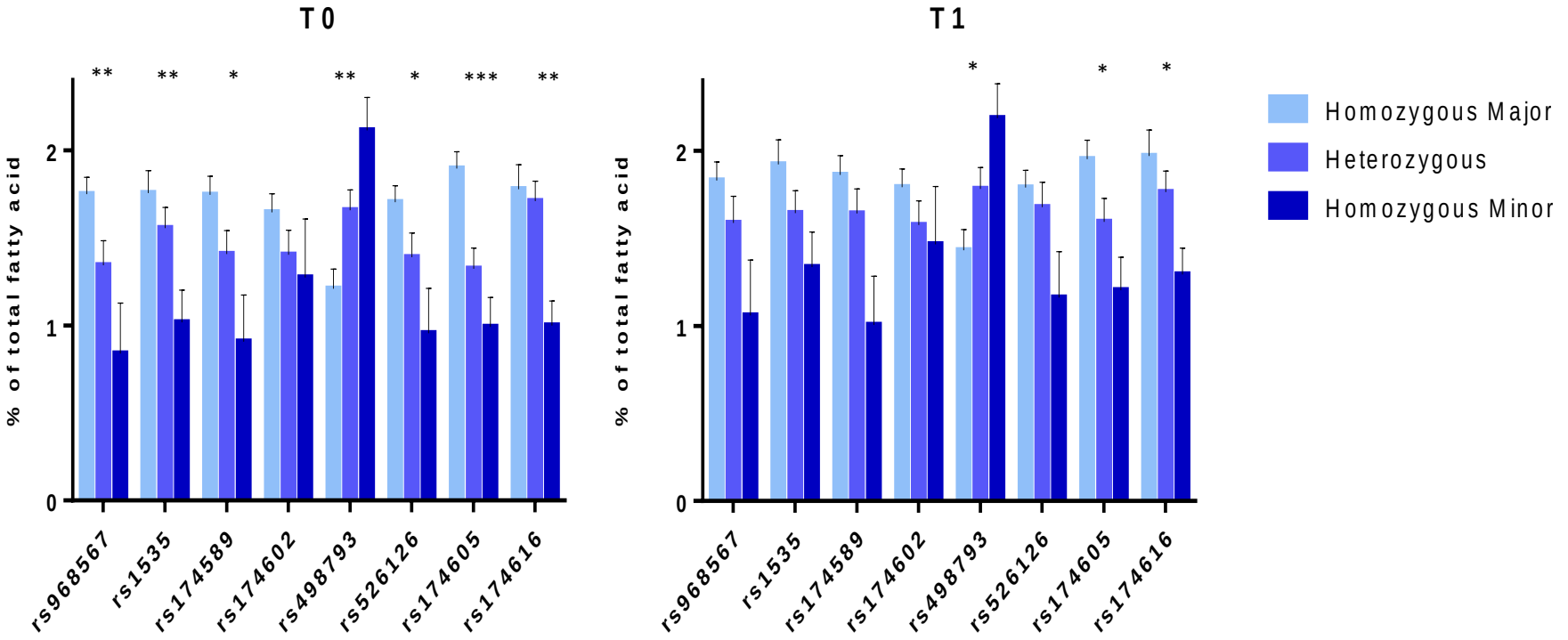
Figure 6.10 shows the influence of the NU-AGE diet on the relationship between the *FADS* genotype and plasma DHA status. At baseline, the DHA status of older adults was significantly associated with rs498793 (p= 0.000), rs174605 (p= 0.003) and rs174616 (p= 0.025). There were also trends towards significance for rs968567 (p= 0.063). Following intervention, only rs498793 was associated with the DHA status significantly (p= 0.003). The difference in DHA levels between participants homozygous for the major allele and those homozygous for the minor allele decreased from 31.0% to 26.9% for the rs498793 SNP following the NU-AGE intervention.

Figure 6.8. The impact of 8 *FADS* SNPs on Arachidonic Acid (% of total fatty acids) pre- (T0) and post- (T1) intervention in older adults in the NU-AGE intervention group



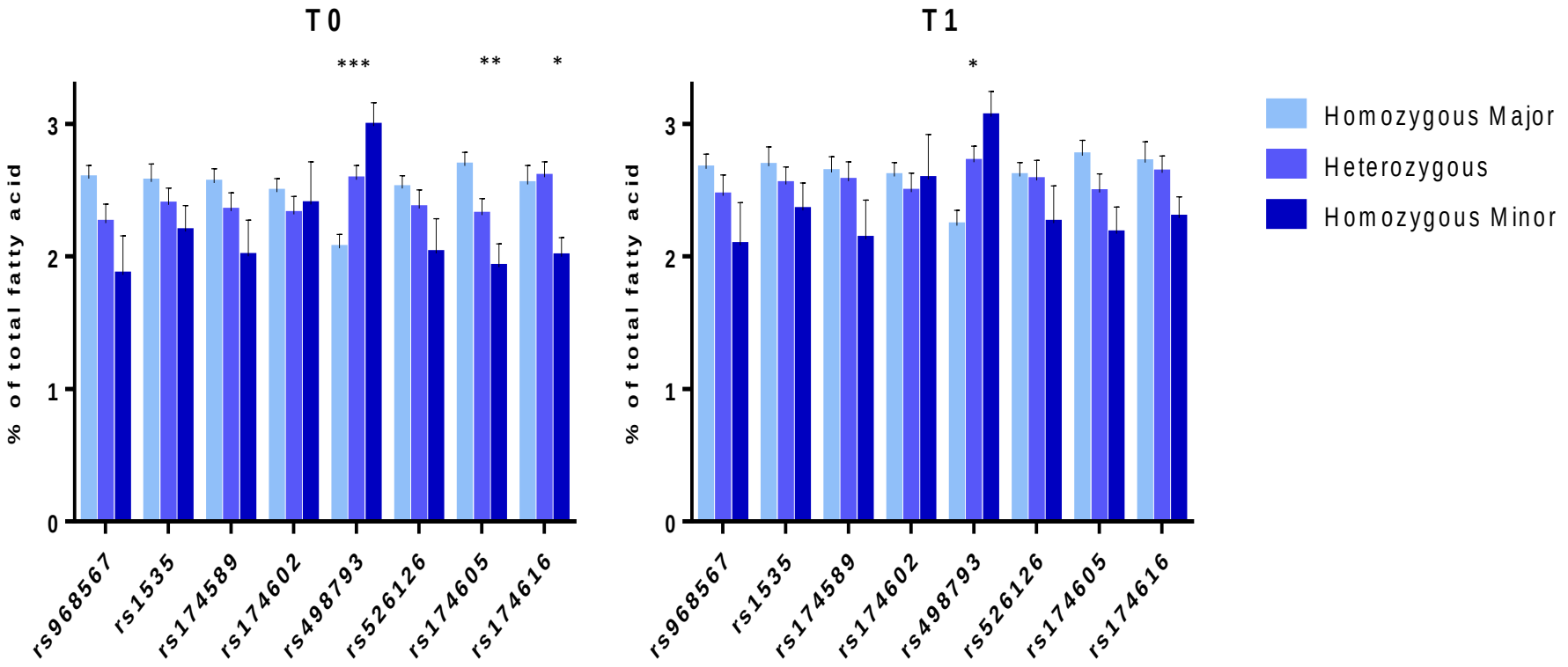
n=63, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

Figure 6.9. The impact of 8 *FADS* SNPs on EPA (% of total fatty acids) pre- (T0) and post- (T1) intervention in older adults in the NU-AGE intervention group



n=63, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

Figure 6.10. The impact of 8 *FADS* SNPs on DHA (% of total fatty acids) pre- (T0) and post- (T1) intervention in older adults in the NU-AGE intervention group

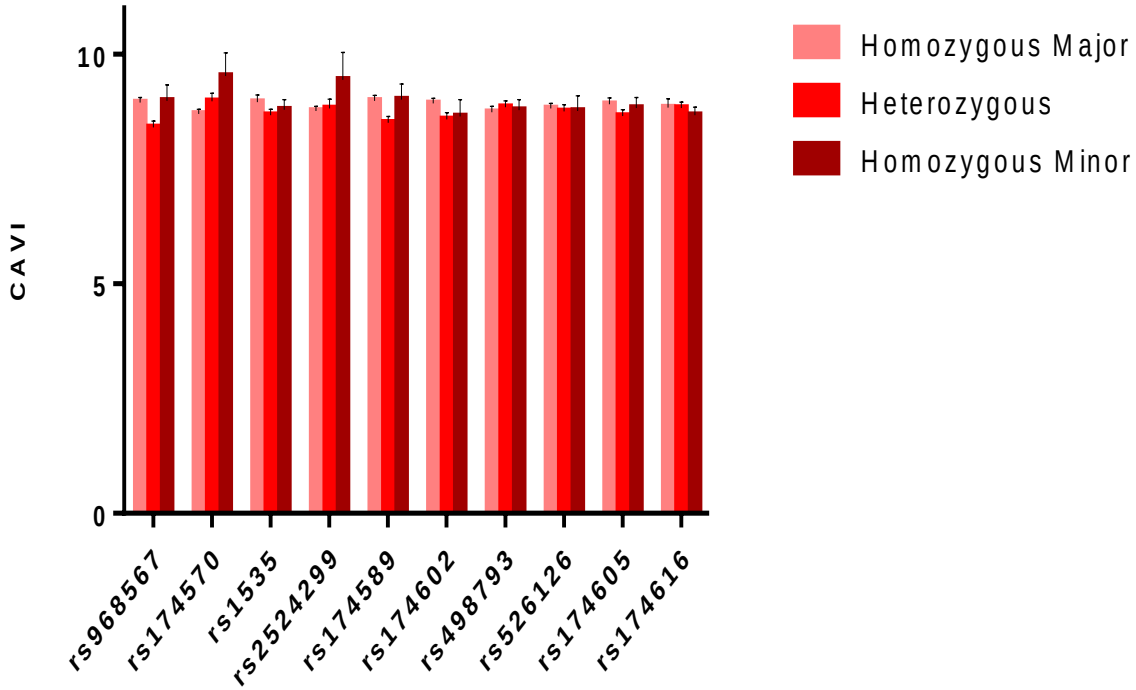


n=63, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

6.3.5 The impact of *FADS* genotype on CAVI

The impact of each of the 10 selected *FADS* tag SNPs on CAVI in older adults is graphically depicted in figure 6.11. There was no significant association for any of the *FADS* SNPs with arterial stiffness. Furthermore, gender specific subgroup analysis showed that there were also no significant associations for any of the *FADS* SNPs with CAVI in either males or females when separately analysed (data not shown). As there was no significant association for the genotype with arterial stiffness, it was unnecessary to examine the impact of the NU-AGE diet on genotype-fatty acid relationships.

Figure 6.11. The impact of 10 *FADS* SNPs on Cardio-Ankle Vascular Index in older adults



n=140, varying frequencies for homozygous major and homozygous minor for each SNP as shown in table 6.2. Data presented as mean ± SEM. 1-way ANOVA was conducted to examine the impact of treatment on each fatty acid. *p<0.05, **p<0.01, ***p<0.001.

6.4 Discussion

6.4.1 Selected tag SNPs and characteristics

Published research to date indicates that genetic variation in the whole *FADS* gene region may be important in terms of its effects on plasma fatty acid status; no particular SNP has emerged as having a much higher association with fatty acid status compared with other SNPs. Therefore, we used tag SNPs methodology to capture the genetic variation in the entire *FADS* chromosomal region and its impact on habitual fatty acid status and response to intervention. Such an investigation has not previously been carried out in older adults. 10 tag SNPs were identified through the use of the HapMap databases and the NU-AGE cohort was genotyped for these 10 SNPs. Table 6.2 shows that the majority of the selected SNPs are intron variants. Introns are the DNA sequences between exons that are removed by splicing and are frequently selected as tag SNPs [435]. Intronic SNPs in the *FADS* gene region may exert effects on plasma fatty acid status, potentially by influencing mRNA splicing and stability (and therefore gene expression). Alternatively, the tag SNP may simply be in LD with the functional SNP [435]. Comparison of the NU-AGE cohort with the European population described in HapMap show that the allele frequencies for all SNPs were generally similar (<10% difference between both populations for all major alleles), as shown in table 6.2 [434].

