The SLOWCARB Project: Investigating the potential influence of whole grains on cardiovascular disease risk in humans

By

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Abstract

Epidemiological evidence suggests that those individuals who consume greater quantities of whole grain are at reduced risk of cardiovascular disease (CVD). The current body of work set out to investigate this relationship via a programme of dietary intervention trials.

In order to develop a novel and robust protocol a range of markers of cardiovascular risk were sought. Pulse wave velocity (PWV) is an established independent marker of CVD risk but was lacking in reproducibility data. Therefore initial method development focused on examining the PWV responses of a cohort reflecting that of the planned targeted population groups (i.e. CVD risk). Within a cohort of men at varying levels of CVD risk \((n=8)\), it was clear that PWV does not change significantly over time when participants were following an habitual daily routine (4-6 weeks duration), nor is it significantly affected by acute metabolic challenges from exercise or the post-prandial state. With the degree of variation below 10% for all scenarios, the whole-body measure of vascular health was proven to be a robust marker of PWV which is not unduly influenced by possible confounding factors.

The intervention phase of the research encompassed three distinct studies. The High Risk Study (Chapter 4) was a randomised, controlled, parallel dietary intervention study in a cohort of individuals at an increased risk of CVD due to obesity \((n=21, \text{aged } 47.6\pm12.0\text{years, BMI } 28.9\pm3.1\text{kg/m}^2)\), it was examined whether increasing the amount of whole grain consumed per day by 48g conferred benefits to health. The study design examined the difference between the effects of consuming whole grain versus the equivalent amount of milled grain, which in turn was compared to an isocaloric control. A unique system of bread rolls were used to deliver a controlled amount of intervention ingredient in a timely manner, participants were required to consume two rolls per day (24g of whole or milled grain per roll) for eight weeks. Focussing on arterial stiffness (via the measurement of PWV) and the reported inflammatory response associated with the establishment of atherosclerosis, no significant beneficial effect was found post-intervention for either the whole or milled grain. Similarly no positive changes in lipid profile, post-prandial glycaemic control or anthropometric outcomes were identified.

To understand the possible benefits of whole grain consumption within the wider population, The Low Risk Study (Chapter 5) replicated the High Risk Study design in a cohort of young men \((n=25, \text{aged } 22.8\pm2.0\text{years, BMI } 23.5\pm3.0)\) at low risk of CVD. Once again no beneficial effects on lipid profile, fasting glycaemic control or anthropometric measures were found following whole grain supplementation. However, a significant reduction in central PWV (carotid-femoral) of 0.3m/s (from
5.8 m/s to 5.5 m/s, $p=0.02$) was detected indicating possible long-term, beneficial changes within the structural function of the arteries.

Finally study 3, the Cross-Over Study (Chapter 6) carried through the same outcome measures to a comparative investigation of 2 key components of whole grains, wheat fibre and inulin versus control. The study cohort ($n=10$) were at increased risk of CVD due to obesity (aged 39.8±9.6 years, BMI 30.2±3.0 kg/m$^2$) and consumed 15g of the active ingredient a day for 28 days per study leg. No significant changes within or between supplement (inulin, wheat fibre or control) were found for arterial stiffness and blood flow, inflammatory markers, lipid profile or anthropometric markers. For glycaemic control, a small significant reduction in glucose area under curve (AUC) was detected following wheat fibre supplementation when expressed as percentage change from baseline and compared to the control.

The key point of the SLOWCARB programme is that it is the first known body of research within the current literature to demonstrate the successful use of an ‘intervention delivery system’ via the use of specially-made bread rolls containing a specified amount of whole grain or fibre. Commonly within previous dietary intervention studies, food replacement strategies were implemented which often resulted in a high level of dietary modifications. Despite no clear pattern of effect from whole grains (and their constituent fibres) being detected, some positive outcomes have been achieved. Therefore additional research focussing on the effects of whole grains on vascular health is justified.
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List of Abbreviations

AUC – Area Under Curve
AIx – Augmentation Index
BMI – Body Mass Index
BSA – Bovine Serum Albumin
CHO – Carbohydrate
CVD – Cardiovascular Disease
C-F PWV – Carotid-femoral Pulse Wave Velocity
CIU – Clinical Investigation Unit
CV – Coefficient Variation
CAD – Coronary Artery Disease
CHD – Coronary Heart Disease
DASH – Dietary Approaches to Stop Hypertension
DEBQ – Dutch Eating Behaviour Questionnaire
ECG – Electrocardiogram
FHMS – Faculty of Health and Medical Sciences
FMD – Flow mediated dilatation
FFQ – Food frequency questionnaire
FFA – Free Fatty Acid
GLUT4 – Glucose Transporter-4
GM-CSF – Granulocyte Macrophage Colony-Stimulating Factor
HPFS – Health Professionals Follow-Up Study
HDL-C – High Density Lipoprotein Cholesterol
(hs)CRP – (high sensitivity) C-Reactive Protein

IR – Insulin Resistance

IFN-γ – Interferon-γ

IL-6 – Interleukin-6

IL-1β – Interleukin-1β

IL-8 – Interleukin-8

IDF – International Diabetes Foundation

ICAM-1 – Intracellular Cell Adhesion Molecule-1

KOL – Key Opinion Leader

LPS – Lipopolysaccharide

LDL-C – Low Density Lipoprotein Cholesterol

MAP – Mean Arterial Pressure

m/s – metres per second

MCP-1 – Monocyte Chemoattractant Protein-1

NCEP – National Cholesterol Education Programme

NDNS – National Diet and Nutrition Study

NICE – National Institute of Clinical Excellence

NO – Nitric Oxide

NHS – Nurses Health Study

OGTT – Oral Glucose Tolerance Test

OxLDL – Oxidised Low Density Lipoprotein Cholesterol

PBMCs – Peripheral Blood Mononuclear Cells

PVD – Peripheral Vascular Disease

(sf)PBS – (sterile, filtered) Phosphate-Buffered Saline
PAI-1 – Plasminogen-Activator Inhibitor-1

PP – Pulse Pressure

PWV – Pulse Wave Velocity

QALYs – Quality-Adjusted Life Years

R-C PWV – Radial-Carotid Pulse Wave Velocity

RIA – Radio-Immuo Assay

RPMI – Roswell Park Memorial Institute Medium

sELISA – sandwich Enzyme-Linked Immunosorbent Assay

SFA – Saturated Fatty Acid

SD – Standard Deviation

TC – Total Cholesterol

TGF-β – Transforming Growth Factor β

TAG – Triglyceride

TNF-α – Tumour Necrosis Factor-α

T2DM – Type 2 Diabetes Mellitus

UK – United Kingdom

USDA – US Department of Agriculture

VCAM-1 – Vascular Cell Adhesion Molecule-1

WHR – Waist:Hip Ratio

WHO – World Health Organisation
Chapter 1

1 Introduction

1.1 Cardiovascular disease

1.1.1 Definition of Cardiovascular Disease (CVD)
Cardiovascular disease (CVD) is a disease process that affects the heart and circulation that can result in angina, myocardial infarctions, stroke, claudication and possibly death (Stanner, 2005). CVD is used as a collective term to describe the development, presence and consequences of atherosclerosis in three separate areas of the vasculature (defined below):

- Coronary heart disease (CHD) – Narrowing of the arteries that supply the heart with blood (coronary arteries). If the blood flow becomes compromised (due to the narrowing) and there is a lack of oxygen to the heart muscle, the individual may experience angina. If the flow is obstructed completely due to a blood clot from a ruptured atherosclerotic plaque, a myocardial infarction will occur (Stanner, 2005).

- Cerebrovascular disease – Narrowing of the arteries that supply blood to the brain (cerebral arteries). The main consequences of this disease process is the ischaemic event of a stroke caused by the blockage of a cerebral artery by a blood clot formed either locally from a ruptured atherosclerotic plaque, or from a blood clot formed elsewhere in the body (Stanner, 2005).

- Peripheral vascular disease (PVD) – Narrowing of the arteries that supply blood to the peripheral organs and tissues of the body (not including the heart or brain). If atherosclerosis is allowed to develop, the arteries supplying the extremities may become narrowed, thus compromising the flow of blood and oxygen. An example of the consequences is claudication (pain on exercise), due to the arteries supplying blood to the legs becoming narrowed. More serious cases can result in death of the tissue in the leg resulting in ulceration and possibly loss of the limb (Stanner, 2005).

1.1.2 Current CVD mortality and morbidity rates, and the impact on the healthcare system
Recognised by the British government as a major risk to public health, targets to reduce CVD over 10 years were created. In 1999, the white paper ‘Saving Lives: Our Healthier Nation’ (DoH, 1999) was produced aiming to tackle health inequalities; specifically targeting CVD mortality rates by setting the
target that for those individuals under 75 years of age, the death rate from CHD and stroke would be cut by 40% by 2010.

Fortunately, mortality rates from CVD in the United Kingdom (UK) have been falling since the 1970's. For the 10 years leading up to 2006, the mortality rate reduced by 40% for those individuals under 75 years of age (thus approximately meeting the government targets) yet this still equates to 198,000 deaths each year (Allender, 2008). In 2006, premature deaths from CVD (before 75 years old) accounted for 30% of all deaths in men and 22% of all deaths in women, totalling 53,000 people (Allender, 2008).

However, whilst death rates may be falling, treating CVD-related illness is extremely expensive to the healthcare system and the wider-economy, with Luengo-Fernandez et al. (2006) estimating the cost of treating CVD in the UK in 2004 to be £29.1 billion. Within this, CHD accounted for £8.5 billion and treating cerebrovascular disease cost £8 billion (Luengo-Fernandez, 2006). The costs calculated include not only the cost of healthcare in the emergency and primary healthcare setting but also the lost time in working due to morbidity and mortality directly related to CVD, and the cost of ‘economically-active’ relatives becoming carers to those affected by CVD (Luengo-Fernandez, 2006).

Given the significant financial, social and health burdens of CHD within the UK population, effective strategies to tackle risk factors for CHD as well as keeping the disease process at the forefront of research is essential in order to ensure the future health of the nation.

1.2 Pathophysiology of CVD

1.2.1 Aetiology of CVD

The risk of developing CVD within a lifetime is dependent on a range of modifiable and non-modifiable risk factors. Ethnicity, gender and age are all non-modifiable CVD risk factors (Stanner, 2005). However there are also modifiable risk factors - such as smoking, hypertension and hyperlipidaemia - that have strong influences on CHD/CVD risk and can account for up to 80% of all CHD cases in middle-aged men (Emerson et al., 2003).

The development of atherosclerosis over time involves a very complex metabolic pathway and can occur in combination with the development of metabolic syndrome and type 2 diabetes due to the nature of the symptoms and the metabolic pathways that inter-cross as the disease process escalates.

(i) Obesity

It is believed that the core driving factor behind atherosclerosis is the development over time of obesity within the individual (Gustafson, 2010) and the presence of obesity can be used to identify
those individuals at risk of developing CVD. Far from being simply an issue of excess and inert fatty tissue it is now widely recognised that the adipose tissue present in the central area of the body (visceral adipose tissue) is metabolically active and has various roles in the chronic inflammatory response that is itself linked to CVD risk (Gustafson et al., 2007, Trayhurn and Beattie, 2001). In combination with the development of adipogenesis (maturation of adipocytes) within the visceral tissue, insulin resistance (IR) usually occurs which is a metabolic state where the expected amounts of insulin are no longer as effective in their roles, such as glucose uptake in the muscle or prevention of free fatty acid release into the blood stream from adipocytes. More and more insulin is required to obtain a qualitatively normal result. This process is recognised as one of the key stages in the pathogenesis of chronic diseases such as Type 2 Diabetes (T2DM) which in turn is associated with an increased risk of CVD (Song and Hardisty, 2008).

It is believed that TNF-α, an inflammatory mediator released from macrophages infiltrated into adipose tissue (Coppack, 2001, Gustafson, 2010) has a role in inducing IR due to its specific action on GLUT4, insulin receptor autophosphorylation and insulin receptor substrate-1, which results in the down regulation of glucose uptake (Coppack, 2001). TNF-α levels are also linked with IR because its expression is directly related to the amount of abdominal obesity present, to circulating levels of insulin and because it is involved in inflammatory processes. Levels of the cytokine then decrease with improvement of insulin sensitivity (normally via weight loss or drug therapy). This scenario has been found in a number of studies (Coppack, 2001, Ryan and Nicklas, 2004, Le Roith and Ziclc, 2001).

It has been recognised that the visceral adipose tissue is associated with macrophage infiltration, and so the occurrence of this infiltration may be the critical mechanism connecting increasing visceral obesity to TNF-α production, insulin resistance and eventual CVD (Weisberg et al., 2003). Weisberg et al. (2003) identified three potentially critical processes, namely that macrophages infiltrate the adipose tissue and then are almost entirely responsible for the production of TNF-α, that mature adipocytes produce the bulk of leptin and that a variety of cells (including both macrophages and adipocytes) produce IL-6. This work was extended by Suganami et al. (2005), who not only confirmed macrophages as the primary source of TNF-α, but also demonstrated that this leads to the release of monocyte chemoattractant protein 1 (MCP-1) and free fatty acids (FFAs) which further exacerbates the inflammatory process. The release of these inflammatory factors have repercussions for the endothelium via the action of MCP-1 (Libby, 2002) and the adipose tissue because of the release of FFAs (which are predominately saturated fatty acids such as palmitate) which can induce further production of TNF-α thus perpetuating the cycle of inflammation (Suganami et al., 2005). Therefore if the levels of TNF-α continue to climb, it is almost inevitable that they will eventually exert their effects on receptors critical in retaining insulin sensitivity. Figure 1.1 describes the inflammatory
pathway that is thought to link obesity with the eventual onset of CVD due to the presence of atherosclerosis.

(ii) Hypertension

Hypertension is an important risk factor for CVD, with many prospective studies having reported the inverse association between the two (Wang et al., 2007). According to the World Health Organisation (WHO), 62% of cerebrovascular disease (stroke) and 49% of coronary heart disease episodes globally are due to the presence of hypertension (WHO, 2002).

The causes of hypertension are many, including such issues as psychological stress, hyperaldosteronism, increased circulating blood volume and renal disease (Stanner, 2005). However, the presence of endothelial dysfunction caused initially by a lack of nitric oxide (the endothelial derived relaxing factor) and increased arterial stiffness are also key factors identified as having a role in not only causing hypertension but also CVD in general (Stanner, 2005).

(iii) Metabolic syndrome

It has been known for some time that CVD risk factors such as obesity, hypertension and hyperlipidaemia tend to cluster and this phenomenon is termed the ‘metabolic syndrome’ (Paoletti et al., 2006). The specific risk factors are hyperglycaemia, abdominal obesity, hyperlipidaemia (raised triglycerides, greater concentration of small dense LDL particles, low HDL-cholesterol) and hypertension (Paoletti et al., 2006, Alberti et al., 2009). The driving forces behind the appearance of metabolic syndrome are thought to be insulin resistance and abdominal obesity (Paoletti et al., 2006).

It has been found that metabolic syndrome is predictive of CVD risk, and as the number of risk factors accumulate, so too does the overall risk of CVD (Sattar et al., 2003). However, it is not yet decided whether the clustering of risk factors presents a higher risk of CVD overall as opposed to the sum of the individual risks associated with each factor. Cameron (2010) certainly states that it does not, and that there is no additional predictive value to CVD risk by identifying the presence of metabolic syndrome (Cameron, 2010). This could be interpreted as saying that metabolic syndrome is ‘just’ a cluster of issues indicating a risk of CVD but there is no multiplicative effect simply because risk factors are classed within one syndrome. However, it is agreed that those individuals diagnosed with metabolic syndrome are twice as likely to develop CVD between 5 and 10 years later than those individuals without (Alberti et al., 2009). The risk is even higher (five-fold) for developing type 2 diabetes in those with metabolic syndrome (Alberti et al., 2009).
Figure 1.1. Diagram to demonstrate the complexity of the proposed pathways of inflammation postulated to be involved in the development of CVD, which is the main concept linking inflammation and arterial stiffness.
To gauge the overall health of an individual and how their current CVD risk factors may be connected, the metabolic syndrome is a useful marker. There are three definitions of metabolic syndrome currently published by the WHO in 1998 (Alberti, 1998), US National Cholesterol Education Program in 2001 (NCEP, 2001) and the International Diabetes Federation in 2005. However in 2009, an agreement was reached to provide a cohesive document setting out a diagnostic criterion for metabolic syndrome that attempted to eliminate the issue of discrepancies between the three separate definitions that were in use. The key opinion leaders (KOLs) involved in composing the ‘Harmonizing the Metabolic Syndrome: A Joint Interim Statement’ (Alberti et al., 2009) are below:

International Diabetes federation Task Force on Epidemiology and Prevention

National Heart, Lung and Blood Institute

American Heart Association

World Heart Federation

International Atherosclerosis Society

International Association for the Study of Obesity

The previous definitions of metabolic syndrome focused on separate areas of the syndrome, with some specific factors highlighted as the primary influence to the overall risk - WHO focused on insulin resistance plus the presence of two other risk factors; NCEP had no specific focus as the underlying risk factor, the diagnosis was based on meeting three out of the five criteria (abdominal obesity, raised triglycerides, reduced HDL-cholesterol, hypertension, impaired fasting glucose); IDF followed the NCEP criteria but abdominal obesity had to be one of the criteria met. The consensus document produced by the six KOLs in 2009 now states that abdominal obesity should not be a mandatory risk factor but be given equal weighting against the other four risk factors. In the case of waist circumference, locally established cut-off points for risk are to be used (i.e. in UK ≥94cm equates to CVD risk for men, whereas in China cut-off point is ≥85cm) and for the other four risk factors, set cut-offs have been established - see Table 1.1- (Alberti et al., 2009).

1.2.2 Endothelial dysfunction and the role of inflammation as a critical driver of CVD

As shown in Figure 1.1, as adipose tissue accumulates within the abdomen due to a positive energy balance, adipokines are released which seem to play a key role in the initial stages of the inflammatory process linked to cardiovascular disease risk and progression.

Evidence has been accumulating to indicate that there is both an innate and acquired immune response connected to CVD involving the activation of macrophages during the development of atherosclerosis (Pickup, 2004). Attention has focused on the investigation of the activity of inflammatory cytokines,
believed to be associated with the pathogenesis of Type 2 Diabetes (T2DM) and other related conditions such as endothelial dysfunction, atherosclerosis, CVD, and metabolic syndrome (Pickup 2004).

Table 1.1. Criteria for Clinical Diagnosis of the Metabolic Syndrome

<table>
<thead>
<tr>
<th>Measure</th>
<th>Categorical Cut Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated waist circumference*</td>
<td>Population- and country-specific definitions</td>
</tr>
<tr>
<td>Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator†)</td>
<td>≥150 mg/dL (1.7 mmol/L)</td>
</tr>
<tr>
<td>Reduced HDL-C (drug treatment for reduced HDL-C is an alternate indicator‡)</td>
<td>&lt;40 mg/dL (1.0 mmol/L) in males; &lt;50 mg/dL (1.3 mmol/L) in females</td>
</tr>
<tr>
<td>Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator)</td>
<td>Systolic ≥130 and/or diastolic ≥85 mm Hg</td>
</tr>
<tr>
<td>Elevated fasting glucose* (drug treatment of elevated glucose is an alternate indicator)</td>
<td>≥100 mg/dL</td>
</tr>
</tbody>
</table>

HDL-C indicates high-density lipoprotein cholesterol.

*It is recommended that the IDF cut points be used for non-Europeans and either the IDF or AHA/NHLBI cut points used for people of European origin until more data are available.

†The most commonly used drugs for elevated triglycerides and reduced HDL-C are fibrates and nicotinic acid. A patient taking 1 of these drugs can be presumed to have high triglycerides and low HDL-C. High-dose ω-3 fatty acids presumes high triglycerides.

‡Most patients with type 2 diabetes mellitus will have the metabolic syndrome by the proposed criteria.

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The Interleukins (i.e. IL-1β, IL-6, IL-8) and TNF-α are biologically active molecules released by multiple sources including mononuclear phagocytes (monocytes and macrophages) and adipocytes to induce autocrine or paracrine effects linked to the escalation of the inflammatory response (Coppack, 2001). Many cytokines have multiple responsibilities and functions, in addition to causing the release of other chemotactic agents involved in atherosclerosis (such as monocyte-chemoattractant protein-1 and vascular cell adhesion molecule-1), however a large number also share the same roles (Coppack, 2001) therefore adding to the enormous complexity of the inflammatory response.
Endothelial dysfunction – a state where there is an imbalance between factors involved in coagulation, inflammation and vasodilation in the vascular endothelium (Poredos, 2002) – has a major role in the development of atherosclerosis and therefore CVD risk.

A ‘healthy’ endothelium relies on the production of Nitric Oxide (NO), to ensure vasodilation, and to prevent platelet aggregation and smooth muscle proliferation (Singhal, 2005). NO is a gas produced throughout the endothelium via the metabolism of L-arginine by endothelial NO synthase (eNOS) (Versari et al., 2009). An increase in inflammatory markers or free radicals (due to smoking, poor diet, lack of exercise or increase in adipose tissue) will degrade NO before it can be effective at the endothelium, thus allowing the process of atherosclerosis to begin (Singhal 2005).