LD is defined as the non-random gametic association of alleles at different loci in a population, and is measured using D' and r^2 values. D is the difference between the actual gametic frequency and the expected gametic frequency when the loci are independent [436]. The D value is standardised by dividing D by its maximum value, given the allele frequencies. Therefore $D' = D/D_{max}$. r^2 is determined by the product of the four allele frequencies. In terms of association studies, r^2 is preferred to quantify and compare the amount of LD between pairs of loci because if its inverse relationship with the sample size [436]. Figure 6.1 shows that a small number of SNPs were in LD with each other as assessed by D' but in low LD with each other as assessed by r^2. This is because the D' value estimates are more likely to be inflated in studies with lower sample size and small allele frequencies [436], and therefore r^2 is a more suitable measure in our study. All SNPs were shown to be in low LD with each other, and therefore provided unique information regarding the genetic variability in the *FADS* locus and therefore none of the SNPs were removed from the analysis.

6.4.2 Impact of *FADS* genotype and haplotypes on fatty acid status in older adults

The results of the SNP and haplotype association analysis with plasma fatty acid levels in the NU-AGE cohort showed that for the majority of the selected *FADS* SNPs, carriers of the minor alleles tend to exhibit higher levels of the essential fatty acid LA (≤ 9.8%), as well as lower levels of the longer chained fatty acids AA (≤ 16.8%), EPA (≤ 47.9%) and DHA (≤ 19.3%). This suggests that, in general, carriers of the minor allele may have a reduced ability to convert precursor fatty acids into the product fatty acids via the *FADS* mediated pathway. Desaturase activity was calculated and was also shown to be ≤ 29.6% lower for participants carrying the minor allele. However there was one exception to this trend; in the case of rs498793 we saw the opposite effect was seen. Subjects that were homozygous for the minor allele of the rs498793 SNP had significantly higher AA (16.8%), DHA (19.0%) and desaturase activity (16.0%). This finding has previously been reported (as discussed below). Similarly, the most common haplotypes were made up of major alleles with the exception of the rs498793 SNP. Five of the SNPs are discussed below; these five were chosen as a result of having either a higher effect size or having an effect on more than one fatty acid.

rs174570

In the NU-AGE group, both AA and desaturase activity were significantly lower (15.7% and 26.4% respectively) in carriers of the rs174570 minor allele compared with subjects that were homozygous for the major allele. Similar results have been seen in younger age groups. For example, rs174570 was also analysed by Schaeffer et al. [130]. The minor allele for this intronic SNP was found to be associated with enhanced LA and αLNA, with decreased AA and EPA. Bokor et al. carried out a study on 3865 European adolescents and also found that carriers of the rs174570 minor allele had increased levels of LA and reduced levels of AA (effect sizes not reported) [133]. This study also reported associations for significantly reduced desaturase activity for carriers of the rs174570 minor allele; 21.4% and 13.5% lower for D6D and D5D respectively. Furthermore, rs174570 has also been shown to be significantly associated with TC and LDL-C levels with regression coefficients of 0.088 and 0.110 respectively [144]. Our lack of a significant association between rs174570 and other fatty acids, particularly EPA, is most likely due to the smaller sample size.

rs1535

Carriers of the minor allele of the rs1535 polymorphism, which tags for 17 *FADS* SNPs, had 6.7% higher LA levels, as well as lower AA (9.7%), EPA (35.5%) and desaturase activity (29.6%) in the NU-AGE cohort. Similar findings have previously been published in cohorts of infants, as well as widely ranged age groups (21-102 years) [134, 139, 437]. Several studies have also

shown a link between the rs1535 and IQ/cognition [136, 438, 439]; specifically that formula milk-fed children that were homozygous for the rs1535 minor allele performed worse than other children that had been on formula milk, while breast fed children tend to perform similarly irrespective of genotype. However, further research is required as the majority of research on rs1535 has been conducted on pregnant women, infants and young children, research should be carried out to determine the impact of the rs1535 on other adult groups. This could be carried out as an additional analysis on the NU-AGE project, as in addition to the genetic data described here cognitive data has been collected.

rs174589

In the case of the rs174589 polymorphism, it was shown that carriers of the minor allele exhibited significantly higher levels of LA (9.8%) and reduced levels of AA (12.1%), EPA (43.3%) and desaturase activity (20.6%) in this older adult cohort. Both Schaeffer et al. and Bokor et al. reported similar findings for this SNP but also found a significant increase in αLNA [130, 133]. Malerba et al. reported a 22.7% decrease in AA in a cohort of patients with CVD, while Aslibekyan et al. showed that although there were significant decreases in both AA (10.0%) and EPA (9.3%) in adipose tissue; these decreases did not translate into a change in inflammatory biomarkers, blood lipids or MI risk [132, 430].

rs498793

Interestingly, the rs498793 SNP was shown to have opposite effects on fatty acid metabolism compared with all of the other analysed SNPs, with the minor allele being significantly associated with increased AA (16.8%), DHA (19.0%) and desaturase activity (16.0%). This finding has previously been reported and investigations show this SNP to be atypical as it is not associated with other HapMap SNPs in the region [132, 133, 135, 440]. Brookes et al. have also shown this SNP to be significantly associated with ADHD (p=0.004) with an odds ratio of 1.6 in 180 ADHD cases compared with controls [440]. The mechanism of action for this SNP has not yet been identified. A number of studies have reported the rs498793 SNP to be an isolated SNP, in negative LD with other SNPs, however resequencing of the immediate gene region would be necessary to investigate the possibility of an alternative functional variant in the *FADS* gene region [133, 135, 440].

rs174605

Lower levels of EPA (39.4%), DHA (19.3%) and desaturase activity (14.3%) were evident in carriers of the minor allele of the rs174605 SNP in older adults. To date, the rs174605 has

only been included in one study in humans [441]. This was a recent Genome Wide Association Study on 8631 participants and examined n-6 PUFAs only. They reported that rs174605 minor allele carriers had higher LA (regression coefficient 0.92) and lower AA (regression coefficient -1.08). This is in line with the trends seen in our results, with a much smaller sample size explaining the lack of significance. Our study is the first to report that this SNP has a significant impact on both EPA and DHA levels.

Haplotypes

Although the impact of the individual FADS SNPs on fatty acid status is of interest, many of these SNPs have previously been examined. A more novel aspect of our analysis was the investigation of the impact of haplotypes, reconstructed from these individual SNPs, on fatty acid status in older adults, which provides a more holistic insight into genetic variation across the whole gene region on fatty acid status. The rs968567, rs174570, rs1535, rs2524299, rs174589 SNPs were all present in 10-, 7- and 5- locus haplotypes, where presence of the minor allele was associated with increased levels of LA and decreased levels of longer chained fatty acids, with the exception of rs174589. For example, the 10 marker haplo.2 was made up of 6 minor alleles and was associated with 29.1% lower EPA and 14.2% lower DHA. Haplo.2 was associated with a 20% lower plasma EPA and DHA combined (actual change from 4.2% to 3.4%). This is clinically significant as previous research has shown that an increase in plasma EPA+DHA from <3.5% to >4.2% is associated with a 72% reduction in risk of sudden death from cardiac causes [442]. Furthermore, combined EPA and DHA plasma levels >3.6% were significantly associated with reduced all-cause mortality over 5.9 years [442]. Research has also shown that a modest increase in oily fish, for example a total 180g of salmon per week can increase combined plasma EPA and DHA by 38% [443]. As different haplotypes are constructed based on selected tag SNPs in each study, it is difficult to make comparisons to other studies.

If the reconstructed haplotype analysis had resulted in impacts on fatty acid status that were stronger than the individual SNPs, this would suggest that tag SNPs, but not the functional SNPs, were analysed [130]. It could also suggest that more than one causal variant was present on a certain haplotype, resulting in an additive effect on fatty acid levels. However, the haplotypes showed equal, if not a lower, impact on fatty acids than the individual SNP associations, suggesting that a particular SNP may explain the associations of other tag SNPs.

Summary

To summarise, we aimed to investigate the impact of 10 selected tag SNPs, tagging the *FADS* gene region, on plasma fatty acid status in 65-79 year olds based in the UK. Research on the impact of the *FADS* gene on plasma fatty acid status in the elderly is limited and previous research has been conducted on studies examining the effects of a small number of SNPs. Results suggest for the majority of selected *FADS* SNPs (excluding rs498793) that carriers of the minor allele may have a reduced ability to convert precursor fatty acids, such as LA, into the longer chained product fatty acids, such as AA, EPA and DHA, of the *FADS* metabolic pathway within the body. This was supported by the results of the calculated desaturase activity, which was also shown to be up to 29.6% lower for participants carrying the minor allele. These effects have previously been reported in the studies containing cohorts of a range of ages mentioned above. However, it is difficult to make direct comparisons between studies as many do not report effect sizes and different studies examine different SNPs. Further research is required to explain the mechanism, which has not yet been elucidated, behind these results. As the majority of our selected *FADS* SNPs were intronic SNPs, it is not possible to determine whether any of the SNPs themselves are the functional polymorphisms responsible for exerting such effects on fatty acid status, or whether they are simply in LD with the functional SNPs. However, haplotype analysis did not result in stronger associations as a result of additive effects of SNPs. The intronic SNPs selected in this study could potentially be directly responsible for effects seen by affecting the folding function or expression of a micro RNA [444]. However further research is required in this area. The rs498793 polymorphism has previously been reported to display opposite effects to other analysed SNPs and this finding was replicated in our study [133, 135]. The cause of such an effect has not yet been determined, but as this SNP is considered to be an isolated SNP it is possible that the rs498793 itself is a functional SNP. The fact that this SNP has opposite effects compared with the majority of the *FADS* SNPs reinforces the importance of using a number of tagging SNPs rather than simply selecting one or two SNPs previously cited in the literature.

6.4.3 The potential of the NU-AGE intervention to overcome the impact of *FADS* genotype on fatty acid status in older adults

By comparing results before and after intervention for participants on the intervention group only, we aimed to determine if the NU-AGE intervention (and in particular altered fatty acid and increased EPA and DHA intakes) could modulate the effect of *FADS* genotype on fatty acid status. This is a novel approach in which a loss of a significant effect post-intervention

could suggest that the negative impact of genotype on EPA and DHA status could be ameliorated by increased EPA and DHA intake. One limitation of this analysis is that the use of the intervention group only results in a reduction of our original sample size and further research will be required in larger sample sizes.

As previously discussed, excess levels of AA can have negative health impacts, such as increased inflammation [147, 148]. We aimed to determine if adherence to the NU-AGE diet (designed to reduce inflammation) for one year could help overcome the impact of the major allele which tends to be associated with increased AA [148]. However, the significant association for the *FADS* SNPs with AA reported in section 6.4 was no longer significant in this analysis due to a reduced sample size and therefore future research will be necessary to investigate this hypothesis. As discussed in section 6.4, the FADS minor alleles were generally associated with reduced EPA and we aimed to examine if the NU-AGE diet could ameliorate the difference between minor allele carriers and those homozygous for the major allele. The results show that the significant differences found for rs968567, rs1535, rs174599 and rs526126 before the intervention were no longer significant following this dietary intervention. For example, the highly significant (p= 0.002) difference of 41.7% in EPA levels between the rs1535 homozygous major and minor allele participants decreased to a non-significant difference (p= 0.199) following intervention of 30.2%. Differences among rs498793, rs174605 and rs17616 were also reduced but remained significant. Therefore despite our small sample size, we saw that adherence to a healthy dietary intervention (including advice to improve EPA and DHA intakes) could help overcome the impact of genotype on EPA status.

The *FADS* minor alleles also tend to be associated with lower DHA levels compared with those that were homozygotes for the major allele, although to a lesser extent than EPA as discussed in section 6.4. The results suggest that the NU-AGE diet could potentially influence the impact of the FADS genotype on DHA status. For example, we saw that the significant impact of rs174605 and rs174616 was no longer significant post-intervention and the significant impact of rs498793 was ameliorated (31.0% to 26.9%), although still significant. In the case of rs174605, the highly significant (p= 0.003) difference in DHA levels between the homozygous major and minor allele participants decreased from 0.77% to a non-significant difference (p= 0.134) of 0.58% following intervention.