As shown in Figure 1.2, if NO is not present at the endothelium (or NO production and bioavailability is at least compromised), then this can allow both vascular cell adhesion molecule (VCAM-1) and intracellular cell adhesion molecule (ICAM-1), which are both membrane-bound to the endothelium, to attract monocytes to adhere and migrate across the endothelium into the intima layer of the arterial wall (Libby, 2002, Lind, 2003). Monocyte Chemoattractant Protein-1 (MCP-1) also facilitates the migration of the monocytes by encouraging a ‘chemoattractant gradient’ (Libby, 2002). Once the monocytes are present in the intima, they modify to become macrophages which then exacerbate the process further by accumulating oxidised lipoprotein (to be now known as foam cells) to form the atherosclerotic plaque (Libby, 2002).

The main risk from the presence of atherosclerosis centres on the stability of the plaque and the fibrous cap that forms over the atherosclerotic plaque over time. Plaque instability has been attributed to the occurrence of microvessels forming within the plaque (angiogenesis) and this activity has been shown to be a predictive factor in plaque rupture (Moreno et al., 2004). In addition, the fibrous cap can become unstable and then suddenly rupture due to high levels of shear stress within the arterial lumen (Crowther, 2005). If the plaque ruptures, this results in a thrombus forming within the lumen of the artery due to the platelets, fibrin and other thrombogenic inflammatory co-factors from the plaque coagulating with the arterial blood to occlude the artery (Crowther, 2005). The consequences of plaque rupture can be fatal as dependant on the location within the vasculature of the thrombus, a myocardial infarction (thrombus in coronary artery) or stroke (thrombus in carotid artery) will result.

In addition to plaque rupture, the process of atherosclerotic plaque formation can cause extensive changes to both the arterial lumen and the integrity and function of the arterial wall (Figure 1.3), which have the potential to markedly alter the flow of blood to the target organs over time thus resulting in ischaemic-related conditions such as peripheral artery disease, claudication, angina and heart failure (Versari et al., 2009).
Figure 1.2. The stages involved in the development of an atherosclerotic plaque

Although this endothelium-level activity can be modelled via *in vitro* cellular techniques, measurement of circulating levels of inflammatory markers provides a clinically useful and practical proxy in establishing the level of cardiovascular risk an individual may be experiencing.
(i) Interleukin-6 (IL-6)

IL-6 is not well understood in terms of its exact actions, however it is known to be one of the main cytokine mediators of the acute-phase response (Libby, 2002) and so plays an important role in driving the inflammatory response linked to atherosclerosis. Known to be released by adipose tissue (Fruhbeck et al., 2001) and from vascular cells (Ikonomidis et al., 1999) it has been found that as IL-6 increases, so too does the number of characteristics of the metabolic syndrome within T2DM and non-DM subjects (Pickup et al., 1997) such as glucose intolerance (Deepa et al., 2006). This is thought to be due to the positive, linear relationship between cytokine activity and insulin resistance (Pickup, 2004). Furthermore, Woods et al. (2000) found that IL-6 stimulates the production of fibrinogen and C-Reactive Protein (from the acute-phase response), reduces HDL-cholesterol, increases platelet aggregation and encourages the expression of adhesion molecules at the endothelium (Woods et al., 2000).

A study by van Hall et al. (2003) found that IL-6 could also be responsible for modulating fat metabolism within humans in addition to increasing fat oxidation and re-esterifying fatty acids (van Hall, 2003). Using an isotope study to trace fatty acid metabolism during infusions of either saline, low or high doses of recombinant human IL-6, it was found that that in those with rhIL-6 infused, lipolysis increased (participants were not hypertriglyceridaemic). However, importantly, there were no changes in catecholamines, glucagon or insulin which indicates that IL-6 may be solely responsible for the lipolytic activity in this case. Cortisol levels were elevated during the infusions but the authors state that this does not affect the results and merely strengthens the findings of the lipolytic effects of IL-6 due to the findings from in vivo studies that glucocorticoids have no effect on lipolysis (Divertie et al., 1991) and in the case of human adipocytes, cortisol has an anti-lipolytic action (Ottesson, 2000).

(ii) Tumour necrosis factor-α (TNF-α)

Tumour Necrosis Factor-α (TNF-α) is seen as a pivotal cytokine within the pro-atherosclerotic inflammatory response due to its ability to induce a cascade of inflammatory cytokines (Osterud and Bjorklid, 2003), to stimulate the expression of adhesion molecules and reduce the bioavailability of nitric oxide (NO) at the endothelium, therefore assisting in the induction of endothelial dysfunction (Willerson and Ridker, 2004).

TNF-α is produced by macrophages found within endothelial tissue and macrophages infiltrated within visceral adipocytes (Mohamed-Ali et al., 1997). In adipocytes, TNF-α has both a paracrine and autocrine function, and together with IL-6 (produced by the adipocytes) increasing levels of TNF-α results in increases in lipolysis and the subsequent release of free fatty acids (FFA) (Gustafson, 2010). Circulating TNF-α levels appear to elevate as adipose tissue and BMI increases (Kern et al., 1995), along with IL-6 (Coppack, 2001), causing the release of MCP-1 (Suganami et al., 2005). According to
Hube & Hauner (1999), TNF-α is associated with the inhibition of lipoprotein uptake into the adipose tissue and with fat cell development, resulting in increased lipolysis and impaired glucose uptake, thus inducing insulin resistance (Hube and Hauner, 1999). Once TNF-α is released into the circulation via macrophages, it stimulates the release of Transforming Growth Factor β (TGF β) which then proceeds to stimulate Plasminogen-Activator Inhibitor-1 (PAI-1) (Birgel et al., 2000, Sakamoto et al., 1999). The presence of PAI-1 is thought to be involved in the further development of insulin resistance (Hauner, 2005) as well as other roles in fibrinolysis and coagulation (Gil-Campos et al., 2004).

Overall, the main target of TNF-α activity is at the endothelium (Ritchie et al., 2004) because it reduces the availability of NO (Gao et al., 2007, Picchi et al., 2006) which in turn reduces vasodilation (Ritchie et al., 2004); without the protection of NO, endothelial dysfunction is likely to follow. As described by Zhang et al. (2009), once NO availability is compromised, TNF-α then has the opportunity to induce the expression of cytokines such as IL-6 and IL-8, in addition to chemoattractants such as MCP-1, ICAM-1, VCAM-1 and E-selectin (Zhang et al., 2009). Matrix metalloproteinases are also produced and involved in vascular remodelling which can have implications relating to the stability of the developing atherosclerotic plaque, facilitated in part by the inflammatory process now taking place at the endothelium. As shown in Figure 1.4, the effects of TNF-α are widespread and in the latter stages of endothelial dysfunction cause reductions in vascular repair as well as the reduction in NO availability and vasodilation already described (Zhang et al., 2009). However, oxidative stress, followed by vascular inflammation, endothelial cell infiltration, atherosclerosis, thrombosis, vascular remodelling and endothelial apoptosis are all up-regulated by the corresponding increasing levels of TNF-α (Zhang et al., 2009). If no action is taken to dampen the production of TNF-α then the cycle of inflammatory marker production becomes self-perpetuating (Bruunsgaard, 2005), having more serious effects with time.

![Figure 1.4. The role of TNF-α in endothelial dysfunction](image)

Those markers in green indicate factors which can inhibit TNF-α production or signalling, markers in orange indicate those factors which encourage TNF-α production. It is clear that TNF-α has a vast effect on endothelial function via many separate pathways. Taken from Zhang et al. (2009) Clinical Science 116, 219-230.
(iii) Monocyte Chemoattractant Protein-1 (MCP-1)

In order for atherosclerosis to progress, increasing numbers of monocytes need to migrate into the intima of the arterial wall and transform into macrophages before taking up oxidised LDL to then become macrophage foam cells (Libby, 2002). This process of monocyte recruitment and activation is facilitated by a number of different chemokines however it is believed that the main influence in creating the crucial chemoattractant gradient is by Monocyte Chemoattractant Protein-1 (MCP-1) (Osterud and Bjorklid, 2003, Ikeda et al., 2002, Libby, 2002). Recent research has also found that MCP-1 may have further effects involving the increased proliferation of smooth muscle cells and associated increase in IL-6 production (Viedt et al., 2002).

The source of MCP-1 production appears to change as the atherosclerotic plaque develops. In the presence of hypercholesterolaemia or similar sources of injury to the endothelium (essentially causing endothelial dysfunction), it is believed that the endothelial cells themselves produce MCP-1 to attract initial numbers of monocytes to the intima. As the plaque progresses and macrophage foam cells are present, they then take over the production of MCP-1, thus ensuring a continuous cycle of monocyte infiltration (Takeya et al., 1993). P-selectin (a chemoattractant) also increases MCP-1 production alongside TNF-α in monocytes that have been exposed to platelet activating factor (PAF) (Weyrich et al., 1995).

Animal studies have found that when the production of MCP-1 is blocked, the progression of the atherosclerotic plaque is dramatically reduced. Ni et al. (2001) found that in apo E knockout mice with a MCP-1 gene deletion mutation at the N-terminal, MCP-1 activity was extremely restricted with atherosclerotic lesions dramatically reduced to virtually none (Ni et al., 2001).

1.2.3 Importance of measuring arterial stiffness as a potential endpoint of inflammation

Arterial stiffness has the capacity to independently predict CVD risk (Laurent et al., 2001) and C-reactive protein (C-RP) has been shown by a number of research groups to have a positive linear relationship with PWV (Yasmin et al., 2004, Okamura et al., 2004, Mattace-Raso et al., 2004). C-RP is an acute phase response protein produced by the liver in response to an inflammatory process occurring within the body and it is a general systemic marker of inflammation (Gaw et al., 2002). C-RP is produced in response to circulating IL-6 (Gaw et al., 2002), which in turn is produced due to the strengthening inflammatory response and the presence of TNF-α and IL-1β (Hansson, 2001).

As shown in Figure 1.1, the inflammatory process and the resultant presence of endothelial dysfunction followed by atherosclerosis describes a possible pathway linking the pro-atherosclerotic inflammatory response to the ‘end marker’ of arterial stiffness (measured as PWV).
Vlachopoulos et al. (2005) supports the causal relationship in terms of acute inflammation resulting in an increase in arterial stiffness. In a cohort of 100 healthy people (55% men) who were neither obese nor smokers, two randomised, double-blinded and sham-procedure controlled studies were completed (Vlachopoulos et al., 2005). For both studies participants were injected with either *Salmonella Typhi* vaccine or the equivalent volume in saline. For 'Study 1', participants had aortic stiffness, wave reflections and inflammatory markers measured at baseline, 8hrs and 32hrs post-injection. ‘Study 2’ involved the same injections but with the addition of 1200mg of aspirin taken at baseline with the same measurements as Study 1 taken at baseline and 8hrs post-injection (Vlachopoulos et al., 2005).

Overall the results showed that the vaccination significantly increased PWV (+0.43m/s at 8hrs, p<0.001) compared to the placebo and that this increase in PWV was significantly correlated with C-RP levels (raised at 32hrs post-injection, p<0.001) and IL-6 (raised at 8hrs post-injection, p<0.01). The addition of aspirin for Study 2 resulted in no significant changes in PWV. However C-RP significantly increased with the vaccine injection (p<0.05) and was still correlated with PWV (p<0.01). This is in contrast to IL-6 which was also significantly increased despite the aspirin (p<0.001) yet it did not correlate with the PWV results (Vlachopoulos et al., 2005). The authors believe that the mechanism behind this apparent effect is the reduction in the bioavailability of nitric oxide (NO) due to the induction of the inflammatory response. However, despite the promising results, only a small number of inflammatory markers were tested – C-RP, MMP-9, IL-6 and CD14. Even though MMP-9 significantly increased post-vaccine for both Study 1 and 2, no correlations with PWV are reported (Vlachopoulos et al., 2005).

This is a relatively new field of research to understand the relationship between arterial stiffness and the inflammatory response. However, given the clear involvement of the inflammatory response in the development of atherosclerosis and the fact that PWV is an independent predictor of CVD, it is logical to conclude that there is an association which warrants further research. This is particularly important as understanding how exactly PWV is affected and how quickly the arterial stiffness develops, in addition to what degree the changes are dependent on activity at the endothelium would be extremely useful data that would have many applications within the research environment.

1.2.4 **Clinical endpoint of inflammation: Arterial stiffness**

Arterial stiffness is in part caused by endothelial dysfunction which is triggered due to a lack of nitric oxide available at the endothelium (Wilkinson et al., 2002, Tomiyama and Yamashina, 2010). These changes, in addition to shear stress found within the aorta and other CVD risk factors like aging and diabetes, then contribute to the loss of the elastic properties of the artery in addition to the distensibility, and the conduit function (Tomiyama and Yamashina, 2010).
Arterial stiffness is identified by an increase in the speed at which the pressure wave travels down the arterial tree such that it is reflected back to the heart during the systole phase as opposed to the diastole phase (Tomiyama and Yamashina, 2010, O'Rourke et al., 2002). This event indicates that the arteries are less compliant with a reduced ability of the artery walls to cushion and control the pressure wave and ensuing blood flow.

1.2.4.1. Assessing Arterial Stiffness via Pulse Wave Velocity (PWV)

Pulse Wave Velocity (PWV) is an indicator of arterial stiffness, and PWV is used as a strong predictor of CVD risk for both healthy (Safar et al., 2002a, McEniery et al., 2006) and ‘unhealthy’ individuals who have conditions such as the metabolic syndrome (Mule et al., 2006) or hypertension (Laurent et al., 2001).

PWV is a measure of the speed at which the pressure wave travels down the arterial tree following the ejection of blood from the heart into the ascending aorta (Safar et al., 2002a). The velocity of this wave is the point of measurement and it is then used as an indicator of the vasculature’s elasticity (Van Bortel et al., 2002). The higher the velocity of the wave, the less elastic the arterial walls, thus indicating increased arterial stiffness (Stehouwer et al., 2008).

To measure peripheral arterial stiffness, the pressure waves are recorded from pulse points on the wrist (radial pulse) and neck (carotid pulse). Central arterial stiffness is measured again at the carotid but then the femoral pulse. When the two pressure waves are recorded, the propagation of the waves is assessed with the difference in time delays and distance between waves measured (Safar et al., 2002a).

PWV is a simple, non-invasive technique using applanation tonometry technology with good levels of reproducibility. Liang et al., (1998) found that when used on 50 healthy volunteers (20 men, 30 women, mean age 46.5 years), central PWV had an intra-individual coefficient of variation (CV) of 3.2%. The PWV results were also significantly correlated with age (p<0.001) and carotid intima-media thickness, p<0.01 (Liang et al., 1998). Further investigation into PWV reproducibility has also been undertaken by Woodman et al., (2005), when an intra-individual CV of 7.6% for central PWV was found to be acceptable considering the range of other techniques used to measure arterial stiffness in the group of participants (15 men with CHD, 15 men without), showed lower levels of reproducibility – namely central pulse pressure with a CV of 25.3%, and brachial pulse pressure with a CV of 8.0% (Woodman et al., 2005). Given that PWV, especially central measurements (involving the carotid and femoral arteries), have high levels of reproducibility, within the current literature it is regarded as being the gold standard technique for quantifying arterial stiffness as a marker of CVD risk (Stehouwer et al., 2008).
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There are a variety of other techniques available to assess the elasticity of the vasculature; with many also applicable as a ‘bedside’ clinical measurement. The only direct measure of arterial stiffness available is an invasive system which measures both aortic pressure and diameter using an intravascular catheter placed directly into the thoracic aorta (Stefanadis et al., 1995). However due to the obvious risks and costs involved in such a methodology it is not practical within a typical research or clinical setting.

As described by Laurent et al., (2006) in the ‘Expert consensus document on arterial stiffness: methodological issues and clinical applications’, arterial stiffness can be measured regionally (Aortic PWV), locally (at a specific artery) or systemically (analysing the shape of the waveform) using methods such as a tonometer for PWV and echotracking for local detection of stiffness.

A number of indices are also calculable from the measurements taken of the key arteries (i.e. brachial, carotid, femoral) during ultrasound scanning. All have a different purpose, for example the change in lumen area during systole, the cross-sectional distensability and compliance coefficients, in addition to the Young’s elastic modulus all provide information on the functional elasticity of the artery as opposed to the propensity of the propagated pressure waves that PWV indicates (Laurent, 2006).

The augmentation index (AIX) has also been suggested as a measure of arterial stiffness although it is a common misconception that, like PWV, this offers a definitive measure of arterial stiffness (Laurent, 2006). In fact it is a ratio of augmentation pressure to pulse pressure calculated from the waveforms recorded during Pulse Wave Analysis (Stehouwer et al., 2008), giving information on the character of the pressure waves, and therefore is an indirect index that gives some indication of arterial stiffness when used in conjunction with PWV (Laurent, 2006). AIX has been found to correlate significantly with central PWV (Yasmin and Brown, 1999) and also, like central PWV, is inversely associated with endothelial function (McEniery et al., 2006).

However, for the purposes of the future planned dietary intervention studies, pulse wave velocity (PWV) remains the most suitable methodology for identifying arterial stiffness and the subsequent risk of CVD within the study participants due to the high reproducibility (Liang et al., 1998), ease of use and its capability to independently predict CVD risk (Laurent et al., 2001). PWV is also proven to be the ‘gold standard’ in measuring arterial stiffness (Stehouwer et al., 2008).

1.2.4.2. Translating PWV results to health

It has been acknowledged that central PWV is independently predictive of all-cause and CVD-related mortality, particularly in those with end-stage renal disease (Safar et al., 2002c) and hypertension (Laurent et al., 2001). However, critically, PWV is also independently predictive of CVD risk in
apparently healthy individuals (Sutton-Tyrrell et al., 2005, Willum-Hansen et al., 2006, Mattace-Raso et al., 2006).

For example, Willum-Hansen et al. (2006) followed-up over 9.4 years a random sample of 1678 people aged 40-70 years, measuring central PWV, pulse pressure (PP) and 24-hour ambulatory PP. Results were adjusted for age, sex, body mass index, mean arterial pressure (MAP), smoking and alcohol. The crucial findings were that even after fully adjusting the PWV, it retained its predictive capacity for CVD-related morbidity and mortality events (comparing against both pulse pressures) with a relative hazard ratio of 1.17 (1.04-1.32 95%CI) when associated with a 1-SD increase in PWV which was equivalent to an increase of 3.4m/s (n=154, p<0.05).

Similarly, Mattace-Raso et al. (2006) found that within a sample of 2835 subjects involved in the Rotterdam Study, the highest risk of a CVD event was found in those in the highest tertile for PWV, even after adjusting for many CVD risk factors such as age, gender, MAP, heart rate, BMI, lipid profile, diabetes, smoking, anti-hypertensive medication, carotid intima-media thickness, ankle-arm index and PP (n=85, hazard ratio 1.93 (1.16-3.2195%CI), p=0.04).

Therefore the current research indicates that, even after adjusting PWV results for many CVD-related factors, PWV retains its predictive capabilities, providing a more robust and dependent marker of risk than other markers such as pulse pressure (Willum-Hansen et al., 2006) and is therefore an important independent predictor of CVD mortality and morbidity risk in healthy individuals.

However, a major limitation of this method within clinical practice is the absence of agreed reference ranges for the healthy population, in addition to appropriate cut offs to indicate increased CVD risk and insufficient data on expected levels of variation. Within the literature there are differing opinions as to the cut-off for PWV at which arterial stiffness can be diagnosed, from as low as 7.9m/s (Sutton-Tyrrell et al., 2005) to 14.6m/s (Mattace-Raso et al., 2006). However, this may in part be explained by the heterogeneity of the study populations from which this data is derived, especially with regards to the average age and the nature of chronic disease or morbidities present within the study groups.

With obvious gaps in knowledge, Khoshdel et al. (2006) completed a meta-analysis to determine age-specific reference intervals for central PWV. The aim was to define acceptable limits of central PWV that indicate vascular health. Should any future results fall beyond the 95th percentile, as determined by this study, then those individuals would be deemed at risk of CVD (Khoshdel et al., 2006). With a combined population of 2008 healthy adults the 95th percentile of the “age-specific reference interval” for central PWV was 10.94 m/s for adults at 20yrs (Khoshdel et al., 2006). For those at 40yrs, central PWV had increased along the curve to 11.86 m/s and for 60yrs it had progressed to 13.18m/s (Khoshdel et al., 2006). The authors note that the reference intervals were not calculated according to gender neither have they been externally validated against further research. However, due to the large
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population involved, this meta-analysis provides some additional guidance and consistency for the assessment of PWV results and their translation into meaningful CVD risk prediction.