To date, limited research has been carried out to investigate the impact of dietary interventions on the relationship between *FADS* SNPs and fatty acid status and this area of

research is worthy of future investigations. Gillingham et al. conducted a randomised crossover trial in which 36 hyperlipidemic subjects were assigned 1 of 3 diets for a 4 week period to examine the impact of αLNA intakes on fatty acids according to *FADS* genotype. A typical Western diet (1.3g αLNA /d) was compared with diets enriched with either flaxseed (20.6g αLNA /d) or canola (2.4g αLNA /d) oils [154]. 5 *FADS* SNPs were chosen based on previous literature, including rs174545, rs174583, rs174561, rs174537 and rs953413, none of which are overlapping with the SNPs analysed in this NU-AGE based research. *FADS* SNPs were not found to be associated with plasma DHA in this study. In addition, Porenta et al. investigated the impact of a 6 month Mediterranean diet intervention on the associations between *FADS* genotype and changes in serum and colonic fatty acids in 108 participants [155]. Again, the *FADS* SNPs (rs174556, rs174561, rs174537 and rs3834458) analysed in this study did not correspond to any of the 10 SNPs analysed in the NU-AGE project. Porenta et al. reported that there was no diet by genotype effect of the intervention on serum fatty acid status. However, this study did show a significant diet by genotype interaction for AA concentrations in the colon; subjects who had all major alleles for *FADS* SNPs and were following the Mediterranean diet intervention had 16% lower AA concentration in the colon after 6 months of the intervention compared with subjects following the control diet.

Overall, dietary advice on EPA and DHA intakes (included as part of the NU-AGE diet) could potentially overcome the deleterious impact of the *FADS* genotype on EPA and DHA status. Although Porenta et al. [155] previously reported results on the interactions of genotype and the Mediterranean diet on changes in fatty acids, this is the first study to show the impact of a one-year whole-diet healthy eating intervention (including advice on intakes of EPA and DHA) on EPA and DHA in the elderly. Compared with the Porenta et al. study, our study contributed to the current scientific literature by using 10 tag SNPs, a 12 month intervention and healthy older adults [155].

The major strengths of this study include the long duration, the use of tag SNPs, as well as the dietary intervention design of the study which is novel in genetic based research. However, there were also several limitations to this research. The likely heterogeneity of the intervention that would have resulted from personalised dietary advice, as well as likely differences in compliance, are major limitations in analysing the impact of the NU-AGE intervention on the relationship between the FADS genotype and fatty acid status. As mentioned previously, changes in fatty acid intakes following the NU-AGE intervention were also unavailable for use in this thesis but will be examined and included in future

publications. In addition, the small sample size also resulted in two SNPs being excluded from the analysis due to lack of sufficient numbers of participants that were homozygous for the minor allele. Future work is required to examine the ability of the diet to modulate the relationship between the FADS genotypes, fatty acids and related health outcomes and such an investigation could be considered as a potential investigation in the entire NU-AGE cohort.

6.4.4 The impact of *FADS* genotype on CAVI

As discussed in section 6.4, the *FADS* gene appears to have a significant impact on fatty acid status in older adults. We also saw that the NU-AGE intervention may have the potential to help overcome the impact of the *FADS* genotype on fatty acid status, as discussed in section 6.5. Our aim was to determine if polymorphisms present in the *FADS* gene region could also potentially impact on arterial stiffness in older adults. To our knowledge, this hypothesis has not previously been tested. Our results show that there was no impact of any of our selected *FADS* SNPs on CAVI in older adults.

Previous research, in relation to the *FADS* gene region and cardiovascular health, has focused on the impact of *FADS* polymorphisms on outcomes including CAD, inflammation and cholesterol levels [131, 142, 143, 423]. Although the results of these studies have not always been consistent, carriers of the minor allele appear to be at reduced risk of CAD [131, 142, 143].

Strengths of this research include the use of 10 tag SNPs and use of CAVI, a novel and validated measure of arterial stiffness. However, there were also limitations. As mentioned before, the power calculations for the sample size used in this thesis were calculated to determine the impact of the NU-AGE intervention on vascular function but did not account for genotype sub analysis. Therefore, we were likely to be underpowered to detect subtle impacts of genotype on vascular function and further research in a larger sample size is warranted to establish the effects of the *FADS* genotype on vascular function.

6.5 Conclusion

Although the impact of many of the individual FADS SNPs on fatty acid status in older adults have previously been examined, this is the first study to examine the impact of FADS tagging SNPs and haplotypes in a healthy elderly cohort on plasma fatty acid status. Our results showed that for the majority of the selected *FADS* SNPs, carriers of the minor alleles tend to exhibit higher levels of essential fatty acid LA, as well as lower levels of the longer chained fatty acids AA, EPA and DHA. Haplotype associations were also in line with these findings. For examples, Haplo.2 was associated with a clinically significant 20% decrease in plasma EPA and DHA combined (from 4.2% to 3.4%). Previous research has shown that an increase in plasma EPA+DHA from <3.5% to >4.2% is associated with a 72% reduction in risk of sudden death from cardiac causes and that combined EPA and DHA plasma levels >3.6% were significantly associated with reduced all-cause mortality over 5.9 years [442]. Research has also shown that a modest increase in oily fish, for example a total 180g of salmon per week can increase combined plasma EPA and DHA by 38% [443]. Therefore, we examined whether the NU-AGE intervention (including advice to consume 250g of fish per week) could modulate the effect of *FADS* genotype on fatty acid status by comparing results before and after intervention for participants in the intervention group only. The results of this analysis suggested that the NU-AGE diet could potentially overcome the impact of the *FADS* genotype on EPA and DHA status. This is the first study to show the impact of a one-year whole-diet healthy eating intervention (including advice on intakes of EPA and DHA) on EPA and DHA in the elderly and the results suggest that offering personalised fatty acid advice could be a useful approach in relation to overcoming the impact of the FADS genotype on EPA and DHA status. Finally, we examined the impact of the *FADS* genotype on arterial stiffness for the first time, as measured by CAVI. However, no significant effects were observed.

These findings significantly contribute to current scientific knowledge and are relevant in the context of public health as the results could contribute to future work in the area of personalised nutrition based on genotype. Endogenous synthesis may be a more viable source of EPA and DHA in individuals that are homozygous for the major allele, while dietary recommendations could inform carriers of the FADS minor allele of their increased requirement for dietary sources of EPA and DHA. Furthermore, the findings should be carefully considered in terms of future research; EPA and DHA blood levels are often used as biomarkers of dietary EPA and DHA exposure in RCT and epidemiological studies. The FADS

genotype should also be taken into account as the relationship between dietary EPA and DHA with plasma EPA and DHA are likely to be influenced by the FADS genotype.

Strengths of this project include the use of tagging SNPs and the reconstruction of haplotypes, as well as the novelty of examining the impact of the whole-diet on the relationship between the *FADS* genotype and fatty acid status in older adults. Examining the impact of the *FADS* genotype on arterial stiffness was also a novel aspect of this work. However, there were also limitations, namely the sample size. Frequencies of some of the minor alleles and several haplotypes were quite low in the cohort, and so a larger sample size could potentially result in significant associations that were not observed in our relatively small sample size. Therefore, future work in this area should include a larger sample size. This data suggests that the NU-AGE diet may have the potential to overcome the impact of the *FADS* genotype and therefore further analysis could be carried out on the entire NU-AGE cohort. In addition, studies examining the effects of the *FADS* genotype on actual desaturase activity, rather than using various calculations for desaturase activity, are also warranted.

Chapter 7

Conclusion

7 Conclusion

7.1 Summary

Currently, 22.9% of the European population is aged over 60 years, while 4.5% are aged over 80 years [8]. Realistic dietary strategies, which focus on the whole-diet rather than individual dietary components, need to be identified to help contribute to increased healthy life years and to reduce the social impact and healthcare costs associated with ill health. Research on whole-diet interactions is vital because foods and nutrients have additive, synergistic or perhaps even antagonistic effects when consumed in combination [177-184], which collectively may have a large impact on risk of disease. Investigations of individual dietary components, in the form of extracts or supplements, may be misleading as the bioactive may have difference effects when consumed in a food matrix or as part of a mixed diet.

CVD causes more than half of all deaths in Europe and the maintenance of cardiovascular health is therefore an important target in the design of dietary interventions [211]. Both vascular function and low grade chronic inflammation are known to be major contributors to CVD pathology and can also be used as early indicators of disease risk. As discussed throughout this thesis, optimal fatty acid status, particularly EPA and DHA plasma and tissue levels, is considered to be beneficial in relation to cardiovascular health outcomes, but at a population level intakes are sub-optimal. In the UK, average EPA and DHA consumption in adults is 244mg per day, which is about 50% of the recommended minimal intake [90]. EPA and DHA status and biosynthesis can be influenced by dietary fatty acid intake and also potentially by other non-lipid components of the diet. For example, previous research suggests there may be an association between moderate wine consumption and n-3 fatty acids, EPA and DHA, in human blood cells and plasma [156]. Furthermore, adequate fatty acid status has been observed in vegetarians and vegans despite negligible EPA and DHA intakes, and confirmed in two studies in animals, suggesting that specific plant derived components, such as flavonoids, may have the potential to improve plasma fatty acid composition [127, 157]. EPA and DHA status can also be influenced by genetics; polymorphisms in the *FADS* 1 and *FADS* 2 gene region have been shown to influence EPA and DHA status.

As discussed in chapter 2, serum, plasma and various tissues from humans and animals that were fed a broad range of compounds (including ACNs, flavan-3-ols, blueberry extract, alcohol and champagne) were analysed to determine whether consumption of these

compounds could stimulate the bioconversion of ALNA to EPA and DHA. This was the first study in which the impact of ACN consumption on fatty acid status was investigated in humans and has significantly contributed to knowledge in the area. The results from this project found no evidence that consumption of ACNs (in addition to other flavonoids and champagne polyphenols) can stimulate fatty acid biosynthetic pathways to produce increased plasma or tissue EPA and DHA, suggesting that some other bioactive component may be responsible for the improved EPA and DHA status observed in red wine consumers and in those who consume a plant-based diet.

The study design for NU-AGE has previously been published [182]. This PhD included data from the Norwich study centre only and the primary aim of this PhD project was to investigate if adherence to the NU-AGE diet for one year could slow the progression of both endothelial dysfunction and arterial stiffness in older adults. Using baseline data, a diet score based on the NU-AGE diet was designed that would be suitable for use in elderly populations. The diet score was created, and validated using TWIN UK data, which included 3262 female participants aged between 18 and 79 years old whose diet was assessed using validated FFQs. Using the TWIN UK data, a significant association for an improvement in CRP concentrations with increasing NU-AGE diet score was observed. The NU-AGE diet score was then used to determine associations between the diet score and various health outcomes in the NU-AGE cohort. No significant associations were observed between the NU-AGE diet score and CRP (although a strong trend was observed), or any of the other cardiovascular related health outcomes measured in the NU-AGE cohort, which consisted of 242 male and female participants aged 65-79 years. A likely explanation for the significant association observed in the TWIN UK cohort but not in the NU-AGE cohort is the large difference in the sample size of the cohorts; analysis using the TWIN UK cohort consisted of 3262 participants and the NU-AGE cohort used consisted of 249 trial participants at baseline. There was also a significant age range difference between the TWIN UK and NU-AGE cohorts. Another potential explanation for the discrepancies in the results could be the difference in methods utilised for dietary assessment; as mentioned previously the TWIN UK data used FFQs as a dietary assessment method whereas NU-AGE dietary data were collected via 7 day food diaries.

This was the first study to assess the impact of a whole-diet intervention for one year on both arterial stiffness and endothelial function in older adults in the UK. There was no significant effect of the NU-AGE intervention on any of the clinical outcomes measured.