1.3 Management of CVD

1.3.1 Current guidelines for the clinical and pharmacological management of CVD

As discussed in Section 1.2.1, three key risk factors targeted in the primary clinical and pharmacological management of CVD are obesity, hypertension and the lipid profile (NICE, 2008). Currently the National Institute of Clinical Excellence (NICE) recommend that individuals aged 40-74 years have their 10 year risk of experiencing a ‘CVD-event’ (i.e. angina, stroke, heart attack) calculated (NICE, 2008). If the risk is greater than 20%, then it is recommended that the individual (as part of ‘primary prevention’) make lifestyle changes, such as implementing a cardio-protective diet, increasing exercise and the cessation of smoking to optimise their health, with a particular focus on controlling blood pressure, obesity and cholesterol levels. If appropriate, statins (cholesterol-lowering drugs) are then commenced alongside the lifestyle modifications. As per the NICE guidance for managing hypertension (NICE, 2006a) and obesity (NICE, 2006b), drug therapy may be implemented if required, however dietary and lifestyle changes in addition to increasing exercise levels are recommended as first-line treatment (NICE, 2006a, NICE, 2006b).

1.3.2 Lowering the risk of CVD through lifestyle changes – diet

There is an abundance of data to show that by changing habitual dietary intake, important CVD risk factors can be ameliorated. For example, the Dietary Approaches to Stop Hypertension (DASH) study showed that in a cohort of 459 adults with moderate hypertension, when compared to a control diet, there was a significant reduction in both systolic and diastolic blood pressure after the participants consumed either a ‘fruits-and-vegetables’ or combination diet (Appel et al., 1997). The diets were carefully constructed to induce specific changes to the dietary intake of the participants. The control diet contained 37% energy from fat, 48% energy from carbohydrate and 9g fibre, in addition to 165mg Magnesium and 1700mg of potassium. The ‘fruits and vegetables’ diet matched the control in terms of percentage energy from fat, carbohydrate and protein (15%) but had approximately three times the amount of fibre (31g), potassium (4700mg) and magnesium (500mg). The combination diet (i.e. low fat and high in fruit/vegetable) provided 27% of energy from fat, 55% energy from carbohydrates and 18% from protein. Fibre, potassium and magnesium levels matched that of the ‘fruits and vegetables’ diet. Sodium was standardised across the three diets at 3000mg/day. After a three week run-in for all participants on the control diet, there followed an eight-week intervention period with the participants randomly assigned to any of the three diets. Overall, when compared to the control diet, the combination diet group showed the greatest reductions in both systolic (5.5mmHg) and diastolic (3.0mmHg) blood pressure (Appel et al., 1997). However, even when comparing against the ‘fruit and vegetables’ diet, the combination diet reductions in systolic (2.7mmHg) and diastolic (1.9mmHg)
blood pressures were still significant (Appel et al., 1997). The current NICE guidance for controlling hypertension states that those with hypertension (160/100mmHg) or those at risk of CVD (10yr risk of +20%) with moderate hypertension (140/90mmHg) should be assessed and receive anti-hypertensive drugs as required. By reducing hypertension, the quality-adjusted life years (QALYs) are considerable – i.e. years of healthy life. For example, by introducing beta-blockers the associated QALY is 9.8, compared to the calcium-channel blockers.

Aside from the effects on hypertension, evidence shows that lifestyle changes may also be beneficial in reducing CVD risk by preventing or at least dampening the established atherosclerotic inflammatory process. Esposito et al. (2004) undertook an intervention trial which evaluated the effects of a ‘Mediterranean style’ diet (increased whole grains, fruit, vegetables, olive oil, nuts) versus a ‘prudent’ diet (CHO 50-60%, protein 15-20%, fat <30%) on the endothelial function and inflammatory markers in individuals with metabolic syndrome. After two years, the intervention (Mediterranean diet) appeared to significantly reduce hs-CRP, IL-6, IL-7, IL-18 and insulin resistance compared to the control group (Esposito et al., 2004). Endothelial function significantly improved and only 40 out of the original 90 Mediterranean diet subjects still had metabolic syndrome at the end of the intervention (Esposito et al., 2004). This study did show that participants significantly increased their wholegrain intake throughout the duration, however there were too many factors involved within the study to be able to clearly show that whole grains had an impact alone in reducing the metabolic syndrome symptoms.

Another study by Lopez-Garcia et al. (2004) also recognised that dietary patterns (including increasing wholegrain intake) are inversely linked to endothelial dysfunction and inflammation (Lopez-Garcia and Hu, 2004). However, they too were unable to pinpoint single causative factors due to the cross sectional design of their study involving 732 women from the Nurses’ Health Study I Cohort.

Dietary research so far has established that implementing ‘whole diet’ approaches and changing key constituents of the diet (i.e. fat content, levels of fibre) can dramatically improve health outcomes (Appel et al., 1997). However, these dietary changes are both difficult to apply in the general population due to the number of changes required and also do not allow the relative benefits of individual dietary components to be identified. Therefore research has also focused on trying to identify the effects of single nutrients to the amelioration of CVD risk in order to identify key changes that are required which could have a substantial impact on risk. Previous research key targets have included saturated fat, essential fatty acids, antioxidants, fruit and vegetables. More recently whole grains have been implicated in the reduction in risk of CVD (McKeown et al., 2002, Jacobs et al., 1998, Wang et al., 2007). However many questions remain as to the mechanism that they may use to exert their effects on health.
Slavin et al., (1999) propose that whole grains have the potential to exert their effects on the body’s metabolism in many ways. Whole grains can be fermented due to the non-digestible carbohydrates that they contain (Slavin et al., 1999) thus producing short-chain fatty acids which have been reported to have a link with reducing plasma cholesterol levels as well as other health risks such as cancer (Cummings, 1992). The actual matrix of the food product and how intact the grain is, may also have an effect via gastric emptying and thus possibly affecting satiety and reducing dietary intake overall. Slavin et al., also indicate that the glycaemic index of whole grain foods compared to refined grains may have an impact as well as the actual antioxidant and vitamin (vitamin E) and mineral content (zinc, manganese, selenium) of the grains (Slavin et al., 1999). It is proposed that there are up to 15 separate metabolically active components or groups of factors within whole grains that could all be having a beneficial effect on the consumers health, with many acting as antioxidants, tumour growth suppressors, enzyme modulators, lowering cholesterol or have hormone-like actions (Slavin et al., 1999). However it is critical to the possible ‘functionality’ of whole grains that milling is kept to a minimum and the grain, or at least the components, are kept in equal proportions as found within the whole kernel to retain the presence of the vitamins, minerals and other factors that may be crucial in exerting health benefits.

1.3.3 Whole grains as a component of the cardioprotective diet

1.3.3.1. Definition of whole grains, current intake and recommendations

The definition of a wholegrain within the UK is recommended by the Whole Grain Working Group of the Institute of Grocery Distribution’s Industry Nutrition Strategy Group – a representative committee of food producers, manufacturers and retailers and other industry experts. Therefore for UK purposes, the definition of a whole grain (used within a food) is referred to as follows:

“edible entire grain after removal of inedible parts....it must include the entire germ, endosperm and bran” (IGD, 2007).

When processing, the committee go further by stating that:

“whole grain also includes grains that have been subjected to processing (i.e. milling, cracking, crushing, rolling, flaking, extrusion) but only if after processing the proportions of the germ, endosperm and bran are present in the same or virtually the same proportions as the original grain” (IGD, 2007).

As shown in Figure 1.5, the composition of a wheat kernel, i.e. the three main parts, are the bran, endosperm and germ. The bran makes up 13-17% of the kernel, forms the outer layer of the grain and provides the bulk of the fibre. The endosperm is the portion of the kernel that contains mainly starch and forms approximately 80-85% of dry weight of the kernel. The germ is a rich source of protein,
minerals, fats, fibre as well as carbohydrate and makes up 2-3% of the wheat kernel mass (Pomeranz, 1988).

It has been shown that ideally, to obtain the benefits of whole grains; three servings must be consumed per day (Lang and Jebb, 2003). Reinforcing the consensus as to the 'ideal' amount of whole grains to consume, the USA is one of only a few countries in the world to give a definitive guideline to the public as to the number of portions of whole grain foods that should be consumed on a daily basis. The US Department of Agriculture (USDA) recommends the consumption of 3 portions of whole grain-containing foods per day which will provide 48g of whole grain (USDA, 2005).

In order to stay healthy and prevent chronic diseases such as Cardiovascular Disease (CVD) and Type 2 Diabetes (T2DM), it is advisable to follow a balanced diet, including fruit, vegetables, oily fish and whole grains. Currently whole grain intake is very low in the UK, with the population consuming less than one portion of whole grains per day (Finch, 1998, Gregory, 1990). The latest data obtained by the National Diet and Nutrition Survey (NDNS) for 2008/2009 shows that the average consumption of non-starch polysaccharide (NSP) is 14g/day for adults when the target is 18g/day (Bates, 2010). The NDNS report states that this lack of NSP in the diet is not helped by the decline in the consumption of wholegrain foods from the previous NDNS survey (Bates, 2010). The most recent data indicates that the average consumption per day of wholegrain-containing products is 127g per day (for males and females aged 19-64yrs). However this amount is taking into account products such as wholemeal bread; brown, granary and wheat germ breads and wholegrain and high fibre breakfast cereals. All of these products may contain wheat-sourced whole grain but to varying degrees and so it is not possible to accurately estimate the amount of actual whole grain consumed on a daily basis due to the heterogeneous groupings of the food products within the NDNS and lack of data showing the nutritional breakdown. The most commonly consumed grains in the UK are wheat followed by rice and maize, in addition to barley, oats, rye used in other cultures (Seal, 2006). As wheat makes such a large contribution to the daily diet within the UK, both as refined and whole grain, it is imperative to quantify any effects that they may have on health.

Figure 1.5. The composition of a wheat kernel (taken from the American Institute for Cancer Research (AICR) website aicr.org. June 2010)
1.3.3.2. Epidemiological evidence for cardioprotective effect of whole grains

The evidence suggests that both the whole grains and the individual components found within the grains may have a beneficial effect on cardiovascular disease risk. There is a range of epidemiological and observational evidence connecting the consumption of whole grains and risk of developing CVD (see Table 1.2). Anderson (2003) completed a meta-analysis of thirteen studies, of which five were directly related to whole grain consumption (studies not identified within meta-analysis by author). It was identified that when comparing the lowest with the highest intakes of whole grain from the five selected studies, there was a risk ratio for developing CVD of 0.71 in those consuming wholegrain (95%CI 0.48-0.94) (Anderson, 2003). This can be interpreted as whole grain consumption conferring a 29% reduced risk of a CHD event (Anderson, 2003). The analysis also showed that out of 8 studies assessing the relationship between type of food/fibre intake and CHD risk, 7 showed a negative association and 6 showed a significantly negative association between whole grains consumption and CHD risk. The author further details that neither cereal fibre nor refined grains provide the level of protection against CVD that is attributed to whole grains.