Gender based subgroup analysis showed that there was a beneficial impact of the NU-AGE diet on CAVI in older females. We also investigated the impact of the NU-AGE intervention on a number of biochemical markers, including markers of vascular function, inflammation and fatty acid status. There were no significant differences between the control and intervention groups for any of these measures. Furthermore, there was no beneficial effect of the NU-AGE diet on a calculated vascular risk score. The PREDIMED study looked at the effects of a low fat diet, a Mediterranean diet supplemented with extra virgin olive oil, and a Mediterranean diet supplemented with nuts on a wide range of outcomes in 7,447 subjects, aged between 55 and 80 years. All 3 diet groups exerted beneficial effects on blood pressure [194], while the Mediterranean diet supplemented with extra-virgin olive oil or nuts also had reduced incidence of major cardiovascular events, reduced PAD incidence, improved plasma NO and improved fatty acid status [178, 195-197]. Therefore in a public health context, it is important that our results are not interpreted to suggest that there is no effect of a whole diet intervention on cardiovascular health in older adults and the limitations of this study (discussed in section 7.2) should be carefully considered.

A secondary aim of this PhD was to examine the impact of polymorphisms in the *FADS* gene region on fatty acid status in older adults, as well as to determine if a dietary intervention could modulate the effect of *FADS* genotype on fatty acid status. The NU-AGE cohort was genotyped for 10 selected tag SNPs and a number of haplotypes were statistically reconstructed. The results showed that for the majority of the selected *FADS* SNPs, carriers of the minor alleles tended to have higher levels of essential fatty acid LA, as well as lower levels of the longer chained fatty acids AA, EPA and DHA. For example, there were significantly lower levels of EPA (39.4%), DHA (19.3%) and desaturase activity (14.3%) in carriers of the minor allele of the rs174605 SNP compared with those homozygous for the major allele. Haplotype associations were also in line with these findings. For example, the 10 marker haplo.2 was made up of 6 minor alleles and was associated with a clinically significant 20% lower plasma EPA and DHA combined (from 4.2% to 3.4%). Previous research has shown that an increase in plasma EPA+DHA from <3.5% to >4.2% is associated with a 72% reduction in risk of sudden death from cardiac causes and that combined EPA and DHA plasma levels >3.6% were significantly associated with reduced all-cause mortality over 5.9 years [442]. Research has also shown that a modest increase in oily fish, for example a total 180g of salmon per week can increase combined plasma EPA and DHA by 38% [443]. Therefore, we examined whether the NU-AGE intervention (which included advice to

consume 250g of fish per week) could influence the relationship between the FADS genotype and fatty acid status. Results suggested that the NU-AGE diet could potentially overcome the impact of the *FADS* genotype on EPA and DHA status. For example, the highly significant (p= 0.002) difference of 41.7% in EPA levels between the rs1535 homozygous major and minor allele participants decreased to a non-significant difference (p= 0.199) of 30.2% following intervention. These findings significantly contribute to current scientific knowledge and are relevant in terms of public health as the results may contribute to the future stratification of dietary recommendations based on genotype, carriers of the FADS minor allele potentially requiring increased intakes of EPA and DHA relative to the wildtype genotype groups. Finally, we examined the impact of the *FADS* genotype on arterial stiffness, as measured by CAVI. However, no significant effects were observed.

7.2 Strengths and limitations

In relation to the work detailed in chapter 2, the major advantages of our investigations on the potential effects of various dietary compounds on fatty acid status include the use of human plasma samples, as well as the examination of a broad range of dietary compounds including ACNs, flavan-3-ols, and different flavonoid-rich products including blueberry extract, alcohol and champagne. Furthermore, we investigated the effects of these compounds on fatty acid status in plasma, as well as in a range of tissues in rodents including the liver, cortex, muscle and heart. However, there were also a number of limitations involved. For example, in the case of the animal study in which the rodents were fed ACN, flavan-3-ols or blueberries, we did not have access to the liver tissue, which is the major site of EPA and DHA synthesis and therefore would be most sensitive to any impact of polyphenols/flavonoids/ACNs on EPA and DHA status. In addition, the diets consumed in the previous rodent studies had relatively low levels of αLNA. To overcome these limitations, our group designed an animal study to test the hypothesis that dietary compounds, specifically ACNs, could increase the bioconversion of αLNA to EPA and DHA. However, the results from this study also suggested that ACNs do not impact on EPA and DHA levels in either blood or tissue [271].

There were also a number of strengths and limitations in relation to the design and validation of the NU-AGE diet score (detailed in chapter 4). For example, a strength of the NU-AGE diet score compared with the EDI, another diet score targeted at older adults, is that it consists of more dietary components previously shown to be beneficial for older adults including nuts, eggs, fluid and cheese. The NU-AGE diet score also has a wholegrain category rather

than just a bread/cereal group which is advantageous considering the importance of fibre and wholegrains in the diet of older adults. In addition, the NU-AGE diet score has been designed for use with both FFQs and food diaries, whereas the majority of previous diet scores have usually been used in conjunction of FFQs. The NU-AGE diet score has a wide ranged scoring system (a range of 0-10) which could result in the NU-AGE diet score being more sensitive than scores involving more narrowly ranged scores. Another advantage of the NU-AGE diet score is that the NU-AGE diet has been used as an intervention diet in the NU-AGE study, therefore when data is available for the full NU-AGE cohort the NU-AGE diet score can be tested on both baseline and follow-up dietary data to determine both adherence to the diet and the effect of changes in the diet and associated score on various health outcomes. As the majority of diet scores have only been used cross-sectionally, this would allow us to determine the sensitivity of the score in detecting dietary changes. This was originally an aim of this PhD, however follow-up dietary data was not ready at the time of thesis submission. There were also several limitations associated with the NU-AGE diet score; the NU-AGE diet score was not associated with any health outcomes when utilised with food diaries and therefore needs to be tested on a larger sample size to determine its functionality with the use of food diaries. In relation to limitations involved in the use of the FFQ, there was no data for oil consumption in the TWIN cohort. Furthermore, a considerable amount of work still needs to be carried out before this diet score can be considered fully validated and submitted for publication and widespread use. The NU-AGE diet score needs to be tested on the entire NU-AGE cohort, both at baseline and follow-up.

In terms of the NU-AGE intervention, this was the first study to investigate the effects of a whole-diet intervention for one year on both arterial stiffness and endothelial function in older adults in the UK. The strengths of this project have previously been discussed and include the use of validated clinical measures to assess vascular health, a diet designed specifically to target the nutritional needs of the elderly, the provision of certain food products and vitamin D supplements. Of particular note in the utilisation of validated clinical measures is the CAVI, a novel measure which is thought to be a particularly useful indicator of arterial stiffness in elderly cohorts because it is independent of blood pressure and is therefore thought to evaluate arterial stiffness more accurately in those taking anti-hypertensive medications or those with masked hypertension [58, 59]. Furthermore, the long duration of the intervention was an additional strength; one year has previously been shown to be sufficient time to improve dietary intakes and observe changes in health

outcomes and also allowed us to avoid seasonal dependent changes in outcomes [414, 415]. However there were several limitations to this project, which may also explain the lack of beneficial effect of the NU-AGE diet on vascular health. The majority of previous research has involved the examination of at-risk populations, for example those with pre-existing hypertension or the metabolic syndrome. Our inclusion criteria did not require participants to be hypertensive or at higher risk of CVD but were representative of a healthy population of that age group. As such, it may have been more difficult to observe beneficial effects in a population that did not have any signs of vascular dysfunction. For example, the NU-AGE cohort had substantially lower PWV figures at baseline in comparison to the age matched European population and so it could be speculated that the NU-AGE population were "too healthy" to see improvements in arterial stiffness. Similarly, 83.7% of the cohort fell within the normal range for PAD risk at baseline and only 6 participants had an average ABI ≤ 0.90, which would therefore make it considerably difficult to observe improvements. However, the use of an apparently healthy population could also be considered a strength of the study as the results can be generalised to older adults of the general population. Another limitation was that the sample size of 150 participants was calculated based on PWV and EndoPAT research and therefore a larger sample size would have been required to observe an effect of the NU-AGE diet on a number of other outcome measures, such as blood pressure and CRP. For example, the NU-AGE power analyses resulted in a sample size requiring 1000 participants in order to detect a difference of 0.6 (± 0.4 SD) unit change in CRP [182]. This work will be carried out as part of the NU-AGE project and results from five study centres will be compiled and analysed. Furthermore, it has recently been speculated that the utilisation of the EndoPAT for the measurement of RHI may be suitable for qualitatively establishing the presence or absence of endothelial dysfunction, but may not be optimal for use in quantitative measurement of endothelial function or in studies examining subtle changes in response to intervention [382], [383]. In addition, neither detailed fatty acid intake data nor the follow-up dietary data had been analysed at the time of thesis submission and so exact changes in nutrient intakes cannot be determined which could have helped to examine compliance in a more detailed manner. These data will be presented in future relevant publications. Until such data are available, the reporting of a null effect of the NU-AGE intervention on vascular function should be interpreted with caution.

In chapter 6, the impact of polymorphisms in the *FADS* gene region on fatty acid status and arterial stiffness in older adults was discussed, as well as the impact of the NU-AGE diet on

the relationship between the *FADS* genotype and fatty acid status. The major strengths of this study include the use of tag SNPs, reconstruction of haplotypes, as well as the intervention design of the study. The utilisation of tag SNPs and haplotyping made it possible to identify genetic variation and phenotype association without the requirement to genotype all SNPs in that chromosomal region [425]. The novelty of examining the impact of the *FADS* genotype on arterial stiffness, as well as the investigations of the effects of a whole-diet intervention on the relationship between the *FADS* genotype and fatty acid status in older adults was an additional strength, as to our knowledge these hypotheses have not previously been tested. However, there were also a number of limitations. For example, the relatively low sample size resulted in low frequencies of a number of minor alleles and several haplotypes. In addition, the sample size was inadequate to detect the impact on AA and other fatty acids when examining the intervention group only.

7.3 Future work

Our results indicated that intakes of dietary compounds, such has ACNs and flavan-3-ols, are unlikely to determine fatty acid status in mammals. However, one consideration is that the volunteers for the human trial were postmenopausal women, who are known to have lower fatty acid bioconversion rates [270]. Therefore, future research may be warranted on the impact of polyphenol consumption on plasma fatty acid status in individuals with a more up-regulated biosynthetic capacity, namely premenopausal women. Future research could also involve the investigation of alternative dietary compounds, such as resveratrol (an anti-oxidant and anti-inflammatory compound also found in fruit and wine), which may increase LC-PUFA biosynthesis or bioavailability. This would be globally beneficial considering the inadequate oily fish intakes and ever depleting fish stocks worldwide; the current production of 1 million tons of fish oils per year is insufficient to meet even the minimum recommended intakes of > 500mg per day [90].

Future work also needs to be carried out to examine associations between the NU-AGE diet score and CRP, as well as other clinical end points or established risk biomarkers, in the whole NU-AGE cohort (n=1,250, using 7 day food diaries) at baseline and following intervention before the NU-AGE diet score can be used as a widespread tool for diet quality assessment in older adults. The NU-AGE diet score could also be used as a potential method of assessing compliance to the NU-AGE diet within the study. Associations between the NU-AGE diet score and a broad inflammatory score could be examined; for example Cassidy et al. used an

inflammatory score that integrated 12 individual inflammatory biomarkers and observed associations between flavonoid intakes and the inflammatory score [445]. However, it is likely that further modifications would be required in order to utilise the NU-AGE diet score in the whole NU-AGE cohort due to the introduction of new foods that are commonly consumed in the other participating countries. Following such modifications, the NU-AGE diet score could potentially be used as a tool to detect CVD risk in older adults for health care professionals. More generally, future work in the area of diet scores should focus on adapting diet scores for use in individuals, potentially through the use of a publicly available online tool.