Another meta-analysis, completed by Mellen et al. (2008), is far clearer in terms of the average intakes of whole grains from the pooled data of the seven observational studies included in the meta-analysis as well as the details of the studies and the level of multivariate adjustment completed as part of the final analyses. Overall, Mellen et al. (2008) found that, from a pooled cohort of 149,000 participants, there was an inverse association between whole grain intake and cardiovascular disease risk. It was found that, on average, the highest level of whole grain intake was 2.5 servings per day as opposed to 0.2 servings per day in the group with the lowest intake (Mellen et al., 2008). Interestingly, for the highest consumers of whole grain within the pooled data, the incident risk of CVD was 37% lower based on demographic-adjusted estimates (Odds ratio 0.63, 95%CI 0.58-0.68). Even with additional adjustment for risk factors, the association remained: 0.79 (0.73-0.85) (Mellen et al., 2008), therefore resulting in a 21% reduction in risk of a CVD event. When assessing the impact of refined grains, it was found that even when comparing high to low intakes, there was no association with reduced risk of CVD (Mellen et al., 2008).

As detailed above in Section 1.2.2, systemic inflammation appears to have a very definite effect on the risk of developing CVD and T2DM. Qi et al. (2006) have investigated the association between whole grains, bran and cereal fibre intakes on markers of inflammation in women with T2DM. Their findings included a significant trend in reducing levels of CVD and TNF-α with increasing quintiles of wholegrain and bran intakes (Qi et al., 2006). The observational nature of this study prevents a causal link from being confirmed yet this study was useful as it distinguishes between different parts of the fibre and whole grains. Over the quintiles, as intake of whole grain increases and of their respective components, significant improvements are seen. For example, increased consumption of cereal fibre
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... (those in the highest quintile) was associated with lower CRP ($p=0.03$). Similarly, those in the highest quintiles of whole grain consumption also had lower CRP ($p=0.03$) and TNF-R2 levels ($p=0.017$), alongside bran, high intakes of which were also associated with 1.33 mg/l lower CRP by ($p=0.007$) (Qi et al., 2006).

However, Jensen et al. (2006) found epidemiological data that opposes the view of whole grains aiding the amelioration of the inflammatory process associated with CVD and T2DM. Data accumulated during the ‘The Health Professionals Follow-Up Study’ (HPFS) and ‘The Nurses’ Health Study II’ (NHS II) was used to evaluate the relationships between certain dietary patterns and key inflammatory markers. The HPFS was a prospective cohort study of initially 51,529 men recruited in 1986, with vocations within the wider healthcare setting. Dietary and lifestyle information plus medical history were assessed every 2-4 years, a blood sample was provided once over the 9 year follow-up. The NHS II Study was again a prospective, cohort study, following 116,671 female nurses recruited in 1989. Diet, health and lifestyle information was collected every 4 years, blood samples were taken between 1996 -1998. The biomarkers chosen to indicate the inflammatory process were CRP, fibrinogen and IL-6 which were not found to differ significantly between the quintiles of wholegrain intake (Jensen et al., 2006). However, given the complexity of the inflammatory process, and the number of potential biomarkers, these studies may have been too limited to identify any relationship between inflammation and wholegrain intake given the absence of data on other markers such as TNF-α, IL-1, IL-2, IL-6, IL-8 IL-18, ICAM-1 or VCAM-1 to name a few. Inferences that can be made are also limited by the observational nature of the study. To draw firm conclusions about the relationship between whole grains and inflammation a randomised controlled trial is required with outside influences, confounding factors and inclusion criteria for the study participants more closely scrutinised and safeguarded.

1.3.3.3. Intervention studies investigating the cardio-protective effect of whole grains

At present there appears to be only a relatively small collection of dietary intervention work looking at the effects of whole grains on insulin resistance, obesity and the risk of CVD. Table 1.3 clearly identifies those pertinent studies and the important outcomes to note.

A number of studies have focused on the effects of whole grain on overall CVD risk (see Table 1.3). Collectively, they have found that when whole grain consumption is increased within the habitual diet, there are reductions in fasting glucose (Jang et al., 2001), fasting insulin (Pereira et al., 2002, Rave et al., 2007), weight and blood pressure (Behall et al., 2006) as well as body fat percentage (Katcher et al., 2008).

For example Jang et al. (2001) reported, from their cohort of 76 males with coronary artery disease, that there was a significant increase in HDL-cholesterol alongside other positive benefits of reductions
in fasting glucose and markers of lipid peroxidation after a 16 week intervention of consuming a whole grain and legume-mix powder. Pereira et al. (2002) and Rave et al. (2007) both employed crossover designs for their dietary intervention studies and focused on obese population groups. After six and four week intervention periods respectively, both studies found significant benefits to health. Pereira et al. (2002) noted reductions in fasting insulin and an improvement in insulin sensitivity; Rave et al. (2007) also noted changes in insulin metabolism with significant reductions in fasting insulin and HOMA-IR. A study by Katcher et al. (2008) implemented an intervention involving increasing whole grain intake in one cohort compared to a control group, however the habitual diets throughout the intervention were also hypocaloric for both groups. Subsequently for both the control and whole grain groups, there were significant reductions in weight, waist circumference, total cholesterol, LDL-cholesterol and PAI-1. However, after making statistical adjustments it was found that the whole grain intervention group had significantly reduced levels of C-reactive protein and body fat percentage compared to the control group (Katcher et al. 2008).

Compared against this backdrop of evidence for significant changes associated with increased whole grain intake, a small number of studies have found no changes for any biochemical or whole-body marker of CVD risk after completing whole grain intervention studies. Andersson et al. (2007) completed a cross-over study in a mixed cohort of men and women who were healthy yet overweight. Despite matching the intervention length of Pereira et al. (2002), with far higher levels of whole grain implemented as part of the intervention (Andersson 112g whole grain p/d, Pereira 20g additional fibre p/d), no significant changes in any parameter (anthropometrics, lipid profile, insulin sensitivity, inflammatory marker) was detected. A study by Brownlee et al. (2010) conducted a large-scale parallel-design intervention study with overweight participants who consumed between 60-120g of whole grain per day for an intervention period of 16 weeks. Using similar outcomes to Andersson et al. (2007), this study also did not show any significant benefits to consuming increased levels of whole grains.

Therefore, at present the most encouraging outcome from the whole grain intervention studies are the observed effects on insulin resistance after whole grain consumption. As described above, three studies (Jang et al., 2001, Pereira et al., 2002, Rave et al., 2007) found that post-consumption of whole grains there were significant reductions in insulin demand and resistance. Behall et al. (2006) also found reductions in blood pressure and so these results combined could indicate a potential mechanism to investigate between whole grain consumption and vascular function. Insulin resistance is known to be induced by the action of TNF-α (Hube and Hauner, 1999), in addition TNF-α also targets the endothelium by reducing the bioavailability of NO which is essential in maintaining vasodilation and preventing the onset of endothelial dysfunction. Therefore increasing the consumption of whole grains may be implicated in a mechanism which ameliorates a complex inflammatory pathway to endothelial
dysfunction which essentially revolves around the reduction in circulating TNF-α. Thus the observation of insulin resistance could potentially be flagged as a marker for an ongoing inflammatory response, driven by TNF-α which could result in endothelial dysfunction.

It is acknowledged that currently the evidence linking greater whole grain consumption to reductions in CVD risk markers is mixed. Despite a seemingly vast array of clinical outcomes measured, two large scale studies did not find an effect from consuming greater quantities of whole grain (Andersson et al., 2007, Brownlee et al., 2010). However, these studies do not measure whole-body measures of vascular function, such as pulse wave velocity or even related indices such as augmentation index or pulse pressure. The range of inflammatory markers analysed is limited to C-RP and study designs (i.e. amount of whole grain consumed, length of intervention) across all the intervention studies described in Table 1.3 is variable. Thus an opportunity has been opened to investigate comprehensively if and how whole grains may bring benefits to health, focusing particularly on the interactions within vascular function and the inflammatory response.
Table 1.2. Epidemiological studies linking the consumption of whole grains with CVD incidence

<table>
<thead>
<tr>
<th>Study</th>
<th>Outcome measures</th>
<th>Cohort and follow-up</th>
<th>WG measure - High &amp; low quantile</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health Professionals Follow-Up Study (Jensen et al., 2004)</td>
<td>CHD, both fatal and non-fatal</td>
<td>42,850 men, aged 40-75yrs, 14 year follow-up</td>
<td>FFQ, daily intake (g) Quartile 1: 3.3g/d [0.02SE] Quartile 5: 49.6g/d [0.23SE]</td>
<td>Strong inverse association between WG intake and risk of CHD. HR: 0.82; 95% CI: 0.70-0.96, p&lt;0.01.</td>
</tr>
<tr>
<td>Iowa Women’s Health Study (Jacobs et al., 1999)</td>
<td>CVD, CHD and stroke-related mortality</td>
<td>34,333 women, aged 55-69yrs, 9 year follow-up, Total CVD cases: 304 (CHD 190, Stroke 38)</td>
<td>FFQ, weekly servings Quartile 1: 1.5 (0-3.3IR) Quartile 5: 22.5 (18.5-105.0) (median with range)</td>
<td>Strong inverse association between WG intake and CVD death CVD (all) – HR: 0.82; 95% CI: 0.66-1.01, p=0.02 CHD – HR: 0.82; 95% CI: 0.63-1.06, p=0.03 Stroke and ‘other’ CVD cause of death = ns</td>
</tr>
<tr>
<td>Nurses’ Health Study CHD (Liu et al., 1999)</td>
<td>CHD events, both fatal and non-fatal</td>
<td>75,521 women, aged 38-63yrs, 10 year follow-up</td>
<td>FFQ, daily servings Quartile 1: 0.13 (0-0.26) Quartile 5: 2.70 (1.77-17.86) (median with range)</td>
<td>Strong inverse association between WG intake and risk of CHD and ischaemic stroke. Cases of CHD across quintiles of WG intake (all) – RR 0.79 (95% CI 0.62-1.01, p=0.07 Non-smokers – RR 0.47 (95% CI 0.27-0.79, p=0.006 Case of Stroke across quintiles of WG intake (all) – RR 0.69 (95% CI 0.50-0.98), p=0.08</td>
</tr>
<tr>
<td>Nurses’ Health Study Stroke (Liu et al., 2000)</td>
<td>Ischaemic stroke events</td>
<td>761 cases of CHD (553 non-fatal MI, 208 fatal CHD) Same cohort, 12 year follow-up, 352 stroke cases</td>
<td>FFQ, daily intake (g) Quartile 1: 4.8 (0-7.8) Quartile 5: 32.6 (25.5-146.0) (median with range)</td>
<td>CVD mortality across quintiles of WG intake RR 0.70 (95% CI 0.46-1.06), p=0.07</td>
</tr>
<tr>
<td>Nurses’ Health Study Type 2 Diabetes Mellitus (He et al., 2010)</td>
<td>CVD mortality in Type 2 Diabetes Mellitus</td>
<td>7822 women with Type 2 Diabetes Mellitus</td>
<td>FFQ, daily intake (g) Quartile 1: 4.8 (0-7.8) Quartile 5: 32.6 (25.5-146.0) (median with range)</td>
<td>Cases of CAD across quintiles of WG intake HR 0.72 (95% CI 0.53-0.97), p=0.05 Cases of ischaemic stroke HR 0.75 (95% CI 0.46-1.22), p=0.15</td>
</tr>
<tr>
<td>Atherosclerosis Risk in Communities Study (ARIC) (Steffen et al., 2003)</td>
<td>Coronary Artery Disease (CAD) and Stroke events</td>
<td>15,792 men and women, aged 45-64yrs, 11 year follow-up</td>
<td>FFQ, daily servings Quartile 1: 0.1 (0-0.2) Quartile 5: 3.0 (2.0-10.5) (median with range)</td>
<td>Significant inverse association between WG intake and common carotid artery IMT p=0.005</td>
</tr>
<tr>
<td>Insulin Resistance Atherosclerosis Study (Mellen et al., 2007)</td>
<td>Progression of carotid intima media thickness (IMT)</td>
<td>1178 men and women, aged 55.2±8.4yrs, 5 year follow-up</td>
<td>FFQ, daily servings Intake - continuous variable, median intake 0.79servings/d</td>
<td>Cases of HTN across quintiles of WG intake RR 0.89 (95% CI 0.82-0.97), p=0.007</td>
</tr>
</tbody>
</table>