Suggestions for future work related to examining the impact of a dietary intervention on vascular function in older adults include the use of an "at risk" population rather than a healthy cohort. The main benefit of using a healthy cohort is that results can be generalised to the older adult population. However this also makes the observation of a beneficial effect of the diet less likely. Furthermore, the NU-AGE diet was designed with the primary outcome of improving inflammation rather than endothelial function or arterial stiffness [182]. Further research on the impact of a whole-diet specifically designed to improve vascular function in healthy older adults is warranted. This diet could emphasise fruits particularly high in ACNs and flavones which have previously been shown to improve arterial stiffness, as well as further restrictions on salt consumption [65]. In addition, a systematic review on the impact of nutrient interventions on PWV states that the lowest daily dose of n-3 fatty acids that resulted in an effect on arterial stiffness was 540 mg EPA combined with 360 mg DHA [65]. This dose is higher than what would be consumed with intakes of two portions of oily fish per week and therefore perhaps n-3 fatty acid supplementation would have needed to be part of the NU-AGE recommendations in order to see an impact on arterial stiffness. Future work involving the analysis of follow-up dietary data may also help interpret results and provide more sensitive measures of compliance. In terms of clinical measures of vascular function, the use of gold standard methodologies to detect changes in endothelial dysfunction is recommended for future studies, specifically the use of FMD rather than EndoPAT. We observed a significant effect of the NU-AGE intervention on arterial stiffness, assessed by CAVI, in females only. However, further research is required in the field of gender specific plasticity of the aging vascular system to determine potential mechanisms that could account for a gender specific response to dietary change, such as differential absorption of nutrients.

Our data suggests that the NU-AGE diet may have the potential to overcome the impact of the *FADS* genotype. However, further analysis should be carried out on the entire NU-AGE cohort to examine the ability of the diet to modulate the relationship between fatty acid genotypes and related outcomes in a larger cohort. Future work involving dietary interventions involved at targeting vascular function in older adults could also investigate the impact of the *FADS* genotype on endothelial dysfunction and arterial stiffness as such analysis has not previously been carried out and our sample size may have been insufficient to detect an effect. Overall, future work in this area should include larger sample sizes. Research is also required to determine potential mechanisms of action for the *FADS* genotype and fatty acid interactions, with a focus on firstly determining the functional SNP(s). Sequencing of the whole FADS gene region would be required to determine functional SNPs and such research may be warranted. In addition, studies examining the effects of the *FADS* genotype on actual desaturase activity, rather than using various calculations for desaturase activity, are also warranted.

The potential contribution of the NU-AGE study to future work for the food industry and EU legislation is extensive; it is planned that the final results of the NU-AGE dietary intervention will contribute to the development of innovative food prototypes/products with enhanced composition that are tailored specifically towards the elderly. The results from NU-AGE may also support EU strategies on nutritional recommendations, and therefore contribute to the implementation of legislation related to nutrition and health claims for elderly in Europe. As previously discussed, the focus of research on the effects of single nutrients has led to many important discoveries, but it is important that more emphasis is placed on the effects of the whole-diet on health outcomes [177, 179, 180, 300]. However, standard dietary recommendations tailored specifically for older European adults do not exist [446]. If beneficial health outcomes are observed as a result of the NU-AGE intervention, the NU-AGE dietary guidelines could be adopted for such use across Europe.

Furthermore, it is important that validated, cost-effective and non-invasive measures of healthy ageing are identified. Previous research has involved the use of measures, such as mortality rates or specific biomarkers including telomere length, as positive outcomes of ageing [446]. The large amount of data that will be gathered from the entire NU-AGE project could help to identify the interactions between nutrition and biological processes involved in healthy ageing, with a focus on inflammation. This could potentially contribute to the construction of an integrated systems biology based model which could be a more efficient

approach to future research compared with the assessment of separate single tissue and organ responses [182].

7.4 Overall Conclusion

In conclusion, the NU-AGE diet did not slow the progression of vascular dysfunction in this UK sub-group of older adults. However, the NU-AGE diet did appear to be successful in slowing the progression of arterial stiffness in females. Further research is required to determine the mechanism of this effect. The impact of various dietary components and the *FADS* genotype on fatty acid status was also examined. Although we did not observe a significant effect of any dietary compounds on n-3 LC-PUFAs, it was clear that the *FADS* genotype may be a significant determinant of plasma EPA and DHA status in older adults. Furthermore, the NU-AGE diet was shown to have the potential to overcome this effect by influencing the relationship between the *FADS* genotype and plasma EPA and DHA status. This work emphasises the importance of considering the impact of the *FADS* genotype when examining the impact of intervention on EPA and DHA status. Future work involving the investigation of the effects of the NU-AGE diet on the cohort of 1,250 older adults on health outcomes, such as blood pressure and CRP, will help to further determine the health benefits of the NU-AGE diet. If successful, the NU-AGE study could help inform the refinement of dietary recommendations which could contribute to improved health and quality of life for older adults in Europe.

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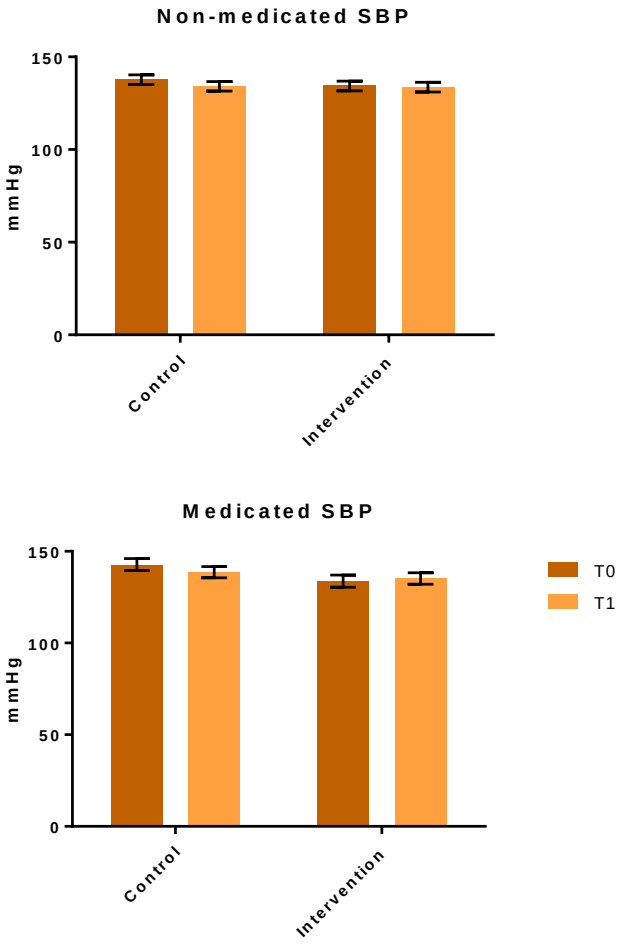
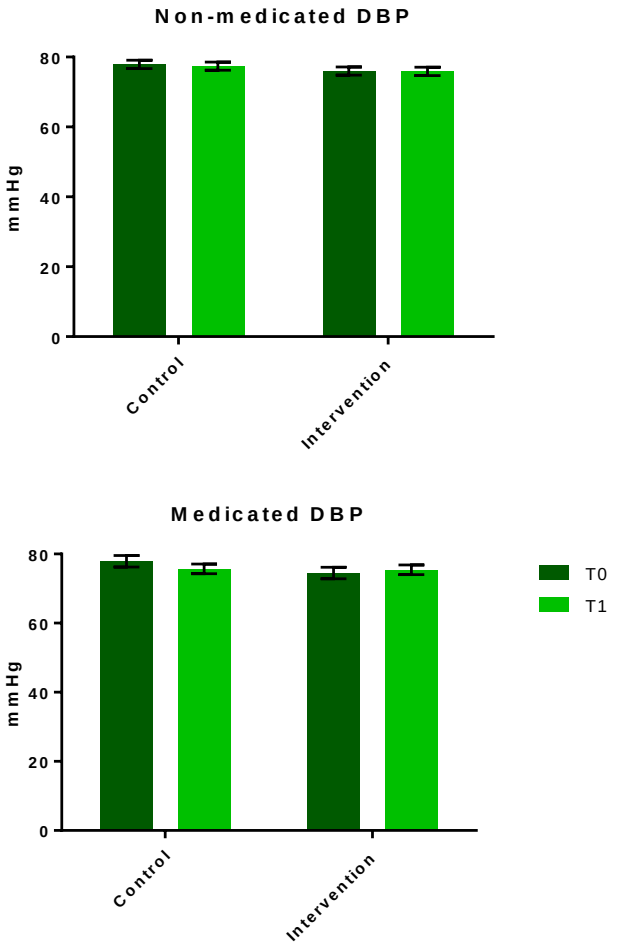
Appendix 1

The differential response of the NU-AGE intervention on systolic blood pressure in medicated and non-medicated individuals



Non-medicated control group: n=43, non-medicated intervention group: n=42, medicated control group: n=30, medicated intervention group: n=29. Data presented as mean ± SEM. Repeated measures ANOVA was conducted to examine the impact of treatment on systolic blood pressure separately in medicated and non-medicated participants.

The differential response of the NU-AGE intervention on diastolic blood pressure in medicated and non-medicated individuals



Non-medicated control group: n=43, non-medicated intervention group: n=42, medicated control group: n=30, medicated intervention group: n=29. Data presented as mean ± SEM. Repeated measures ANOVA was conducted to examine the impact of treatment on systolic blood pressure separately in medicated and non-medicated participants.

Annex 1

NU-AGE pre-study health screening questionnaires, part 1

New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe

(FP7-KBBE-2010-4)

ADMISSION QUESTIONNAIRE PART I

(to be filled in by the interviewer)

To be filled in by NU-AGE staff

Subject Code: | S | C |

Interviewer: _____

Date of interview (dd/mm/yy): _____

Intervention Time: Code

1. Gender: Male ☐ 1 Female ☐ 2

2. What is your date of birth? Day: _____ Month: _____ Year: _____
Age_____

3. What type of housing do you live in?

a. House (incl. town/farm house), apartment, bungalow Yes ☐ 1 No ☐ 2

b. Nursing home or residential care Yes ☐ 1 No ☐ 2

1. **Do you live at home independently (without the help of a caregiver)?**
Yes ☐ 1 No ☐ 2

5. During the last five years, have you been treated or followed by your doctor for a cancer?

Yes ☐ 1 No ☐ 2

If "Yes" please give brief details
_____
_____

a. If "Yes" have you ever been under treatment**?** Yes ☐ 1 No ☐ 2

b. If "Yes" are you still under treatment**?** Yes ☐ 1 No ☐ 2

c. If your treatment has stopped, how long ago did it end? (specify number of months)

6. Have you ever had a heart attack (myocardial infarction - AMI)? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details
_____

a. If "Yes" can you walk about a half to one kilometre or climb 2 flights of stairs without symptoms (chest pain, dyspnea, abnormal sweating)? Yes ☐ 1 No ☐ 2

b. If "Yes" do you have swollen ankles? Yes ☐ 1 No ☐ 2

7. Have you ever had a heart insufficiency? Yes ☐ 1 No ☐

a. If "No" can you walk about a half to one kilometre or climb 2 flights of stairs without symptoms (chest pain, dyspnea, abnormal sweating)? Yes ☐ 1 No ☐ 2

b. If "No" do you have swollen ankles? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

8. Do you have a chronic respiratory disease (chronic bronchitis, emphysema, asthma)?

Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

a. If "Yes" do you need oxygen therapy? Yes ☐ 1 No ☐ 2

b. If "Yes" do you have difficulty in breathing, especially when walking about a half to one kilometre or climbing 2 flights of stairs? Yes ☐ 1 No ☐ 2

9. Do you have a chronic liver disease or liver cirrhosis? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

10. Have you ever had a Hepatitis B or C viral infection or other chronic infectious disease such as HIV infection? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

11. Do you usually drink alcoholic beverages (beer, wine, vodka, cognac, whisky etc.)?

Yes ☐ 1 No ☐ 2

a. If "Yes", how many glasses of alcohol do you drink at the weekend?

Number of glasses: _____

b. If "Yes" how many glasses of alcohol do you normally drink during the week (Mon-Fri)?

Number of glasses: _____

c. Total number of glasses per week: _____

12. Do you have any chronic kidney disease? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

a. If "Yes", are you on haemodialysis or peritoneal dialysis? Yes ☐ 1 No ☐ 2

13. Have you had a stroke or a TIA (Transient Ischemic Attack) in the last year?

Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

14. Do you have diabetes? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

a. If "Yes", do you use insulin? Yes ☐ 1 No ☐ 2

15. Do you have a food allergy/intolerance? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

16. Do you have any disease or condition that requires a special diet?

Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

17. Do you use cortisone-based medicine regularly (chronic use) e.g. immunosuppressive drugs? Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

18. Have you been prescribed and taken a course of antibiotics in the last 2 months?

Yes ☐ 1 No ☐ 2

Date course completed or due to be completed (if applicable) _____

19. Do you use a medicine for high cholesterol? Yes ☐ 1 No ☐ 2

a. If "Yes" have you been taking it for more than 3 months? Yes ☐ 1 No ☐ 2

20. Do you take thyroid hormones? Yes ☐ 1 No ☐ 2

a. If "Yes" have you been taking them for more than 3 months? Yes ☐ 1 No ☐ 2

21. Do you take aspirin as cardiovascular prevention (not as a painkiller or as an anti-inflammatory drug)? Yes ☐ No ☐

a. If "Yes" have you been taking it for more than 3 months? Yes ☐ 1 No ☐ 2

22. Have you changed your habitual prescribed medication use (e.g statins for high cholesterol, thyroxine for hypothyroidism, or aspirin for cardiovascular prevention) in the last 3 months?

Yes ☐ 1 No ☐ 2

If "Yes" please give brief details

_____
_____

23. Do you use any prescribed medicine on a regular basis? Yes ☐ 1 No ☐ 2

If "Yes" fill in the following scheme on use of prescription medicine.

Name of Medicine	**For which disease?**	**N° of intake**	**ATC codes** (to be filled in by interviewers)	**Start of the treatment (date)**

24. Are you currently taking part in any other nutritional or medical research trials?

Yes ☐ 1 No ☐ 2 If "Yes" please give brief details _____

_____

25. Is the volunteer eligible to proceed in the study? Yes ☐ 1 No ☐ 2

Annex 2

NU-AGE pre-study health screening questionnaires, part 1

New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe

(FP7-KBBE-2010-4)

ADMISSION QUESTIONNAIRE PART II

(to be filled in by the interviewer)

To be filled in by NU-AGE staff

Subject Code | S | C |

Interviewer: _____

Date of interview (dd/mm/yy): _____

Intervention Time: Code

1. ANTHROPOMETRIC MEASUREMENTS

a. Measured total height (10^{-1} cm): _____ cm

b. Measured knee height (10^{-1} cm): _____ cm

c. Measured weight (without shoes and heavy clothes) (10^{-1} kg): _____ kg

(Body weight will be measured to the nearest 0.1 kg)

d. Has the subject been measured in his/her undergarment?

Yes ☐ 1

No ☐ 2

Irrelevant ☐ 3

e. BMI (weight /height^2 **):**_____ Kg/m^2

BMI < 18.5 kg/m2 in elderly is a symptom of severe undernutrition which requires specific investigations and nutritional management and should be considered an exclusion criterion.

f. Weight 6 months ago _____ Kg **g.** Weight change (6 months)**:** _____ Kg

h. Weight a year ago: _____ Kg **i.** Weight change (1 year) **:** _____ Kg

An unintentional body weight loss more than 10% in the last 6 month is a symptom of undernutrition and should be considered as an exclusion criterion

2. FRAILTY ASSESSMENT (Fried's Criteria)

2.1 SHRINKING

In the past 12 months have you lost 4.5 Kg (10 lb) or more <u>unintentionally</u> (i.e. not due to dieting or exercise)? Yes ☐ 1 No ☐ 2

2.2 WEAKNESS

Hand grip test (grip strength <u>dominant hand</u>):

This exercise tests the strength in your forearms and your hands. I will now ask you to stand up and squeeze the handle of this instrument as hard as possible – three times with each hand. One practice trial before the three measurements is allowed.

a. What is the subject's dominant arm? **1**. Left arm ☐ **2.** Right arm ☐

Record the distance between grip base and base of frame:

Right hand: _____ cm

Left hand: _____ cm

b. Right hand: _____ kg _____ kg _____ kg

Left hand: _____ kg _____ kg _____ kg

c. Mean of 3 measurements (dominant hand): _____

d. Did the subject complete the test? Yes ☐ 1 No ☐ 2

BMI/Male (kg/m^2)	Cut-Off (kg)*	BMI/Female (kg/m^2)	Cut-Off (kg)*
≤ 24	≤ 29	≤ 23	≤ 17
24.1–26	≤ 30	23.1–26	≤ 17.3
26.1–28	≤ 30	26.1–29	≤ 18
> 28	≤ 32	> 29	≤ 21

Grip Strength, stratified by gender and body mass index (BMI) quartiles

2.3 POOR ENDURANCE (SELF-REPORTED EXHAUSTION):

Evaluation of 2 items of CED-Depression scale. Now I'll read you two statements:

a) I felt that everything I did was an effort.

How often in the last week did you feel this way? _____

b) I could not get going.

How many times in the last week did you feel this way? _____

Scores: **0=** rarely or none of the time (< 1 day)

1= some or a little of the time (1-2 days)

2= a moderate amount of the time (3-4 day)

3= most of the time

2.4 SLOWNESS (GAIT SPEED TEST)

Starting Position: Patient is set-up in a standing position at the beginning of the course.

Volunteers are asked to walk across the course at their "usual, comfortable speed." Time is started when the subject's foot crosses the black tape line indicating the beginning of the course (15 feet (4.57 m.) distance). One practice trial is performed prior to testing to ensure the patient understands the task. The test should be performed twice.

Adaptation: Use of the volunteer's habitual assistive device (cane) is permitted.

Scoring: The fastest of both trials should be considered for scoring using the cut-offs below.

Gait speed (time in seconds over 15 feet (4.57 m.) distance):

Trial 1_____seconds

Trial 2_____seconds

2.4a Fastest _____seconds

Height/Men (cm)	Cut-Off (s)*	Height /Women (cm)	Cut-Off (s)*
≤ 173 cm	≥ 7 seconds	≤ 159 cm	≥ 7 seconds
> 173 cm	≥ 6 seconds	> 159 cm	≥ 6 seconds

Walk Time, stratified by gender and height (gender-specific cutoff a medium height).

2.5 LOW ACTIVITY

Please indicate your average level of physical activity for the last year, from the following categories:

1. ☐ **Virtually no activity** (or nearly bedridden).
2. ☐ **Sitting for most of the time** (occasionally a short walk or other non-demanding activity).
3. ☐ **Light intensity exercise** (walking, dancing, fishing or hunting, shopping without a car) at least 2-4 hours per week.
4. ☐ **Moderate intensity exercise** (running, walking uphill, swimming, gymnastics, digging in the garden/yard, riding a bike uphill, etc.) for 1-2 hours/week, or light intensity (see point 3) for more than 4 hours/week.
5. ☐ **Moderate intensity exercise** for more than 3 hours per week
6. ☐ **Intense exercise regularly** (several times a week).

Light intensity exercise: exercise that is not accompanied by sweating and can be carried out whilst talking with another person.

Moderate intensity exercise: exercise that is accompanied by sweating and does not allow a conversation at the same time.

Intense exercise: maximum tolerable exercise.

PRESENCE OF FRAILTY
Positive for frailty phenotype: ≥ 3 criteria present

2.6. Is the volunteer eligible to proceed in the study? Yes ☐ 1 No ☐ 2

Annex 3

NU-AGE 7 day food diary (condensed to one day)

New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe

NU-AGE 7 DAY FOOD DIARY

To be filled in by NU-AGE staff

Subject Code ☐☐☐☐☐☐ S ☐ C ☐

Diary start date (dd/mm/yy): _____

Diary end date (dd/mm/yy): _____

The home visit is scheduled for :_____

Intervention time: Code ☐

As part of your participation in our research, we would like to know more about your dietary habits. For this, we ask you to keep a food diary for seven consecutive days.

INSTRUCTIONS

Carry the diary with you and write down everything you eat or drink immediately after doing so to avoid forgetting any items. Do not forget to write down things that you eat or drink in between meals (e.g. apples, nuts, cups of tea etc.) and things you have during the night (e.g. a few sips of water). Meals outside the home should also be recorded.

The diary should be recorded in the following way:

- Please write down the date and day of the week in the space provided at the top of the page at the start of each day that you record your diary.
- Start each day with the page titled 'before breakfast'.
  There are 8 sections available for each day; before breakfast, breakfast, during the morning, lunch, during the afternoon, evening meal, evening snack, during the night.
- If nothing is eaten or drunk during one of these sections, draw a line through that section.
- Fill in the diary using the headings provided; time, place, description of foods and drinks and portion size.

Time: In this box write the time you ate or drank the item/meal.

Place: Write down the place where you ate the item or meal (e.g. home, restaurant, cafeteria at work, friend's house etc.)

Description of foods and drinks: Write down a clear description of the food or beverage that you have consumed. It is important to use exact names and descriptions and whenever the product has a **brand name**, please, write that down too (e.g. Tropicana smooth orange juice, Tesco light choices cottage cheese, Hovis wholemeal farmers loaf). Also write down any additions you add to the food or drink such as sugar or salt.

When describing a dish, write down the method of preparation (e.g. boiled or fried or grilled). If fat was used in the preparation, write the type of fat used (e.g. meatballs fried in vegetable oil). Also write down whether the food is home-made or bought ready-made.

If you make a meal that involves a recipe please make a precise note of the recipe including all the ingredients, their quantities and the main cooking methods involved. It is important to give full details of ingredients (e.g. chicken breast, no skin). Please also note the number of portions that the recipe served and indicate how much you ate from this. If someone else made the meal, ask them for the details. There is additional space for recipe notes at the end of each day.

Meals eaten outside the home should also be recorded. When possible ask the cook or a member of the restaurant/canteen staff for information about the dish including the main ingredients and approximate quantities.

Amounts / portion sizes: Write down the portion sizes of the foods and beverages as you list them. Please weigh your food or use household measures such as coffee mugs, tablespoons, millilitres or grams to help you with this.

Use the following to help you estimate portion sizes:

Beverages: state the volume if known (e.g. 300ml) or describe using a description of the size of the glass, cup, mug etc. (e.g. large mug). Milk or cream added to coffee or tea should be measured in teaspoons or tablespoons. If you had a drink from a bottle, carton or can make a note of the size stated on the packaging, and how much you drank (e.g. "250ml bottle. Drank all").

Bread: write the number of slices of bread eaten. Describe the loaf as small, medium or large, or give the total loaf weight. Describe the slice as thin, medium, thick or extra thick (most pre-sliced loaves state the loaf size and the slice thickness on the packaging). (e.g. "2 medium slices, 800g loaf").

Breakfast cereal: describe the portion using small, medium or large bowl sizes or use tablespoons. Describe the amount of milk had on cereal using pints or millilitres or say if it was a 'large', 'medium' or 'small' amount. (e.g. 4 tablespoons of cornflakes with ¼ pint skimmed milk).

Cheese: for hard cheeses write down the number and thickness of slices. Use tablespoons to measure grated cheese. For soft cheeses such as Philadelphia measure in teaspoons or tablespoons.

Confectionary (sweets and chocolate): describe using the weight on the packet, bar weight, number of pieces or individual sweets.

Fruit: record the number of whole fruits, segments or slices. For canned fruit give the can weight and the proportion of which you ate.

Ice cream, cream and dairy desserts: use scoops or tablespoons. Where whole items are eaten give the pot or packet weight.

Meat and fish dishes: record the number or weight of meat or fish portions (e.g. "one chicken breast", "3 rashers of unsmoked back bacon" or "1/4 of 515g pack, lean beef mince, raw weight"). Remember to state if you are recording the weight as the cooked or raw weight. Also note if the meat or fish includes skin or fat and if this was eaten.

For roast meats and cold cuts of meat state the number and thickness of slices (e.g. 2 slices of Tesco wafer thin cooked ham).

Oils, butter and margarine: use teaspoons or tablespoons. When spreading on bread or toast state if the layer was thin, medium or thick.

Pasta, spaghetti: Describe the weight using a proportion of the packet weight (e.g. 1/10 of 1kg packet of dried wholemeal fusilli) or measure in tablespoons. Remember to state if you are giving the dried weight or cooked weight.

Puddings and desserts: use tablespoons, or slices with a description of small, medium or large. For commercial items describe as a proportion of the packet weight (e.g. Sainsbury's strawberry cheesecake 530g. Ate 1/5).