Key: CHD – coronary heart disease; MI – myocardial infarction; CAD – coronary artery disease; FFQ – food frequency questionnaire; WG – whole grain; HR – hazard ratio; RR – risk ratio.
Table 1.3. Intervention studies linking the consumption of whole grains (sourced from wheat) with CVD risk

<table>
<thead>
<tr>
<th>Study title</th>
<th>Cohort and study design</th>
<th>Outcome measures</th>
<th>Intervention</th>
<th>Significant results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consumption of whole grain and legume powder reduces insulin demand, lipid peroxidation and plasma homocysteine concentrations in patients with Coronary Artery Disease (Jiang et al., 2001)</td>
<td>76 men with CAD. Parallel study with 4 week control diet run-in, followed by 16wks consuming either a whole grain and legume powder or continue with the control diet</td>
<td>Anthropometrics, blood pressure, lipid profile, OGTT, HOMA. Plasma levels of Hcy, MDA, vitamins, carotenoids, phospholipids</td>
<td>70g of whole grain and legume powder dissolved in water and taken at breakfast in place of refined grain sources. Powder composition: 66.6% whole grain, 22.2% legumes, 5.6% seeds, 5.6% vegetables. Powder isoallor to 150g cooked refined rice (220kcal)</td>
<td>Comparing to the control group, there were the following significant changes for the WG group (calculated comparing the net difference between two groups): Increases in HDL-Cholesterol, p=0.001; Retinol, p=0.008; α-tocopherol, p=0.006. Reductions in fasting glucose, p&lt;0.001; MDA, p=0.028; urinary 8-Epi-PGF&lt;sub&gt;2α&lt;/sub&gt;, p=0.003; Hcy, p=0.03. OGTT: Non-DM subjects, reduction in HOMA-IR (p=0.001) and increase in HOMA-B/IR (p=0.003) for WG.</td>
</tr>
<tr>
<td>Effect of whole grains on insulin sensitivity in overweight, hyperinsulinaemic adults (Perreira et al., 2002)</td>
<td>Six women, six men with BMI 26-35kg/m&lt;sup&gt;2&lt;/sup&gt;. Randomised, controlled cross-over study (refined and whole grain diets). Two 6-wk feeding periods, 6-9wk washout.</td>
<td>Anthropometrics, OGTT, HOMA. Erythrocyte hyperinsulinaemic clamp. Study days at baseline, then wks 2, 4 &amp; 6 of intervention stage</td>
<td>Subjects follow either a WG or RG diet for 6 weeks each, using menu plans matched in CHO, fat and protein. In the WG diet, there was 10.2g extra of dietary fibre, 8.9g extra of insoluble fibre and 1g extra of soluble fibre than the RG diet.</td>
<td>Over the course of the visits (wks 2, 4, 6), there were the following significant changes for the WG intervention when comparing against the RG intervention: Reductions in fasting insulin, p&lt;0.01; HOMA-IR, p&lt;0.01, Increase in M value for clamp (indicating 18S), p&lt;0.05.</td>
</tr>
<tr>
<td>Blood pressure reduced by whole grain diet containing barley or whole wheat and brown rice in moderately hypercholesterolaemic men (Hallfrisch et al., 2003)</td>
<td>Sixteen normotensive men, aged 28-62yrs, with elevated cholesterol levels. Run-in diet followed by 3 diets running for 5wks inlatin square design</td>
<td>Anthropometrics, blood pressure, 24-hr urine collection</td>
<td>Initial run-in of 2wks following Step 1 AHA diet. Step 1 diet then continued with addition of either brown rice/whole wheat, barley or a combination of both for 5wks each in latin square design.</td>
<td>Compared to the initial run-in diet, at the end of each intervention diet, SBP, DBP and MAP were all reduced, however not all were significantly reduced.</td>
</tr>
<tr>
<td>Consumption of whole-grain cereals during weight-loss: Effects on dietary quality, dietary fibre, magnesium, vitamin B&lt;sub&gt;6&lt;/sub&gt; and obesity (Melanson et al., 2006)</td>
<td>134 men &amp; women, aged 42.3±1.2yrs, BMI 30.9±2.4kg/m&lt;sup&gt;2&lt;/sup&gt;. Randomised, controlled, parallel 24wk diet and exercise intervention.</td>
<td>Anthropometrics</td>
<td>Participants assigned to one of three intervention groups: exercise only, exercise plus hypocaloric diet containing WGs or exercise plus hypocaloric diet not containing WGs. First 12wks, participants had weekly counseling visits. Final 12wks, no counseling visits.</td>
<td>Both hypocaloric diets induced significant weight loss when compared to the exercise only group p&lt;0.001. No significant difference between the hypocaloric diet groups.</td>
</tr>
<tr>
<td>Whole-grain diets reduce blood pressure in mildly hypercholesterolaemic men and women (Behall et al., 2006)</td>
<td>9 pre-menopausal women, 9 post-menopausal women, 7 men. Latin square design study – 3 diets</td>
<td>Anthropometrics, blood pressure, lipid profile, energy intake</td>
<td>2wk run-in diet using the Step 1 (AHA) diet then 5wk rotation of 3 diets replacing RG foods with WG – diets: barley, whole wheat/brown rice, half barley/half whole wheat/brown rice. Total dietary fibre in WG diets = 9.7-11.9g/1,000kcal.</td>
<td>Consumption of all WG diets – significant reduction in SBP (p&lt;0.003), DBP (p&lt;0.003), MAP (p&lt;0.05), body weight (p&lt;0.01). Significant increase in energy intake on WG diets, p&lt;0.036.</td>
</tr>
<tr>
<td>Study title</td>
<td>Cohort and study design</td>
<td>Outcome measures</td>
<td>Intervention</td>
<td>Significant results</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------------------------</td>
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<td>----------------------------------------------------------------------------------</td>
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<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Whole-grain foods do not affect insulin sensitivity or markers of lipid peroxidation and inflammation in healthy, moderately overweight subjects</td>
<td>(Andersson et al., 2007)</td>
<td>Anthropometrics, blood pressure, lipid profile, euglycaemic hyperinsulinemic clamp. Plasma levels of vitamins, and inflammatory markers</td>
<td>Subjects continued with habitual diets but also included a set number of portions of WG or RG foods each day. WG diet provided 112g WGI/day.</td>
<td>No significant effect found on any outcome markers.</td>
</tr>
<tr>
<td>Improvement of insulin resistance after diet with a whole-grain based dietary product: results of a randomised, controlled cross-over study in obese subjects with elevated fasting blood glucose</td>
<td>(Rave et al., 2007)</td>
<td>Anthropometrics, blood pressure, lipid profile, HOMA, fructosamine</td>
<td>Two week run-in diet followed by a 4wk intervention period consuming either a WG product from double-fermented wheat or a meal replacement product. Products were intended to replace two daily meals plus snacks, totalling 200g of product per day.</td>
<td>Adjusting results for weight loss, fasting insulin and HOMA-IR were significantly reduced for the WG product compared to the MR product.</td>
</tr>
<tr>
<td>The effects of a whole grain-enriched hypocaloric diet on cardiovascular disease risk factors in men and women with metabolic syndrome</td>
<td>(Katcher et al., 2008)</td>
<td>Anthropometrics, blood pressure, lipid profile, OGTT, DEXA, inflammatory markers, Apo A1, Apo B-100</td>
<td>Participants consumed either a hypocaloric WG or RG diet for 12wks. Participants also requested to follow set guidance on other parts of the diet i.e. fruit/vegetable portions, low-fat dairy.</td>
<td>Body fat % significantly reduced in WG group compared to RG group (p=0.03). Weight, WC, total, LDL and HDL cholesterol plus PAI-1 all significantly reduced for both groups from baseline.</td>
</tr>
<tr>
<td>Effects of the regular consumption of wholemeal wheat foods on cardiovascular risk factors in healthy people</td>
<td>(Giacco et al., 2010)</td>
<td>Anthropometrics, blood pressure. Test meal: post-prandial lipids, glucose, insulin, ApoA1/B-100, hyperinsulinemic clamp, gut hormones, antioxidants, breath hydrogen</td>
<td>Two-week run-in diet then randomly assigned to one of two isoenergetic diets for 3wks, followed by the other diet plan. One diet contains WG-based CHO, the other RG-based CHO. Test meal at each study session (to match the diet just followed)</td>
<td>Compared to run-in diet, both diets had significant reductions in total and LDL-cholesterol. WG diet reduced total and LDL-cholesterol significantly more than RG. Test meal: glucose at 240mins lower for RG than WG (p&lt;0.04). GIP IAUC higher after WG meal (p=0.001). At 120mins and 240mins, Apo A1 and Apo B-100 lower for WG meal (p&lt;0.01).</td>
</tr>
<tr>
<td>Markers of cardiovascular risk are not changed by increased wholegrain intake: the WHOLEHEART study, a randomised, controlled dietary intervention</td>
<td>(Brownlee et al., 2010)</td>
<td>Anthropometrics, blood pressure, lipid profile, insulin sensitivity, inflammatory markers</td>
<td>Participants randomised to either the control group or 60g WG per day for 16wks or 60g WG per day for 8wks, followed by 120g WG per day for 8wks.</td>
<td>No significant effect found on any outcome markers.</td>
</tr>
</tbody>
</table>

Key: CAD = coronary artery disease; OGTT = oral glucose tolerance test; HOMA = homeostasis model; IR = Insulin resistance; Hcy = homocysteine; MDA = malondialdehyde; 8-Epi-PGF2α; 8-epi-prostaglandin F2α; BMI = Body mass index; CHO = carbohydrate; WG = whole grain; RG = refined grain; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; M = male; F = female; WC = waist circumference; PAI-1 = plasminogen activator inhibitor-1.
1.3.3.4. The potential independent roles of fibrous components of the whole grain – inulin and wheat fibre

Dietary fibre is defined as follows: “the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.”