Rice: Describe the weight using a proportion of the packet weight or use tablespoons. Remember to state if you are measuring cooked or raw rice.

Sauces, gravy and dressings: use teaspoons or tablespoons. Note that even the amount of meat sauces such as Bolognaise sauce should be estimated this way (e.g. "one teaspoon of sweet chilli sauce").

Soups: use bowls to describe the size of the portion as a small, medium or large bowl. Or if you are using canned or carton soups note the size marked on the packaging and state how much you ate (e.g. "440g can, ate half").

Sugar: use teaspoons or tablespoons or if the sugar is cubed state the number of cubes. Remember to mention sugar sprinkled on top of cereal and sugar in tea etc.

Vegetables and salad items: use whole vegetables or salad items, slices or tablespoons as measures (e.g. "4 cherry tomatoes, 5 thin slices of cucumber and 2 tablespoons lettuce").

Other foods: use the information given on the packaging whenever possible. The weight should be included on the packet information. If you don't eat the whole packet note the total weight of the packet followed by the amount you think you ate (e.g. if you had a packet of walnuts, "100g packet, ate ¼ of the packet").

If in doubt about how to describe a portion, write as much detail as possible. The portion can then be further discussed with the research assistant once the diary is complete.

Leftovers: We want to know the amount that was actually eaten, this means leftovers need to be taken into account. This can be done in two ways:

1. If any leftovers remained on your plate from the originally stated portion in your food diary, please make a note of this (e.g. "1/4 of lasagne recipe, *3 small boiled potatoes*. *Only ate 1 of the boiled potatoes").*

2. Alternatively, you can just record the actual amount eaten (e.g. "1/4 of lasagne recipe, *1 small boiled potato*").

Labels/wrappers: Labels are a very useful source of information for us. When possible please save any labels or packaging that shows the product information for an item you have consumed. This is especially useful for foods or brands you record which are perhaps lesser known or uncommon.

Comments: At the end of each day there is space to write any comments you might feel are relevant. For example this may be to inform us if the day was not a typical day or if there was any reason why you might have eaten more or less than usual.

The dietary records will be discussed with the research assistant during your visit to the UEA to be sure that you haven't forgotten anything and to verify whether you have given enough detail.

Example day

EXAMPLE			
Date: 07/05/2012		Day of the week: Monday	
BEFORE BREAKFAST			
Time	Place	Description of foods and drinks	Portion size
07:30	Home	Cup of Nescafe instant coffee semi skimmed milk	1 large mug 1 tablespoon
BREAKFAST			
Time	Place	Description of foods and drinks	Portion size
08.00	Home	Kellogg's fruit n fibre with Semi skimmed milk Tesco orange juice from concentrate	30g 1/5 pint 150ml
DURING THE MORNING			
Time	Place	Description of foods and drinks	Portion size
09.30 11.00	Friend's House Home	Cup of Tetley decaffeinated tea with semi-skimmed milk Water	Medium mug tablespoon Tall glass, 250ml
LUNCH			
Time	Place	Description of foods and drinks	Portion size

13.00	Home	Chicken salad sandwich: Hovis wholemeal medium sliced,800g loaf. Flora light low fat vegetable spread Tesco sliced roast chicken (240g pack) Tesco salad tomatoes Tesco baby leaf and rocket salad (90g pack) Banana Robinson orange squash, no added sugar diluted with tap water	 2 slices 1 teaspoon 1 slice 1 tomato 1/6 of packet 1 medium 50ml 200ml
DURING THE AFTERNOON			
Time	Place	Description of foods and drinks	Portion size
15.00	Starbucks coffee shop	Green tea Blueberry muffin	Regular Ate half
EVENING MEAL			
Time	Place	Description of foods and drinks	Portion size

18.30	Home	Wholemeal pasta Homemade bolognaise sauce (see recipe) Grated cheddar cheese Tap water Strawberries Tesco low fat natural yogurt (500g tub)	150g (cooked weight) 1/8 of recipe 1 tablespoon Large glass, 300ml 6 medium 2 heaped tablespoons
EVENING SNACK			
Time	Place	Description of foods and drinks	Portion size
20.30	Home	PG tea with Semi-skimmed milk	Medium mug 1 tablespoon
DURING THE NIGHT			
Time	Place	Description of foods and drinks	Portion size

Recipe notes

Please write the recipe or list the ingredients of any dishes that may require more information to be given. This might include home-made dishes, take-away meals etc that you have mentioned but not described previously. Where applicable please list amounts of ingredients and brand names. Please indicate the amount or proportion actually consumed by you.

Name of dish: Bolognaise sauce

Number of portions the recipe serves (if applicable): 8 portions

Ingredient	Amount	Ingredient	Amount
Tesco lean beef mince	500g	Napoli chopped tomatoes	400g can
Garlic	2 cloves	Tesco tomato puree	1 tablespoon
Red onion	1 medium	Tesco mild olive oil	1 tablespoon
Red pepper	1 medium	Dried mixed herbs	2 teaspoons
Yellow pepper	1 medium		
Courgette	1 medium		

Brief description of the cooking method:
Fry onion & garlic in oil, add mince and fry until brown.
Add peppers, courgette, tomatoes, puree & herbs.
Simmer for 30 minutes

Any additional comments:

I ate 1 portion from the above recipe.

END OF EXAMPLE

Start of diary

If you have any questions or problems, contact the researchers during regular office hours.

Tel: 01603 591568 or email: nuage@uea.ac.uk

DAY 1			
Date: / / Day of the week:			
BEFORE BREAKFAST			
Time	Place	Description of foods and drinks	Portion size
BREAKFAST			
Time	Place	Description of foods and drinks	Portion size
DURING THE MORNING			
Time	Place	Description of foods and drinks	Portion size
LUNCH			
Time	Place	Description of foods and drinks	Portion size

DURING THE AFTERNOON			
Time	Place	Description of foods and drinks	Portion size
EVENING MEAL			
Time	Place	Description of foods and drinks	Portion size

EVENING SNACK			
Time	Place	Description of foods and drinks	Portion size
DURING THE NIGHT			
Time	Place	Description of foods and drinks	Portion size

Recipe notes

Please write the recipe or list the ingredients of any dishes that may require more information to be given. This might include home-made dishes, take-away meals etc that you have mentioned but not described previously. Where applicable please list amounts of ingredients and brand names. Please indicate the amount or proportion actually consumed by yourself.

Name of dish: Number of portions the recipe serves (if applicable):			
Ingredients	Amount	Ingredients	Amount
Brief description of the cooking method			

Any additional comments:

Repeat x7 days

Additional space for notes and comments that may be useful to the researchers:

Thank you for completing your 7 day food diary

Annex 4

British Dietetic Association information sheet for control group

4. Meat, fish, eggs and pulses (beans and lentils) should be eaten in moderate amounts. Choose lean cuts of meat or remove excess fat and remove the skin from chicken. Avoid frying where possible. Try to include two portions of fish each week, one of which should be an oily fish, for example: mackerel, trout, sardines, kippers or fresh tuna.

5. Dairy - aim for a pint of milk a day, or the equivalent. For example the following contain the same amount of calcium as 1/3 pint of milk:

- a small pot of yoghurt
- a small matchbox size piece of cheese
- six and eight cups of tea or coffee, with milk a day

Try to choose reduced fat versions where you can – for example semi-skimmed milk, low fat yoghurt, cottage cheese and half fat Cheddar cheese or Edam.

6. Sugar and fat should be limited so watch your intake of foods high in fat and sugar, choosing low fat or reduced sugar foods where possible. When using a spreading fat choose one that is low in saturated fat. Saturated fat increases the low density (LDL) lipoproteins or 'bad' cholesterol in your blood which can lead to heart disease. Instead choose unsaturated fats which contain high density lipoproteins (HDL) or 'good' cholesterol that is beneficial for your body.

Saturated fat (BAD)	Unsaturated fat (GOOD)
Butter ghee and lard coconut oil and palm oil.	Polyunsaturated fat Sunflower, soya, corn or safflower oil/soft spreads/ margarines, and fish oil. Monounsaturated fat Olive and rapeseed oil.

"Choose low fat or reduced sugar foods where possible."

Summary

Eat a range of foods from the five food groups to make sure you have a balanced diet. Eat the right amount of food for how active you are. Most of all – enjoy your food!

Other Food Fact Sheets on similar topics such as Fruit and Veg – How to Get Five a Day and Cholesterol can be downloaded at www.bda.uk.com/foodfacts

Annex 5

Questionnaire used to assess compliance in the NU-AGE intervention group

Dietary Goals

1	**How many servings of whole grains do you consume per day?** (1 serving = 1 slice bread, 28g (1oz) uncooked rice or pasta, 28g (1oz) breakfast cereal or 120g (4oz) porridge)	
2	**How many servings of whole grain rice or pasta do you consume per week?** (1 serving = 28g (1oz) uncooked rice or pasta or 150g (5oz) cooked rice or pasta)	
3	**How many portions of fruit do you consume per day?** (1 portion = 1 apple, 1 banana, 2 plums, 7 strawberries, 150ml fruit juice or 1 tablespoon of dried fruit)	
4	**How many portions of vegetables do you consume per day?** (1 portion = approximately 80g (3oz))	
5	**How many portions of legumes do you consume per week?** (1 portion = approximately 80g (3oz))	
6	**How much milk or yoghurt do you consume per day?**	
6a.	**What type of milk and yogurt do you usually consume?**	
7	**How many servings of cheese do you consume per day?** (1 serving = 30g (1oz))	
7a.	**What type of cheese do you usually consume?**	
8	**How many servings of white fish or shellfish do you consume per week?** (1 serving = 125g (4oz))	

8a.	**How many serving of oily fish do you usually consume per week?** (1 serving = 125g (4oz))	
9	**How many servings of red meat do you consume per week?** (1 serving = 125g (4oz))	
11	**How many servings of nuts do you consume per week?** (1 serving = 20g (1oz))	
12	**How many eggs do you consume per week?**	
13	**How many tablespoons of olive oil do you consume per day?**	
14	**How many glasses of alcohol do you consume per week?**	
15	**How many glasses of red wine do you consume per week?**	
16	**How many glasses or cups of water, milk, decaffeinated tea or coffee, fruit juice or fruit squash do you drink per day?** (1 glass/cup=200ml)	
17	**How many cups of caffeinated tea or coffee drink per day?** (1 cup=200ml)	
18	**How often per week do you add salt to your food when cooking or at the table?**	
19	**How often per week do you add sugar to drinks or foods?**	
20	**How many times per week do you consume cakes, biscuits or desserts?**	

Annex 6

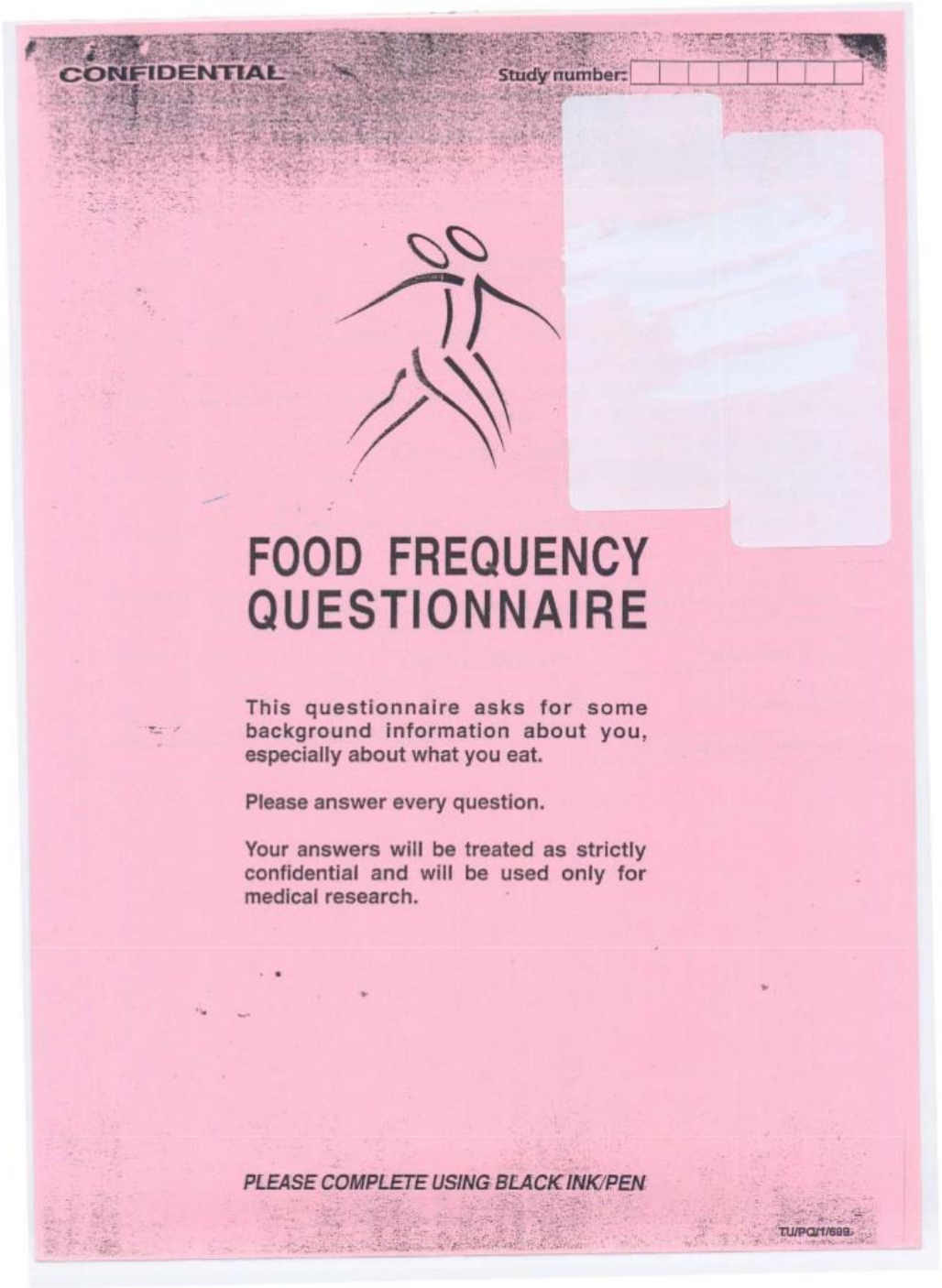
Overview of parameters and measurements (adapted from NU-AGE protocol)

Health Category	Measures	Additional details
Inflammatory status	C-reactive protein (hsCRP), and cytokines, including IL-1Beta, IL-12, INF gamma, IL-6, sIL-6R, IL-1RA, TNFalpha, IL-17, IL-8, IL-10, TGF-beta1	Measured in plasma
Immune health	positivity for HCMV Further characterisation of immune function conducted at IFR	Measured in serum Cytokines, cell proliferation, CD8+ count, using techniques such as flow cytometry
Insulin sensitivity	glucose, insulin, and Hba1C	Measured in serum or whole blood as appropriate, Hba1C measured by accredited laboratory
Liver function status	ALAT, GGT, alkaline phosphatase, creatinine	Measured in serum
Hormonal function status	leptin and adiponectin	Measured in plasma
Bone health	Bone mineral density 25-OH vitamin D, parathyroid hormone	Dual-energy X-ray Absorptiometry (DXA) Measured in serum
Cardiovascular health status	Lipid profile (triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol) Additional lipids, fatty acid status, nitric oxide & vascular health markers Vascular health & function Blood pressure	Measured in plasma Measured in plasma & other blood fractions Multiple measures including pulse wave velocity, EndoPAT, CAVI Using an electronic blood pressure monitor
Cognitive status	CERAD (MMSE, Boston Naming, Word recall and recognition etc), plus additional domain specific tests (Babcock recall, patterns, numbers, trials)	Global measure of cognitive function, Attention and Executive function, depression, information processing speed, executive function and memory, language, processing speed and executive functioning and attention and working memory respectively.
Mental health and quality of life	Depression and health related quality of life	Geriatric Depression Scale (GDS), SF-36v2
Physical functioning	Hand grip strength Physical performance Physical activity	Measured to the nearest 0.1 kg using a Hand Dynamometer Activities of Daily Living (ADL) scale, Instrumental Activities of Daily Living scale (IADL) scale, PASE questionnaire , SPPB ActiGraph activity monitor
Digestive health status	Bowel function, gastrointestinal disturbances, evacuation frequency	Short digestive health questionnaire (in general questionnaire)
Anthropometry	Height Weight Body Mass Index Waist- and hip circumference Body composition	Person standing erect, wearing no shoes, to the nearest 0.1 cm. Person wearing light garments, no shoes and empty pockets, to the nearest 0.5 kg Calculated from height and weight measurements Waist: either at the narrowest circumference of the torso or at the midpoint between the lower ribs and the iliac crest. Hip: measured horizontally at the level of the largest lateral extension of the hips or over the buttocks. Standard protocol and repeat measurements Dual-energy X-ray Absorptiometry (DXA)
Nutritional status	Micronutrient status, including vitamin B12, folate, and 25-OH vitamin D	Measured in serum or other blood fractions as appropriate

	Iron status (Hb, ferritin, transferrin receptor)	Full blood count by accredited laboratory and serum analysis of ferritin and sTfR
Polymorphisms	DNA	From whole blood, using SEQUENOM MassARRAY: Appropriate polymorphisms related to ageing and inflammation or disease pathways including APOE, e2, e3, e4, IL-6-174 G/C, TNFa-318 A/G; TLR4+896 A/G; IL-10-1082A/G; TGFb1G/C915, PPARg2 Pro12AIa, PON1 Q192R, TP53 codon 72 C/G, SIRT3 72bp VNTR, HSP70-1-110 A/C, KLOTHO KL-VF, IGF1R and PIK3CB will be analysed. Additional vascular function related polymorphisms such as FADS1 and FADS2
Biomarkers of ageing	Telomere Length	Real Time PCR on DNA from whole blood
Metabolics	Metabolic profiles NMR and MS metabolomics	Oxido-lipidomics analysis in urine In serum/plasma and urine samples
Immune and inflammation	Genes Proteasome and immunoproteasome composition and activity	mRNA from PBMCs will be analysed by means of transcriptomics analysis. Western blot analysis and fluorimetric assay on protein from PBMC, beta5i, beta2i, beta1i.
Intestinal health	Microbiota	rRNA gene amplicons, phylogenetic microarray profiling (HitChip), pyrosequencing, illumine sequencing in faeces. Cy3, Cy5
Dietary information	Habitual dietary intake Compliance to the diet Food choices	Will be estimated using 7 day diaries Will be estimated by means of 3 day food diaries (2 weekdays, 1 weekend day)
Other	Smoking status Medical history and medicine use Education level Social economic status	General questionnaire

Annex 7

TWIN UK FFQ



1. **YOUR DIET LAST YEAR**

For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick (✓) in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the past year**.

EXAMPLES

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
POTATOES, RICE AND PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

For very seasonal fruits such as strawberries and raspberries you should estimate your average use when the fruits are in season, so if you ate strawberries or raspberries about once a week when they were in season you should put a tick in the column headed "once a week"

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
FRUIT (1 fruit or medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Strawberries, raspberries, kiwi fruit			✓						

Please estimate your average food use as best you can, and please answer every question - do not leave ANY lines blank. PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry eg. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, eg. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver paté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, eg. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, eg. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, eg. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread, eg. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal such as cornflakes, muesli etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, eg. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, eg. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream(tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, eg. Stork, Krona (teaspoon)									
Polyunsaturated margarine (tub), eg. Flora, sunflower (teaspoon)									
Other soft margarine,dairy spreads (tub), eg. Blue Band, Clover (teaspoon)									
Low fat spread (tub), eg. Outline, Gold (teaspoon)									
Very low fat spread (tub) (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
SWEETS AND SNACKS (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate , eg. digestive (one)									
Sweet biscuits, plain, eg. Nice, ginger (one)									
Cakes eg. fruit, sponge, home baked									
Cakes eg. fruit, sponge, ready made									
Buns, pastries eg. scones, flapjacks, home baked									
Buns, pastries eg. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, eg. rice, custard, trifle									
Ice cream, choc ices									
Chocolates, single or squares									
Chocolate snack bars eg. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, eg. Wotsits									
Peanuts or other nuts									
SOUPS, SAUCES, AND SPREADS									
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, eg. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

6

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, eg. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, eg. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, eg. Coca cola, lemonade (glass)									
Pure fruit juice (100%) eg. orange, apple juice (glass)									
Fruit squash or cordial (glass)									
FRUIT **For seasonal fruits marked *, please estimate your average use when the fruit is in season**									
Apples (1 fruit)									
Pears (1 fruit)									
Oranges, satsumas, mandarins (1 fruit)									
Grapefruit (half)									
Bananas (1 fruit)									
Grapes (medium serving)									
Melon (1 slice)									
* Peaches, plums, apricots (1 fruit)									
* Strawberries, raspberries, kiwi fruit (medium serving)									
Tinned fruit (medium serving)									
Dried fruit, eg. raisins, prunes (medium serving)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

7

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
VEGETABLES Fresh, frozen or tinned (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Colesiaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu , soya meat, TVP, Vegeburger									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

YOUR DIET LAST YEAR, continued

2. Are there any **OTHER** foods which you ate more than once a week? Yes ☐ No ☐

If yes, please list below

Food	Usual serving size	Number of times eaten each week

3. What type of milk did you most often use?

Select one only

Full cream, silver ☐ Semi-skimmed, red/white ☐
Skimmed/blue ☐ Channel Islands, gold ☐
Dried milk ☐ Soya ☐
Other, *specify* _____ None ☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals etc?

None ☐ Three quarters of a pint ☐
Quarter of a pint ☐ One pint ☐
Half a pint ☐ More than one pint ☐

5. Did you usually eat breakfast cereal (excluding porridge and Ready Brek mentioned earlier)? Yes ☐ No ☐

If yes, which brand and type of breakfast cereal, including muesli, did you usually eat?
List the one or two types most often used

Brand *e.g. Kellogg's*	Type *e.g. cornflakes*

6. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter ☐ Solid vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

If you used vegetable oil, please give type eg. corn, sunflower _____

7. What kind of fat did you most often use for baking cakes etc?

Select one only

Butter ☐ Solid vegetable fat ☐
Lard/dripping ☐ Margarine ☐
Vegetable oil ☐ None ☐

If you used margarine, please give name or type eg. Flora, Stork _____

8. How often did you eat food that was fried at home?

Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
Less than once a week ☐ Never ☐

9. How often did you eat fried food away from home?

Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
Less than once a week ☐ Never ☐

10. What did you do with the visible fat on your meat?

Ate most of the fat ☐ Ate as little as possible ☐
Ate some of the fat ☐ Did not eat meat ☐

11. How often did you eat grilled or roast meat? ☐☐ times a week

12. How well cooked did you usually have grilled or roast meat?

Well done /dark brown ☐ Lightly cooked/rare ☐
Medium ☐ Did not eat meat ☐

13. How often did you add salt to food while cooking?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

14. How often did you add salt to any food at the table?

Always ☐ Rarely ☐
Usually ☐ Never ☐
Sometimes ☐

15. Did you regularly use a salt substitute (eg LoSalt)? Yes ☐ No ☐

If yes, which brand? ☐

16. During the course of last year, on average, how many times a week did you eat the following foods?

Food type	**Times/week**	**Portion size**
Vegetables (not including potatoes)	☐☐	medium serving
Salads	☐☐	medium serving
Fruit and fruit products (not including fruit juice)	☐☐	medium serving or 1 fruit
Fish and fish products	☐☐	medium serving
Meat, meat products and meat dishes (including bacon, ham and chicken)	☐☐	medium serving

17. Have you taken any vitamins, minerals, fish oils, fibre or other food supplements during the past year? Yes ☐ No ☐ Don't know ☐

If yes, please complete the table below. If you have taken more than 5 types of supplement please put the most frequently consumed brands first.

Vitamin supplements	**Average frequency** Tick **one** box per line to show how often on average you consumed supplements									
Name and brand Please list full name, **brand** and strength	**Dose** Please state number of pills, capsules or teaspoons consumed	Never or less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Thank you for your help