American Association of Cereal Chemists. Published in: (Jones, 2000)

(i) Wheat fibre (insoluble fibre)

Fibres contained within the whole grain kernel include inulin - a polyfructose (fructan), and wheat fibre - an insoluble fibre mainly consisting of cellulose and hemi-cellulose (Jones, 2000). The purpose of insoluble fibre is widely regarded in simple terms to provide bulk and aid appropriate stool frequency, however the evidence also shows that insoluble fibre has a role in peripheral insulin sensitivity (Fukagawa et al., 1990, Wolever et al., 2004). Conversely, Jenkins et al. (2002) showed that when their 23 subjects (all with T2DM) completed the 2 phases of 3 months (per phase) within a cross-over design, the high-fibre (wheat bran) diet had no significant effects on any markers of CVD (Jenkins et al., 2002). This included markers of the lipid profile, inflammation and glycaemic control. The authors do note that the intervention may have been too short to show measurable change and that the pursuit of insoluble fibre to distinguish its metabolic effects is still warranted (Jenkins et al., 2002).

There is only a very small amount of literature assessing the effects of insoluble fibre on overall CVD risk factors. Giacco et al. (2010) attempted to elucidate whether a diet rich in cereal fibre (insoluble fibre) as opposed to refined grain would improve CVD risk factor markers such as glucose and lipid metabolism, antioxidants, in addition to inflammatory markers. Fifteen overweight participants were recruited (12 male, 3 female), aged 54.5±7.6yrs to take part in a cross-over study design involving insoluble and refined fibre. The participants consumed a run-in diet then for 3 weeks followed either a diet rich in insoluble fibre (cereal fibre 23.1g/d, total dietary fibre 32g/d) or refined grains (cereal fibre 9.8g/d, total dietary fibre 20g/d - matches run-in diet).

Despite measuring many markers at fasting and for four hours post-prandially after a test meal at the end of each interventions phase, no significant differences were found between the diets, except for the lipid profile. After consuming the cereal fibre intervention, there were significant reductions in total (TC) and LDL-cholesterol (LDL-C) from fasting samples (TC p<0.03 and LDL-C p<0.04) compared to the refined grain intervention (Giacco et al., 2010). Therefore despite the lack of a 'global' effect of insoluble fibre, the reduction in both total and LDL-cholesterol is certainly
encouraging, especially as soluble fibre has also been found to be effective at lowering the lipid profile.

Even though there is not a substantial amount of data as to the effectiveness of including insoluble fibre in the diet in ameliorating CVD risk factors, there is still some encouraging evidence that appears to show that justification in pursuing with this direction in dietary research. There are still many questions to be answered and there is also a large discrepancy between research groups as to how much insoluble fibre is used in the intervention diets and also how well matched the diets are when being used in comparison, such as a whole grain versus refined grain scenario.

A prospective cohort study (Erkkila et al., 2005) indicates that overall, the increasing consumption of cereal fibre (insoluble fibre) is associated with reducing risk of coronary artery disease (CAD). In a cohort of 229 post-menopausal women, it was found that those that consumed the least amount of cereal fibre were at greater risk of CAD. Those that consumed greater than 3g of cereal fibre per 1000kcal (equivalent to >6 servings of whole grains per week) were at reduced risk of CAD which was classified as a reduction in progressive reduction in coronary artery diameter (Erkkila et al., 2005). The authors also noted that the higher consumers were at lower risk of stenosis however this was not significant. The amount of fruit, vegetables and refined grains consumed did not appear to affect CAD risk and did not linked with the progression of the disease process (Erkkila et al., 2005).

(ii) Inulin

Inulin is a known prebiotic, with only 10-15g per day apparently sufficient to induce a significant increase in bifidobacteria production in the bowel (Gibson et al., 1995) leading to suppression of harmful bacterial strains such as enterococci (Kleessen et al., 1997) E. coli and clostridia (Wang and Gibson, 1993).

It has been found in rats that have very high dietary inulin levels (50-200g inulin per kg of rat chow) was able to induce reductions in their fasting triglyceride levels (Roberfroid, 1993). Reductions in cholesterol are also possible, however this was not evidenced in the shorter term animal feeding studies (Delzenne, 1993). It is believed that the mechanistic pathway in rats via which inulin exerts its TAG-lowering effects is essentially via a reduction in the production of VLDL particles from the liver (Kok et al., 1996).

In humans, current evidence from inulin-based dietary interventions appears to show that increased consumption is also associated with beneficial changes in lipid profiles which would be linked to a lowering of CVD risk.
Chapter 1: Introduction

A review of the literature by Williams & Jackson (2002) collated evidence from human dietary intervention studies involving inulin. From 1984 to 2000 they note six intervention studies took place (four involving inulin (Davidson, 1998, Brighenti, 1999, Causey, 2000, Jackson et al., 1999), and two looking at oligofructose from sucrose (Hidaka, 1991, Yamashita, 1984) – all fructans), in primarily hyperlipidaemic subjects. Doses ranged from 8g fructans/day for the two oligofructose trials to 9-18g inulin/day with a mean intake of 12.2g/day and a mean study duration of 4.6 weeks (Williams and Jackson, 2002).

The review concluded that oligofructose was associated with a significant reduction in total cholesterol, with one study also finding a concurrent reduction in LDL-cholesterol (Williams and Jackson, 2002). For inulin-based studies, improvements in lipid profile were reported across all four studies, with reductions in one or more lipid parameter (total cholesterol, LDL cholesterol and triglycerides) (Williams and Jackson, 2002). In addition one study conducted by Jackson et al. (1999) found a significant reduction in fasting insulin after the consumption of 10g inulin for 8 weeks (Jackson et al., 1999).

However, three studies conducted between 1996 and 1999 (Luo et al., 1996, Pedersen et al., 1997, Alles et al., 1999) found that with a mean intake of 16g of fructans per day for an average of 3.7 weeks, no significant changes in lipid profile, fasting glucose or insulin were detected. This obvious discrepancy in results between studies may be explained by differences in the way in which the inulin was incorporated into the diets of the participants, with vehicles including coffee drinks, powders added to drinks, ice cream and cereals, biscuits, yoghurt and even margarine. Therefore the fat, carbohydrate and protein, not including the impact of background dietary fibre levels could all be having an effect on the efficacy of including the inulin in the diet and therefore impact on the results.

Intervention studies completed after the review by Williams and Jackson (2002) continue to focus on the effects of inulin on lipids. A study by Letexier et al. (2003) provides evidence for a mechanism for this effect, specifically supporting the existence of the pathway previously identified in rats (Roberfroid, 1993). Assuming this animal data can be translated into humans a more recent study reports that a 5% inulin-based diet was linked to a 28% reduction in parametrial fat mass compared to a 10% cellulose-based diet (Jamieson et al., 2008). If human adipose tissue can also be altered via the consumption of inulin, then this has critical repercussions for the inflammatory response linked to atherosclerosis and overall CVD risk, and therefore confirms the importance of further research into the risk reduction potential of inulin in humans.
1.4 Summary

Cardiovascular disease (CVD) has a complex aetiology, combining the occurrence of many risk factors that occur both concurrently and consecutively, such as obesity, insulin resistance, hypertension and the production of inflammatory markers (see Figure 1.6). PWV is an independent predictor of CVD and gauges the extent of CVD risk by measuring the compliance of the conduit arteries. If the consumption of whole grains (or insoluble fibre or fermentable carbohydrate) is accompanied by a significant reduction in arterial stiffness (measured as pulse wave velocity) after a long-term intervention then this would indicate that CVD risk has reduced. Since chronic inflammation is reported to be one influence on the causal pathway for CVD (particularly atherosclerosis, see Figure 1.1), it is expected that a wholegrain intervention would result in a reduction in the levels of inflammatory markers, indicating a reduction in CVD risk. As the inflammatory pathway involved in CVD is so complex, a range of inflammatory markers related to endothelial activity need to be monitored in order to determine the pathway via which whole grains ameliorate CVD risk.

Figure 1.6. The proposed mechanism for CVD progression

A diagram to describe the many factors involved in the development of CVD risk. Diet, lifestyle and environment are modifiable factors, genetics are unmodifiable. When these factors combine to become detrimental to health (i.e. smoking, excess alcohol, lack of exercise, poor diet), the CVD risk factors may develop and if not restricted, will continue on into CVD. Each circle denotes CVD risk factor and the method of measurement.
1.5 Hypothesis

It is hypothesised that the addition of whole grains (including the whole grain constituents wheat fibre and inulin) to the habitual diets of population groups at both low and high risk of cardiovascular disease will ameliorate CVD risk as indicated by a reduction in the key inflammatory markers described, and a reduction in arterial stiffness as measured by PWV.

1.6 Aims and objectives of the current research

1.6.1 Research aim

The main aim of this research is to establish, within population groups at different levels of cardiovascular disease risk, whether the additional inclusion of whole grains (both intact and crushed, and their components) into the regular dietary intake of those individuals may ameliorate the risk factors associated with cardiovascular disease over time i.e. arterial stiffness and the inflammatory response.

There is a lack of intervention data within the current literature examining the effects whole grains may have on health in comparison to refined grains. The effects of the constituent insoluble fibre and fermentable carbohydrate (inulin) found within the whole grain will also be investigated. This programme of research will be supported by the introduction of a direct delivery system using baked goods containing controlled quantities of the intervention (i.e. 24g of whole grain per bread roll) which will be invaluable in terms of the standardisation of the intervention.

1.6.2 Research objectives

The objectives for this research centre on the investigation of how whole grains, insoluble fibre and fermentable carbohydrate impact on various risk factors in both acute and chronic settings, in healthy and at-risk population groups. Over the course of the intervention studies, the following primary factors will be monitored both pre and post-dietary intervention:

Vasculature

The level of arterial stiffness will be measured via the use of Pulse Wave Velocity (PWV).

Blood flow will be measured via the use of both static and 24-hour ambulatory blood pressure, in addition to measurement of the related indices of Mean Arterial Pressure (MAP) and Pulse Pressure (PP).
Inflammatory Markers

Levels of circulating inflammatory markers and chemo-attractant agents that are linked with being involved in the progression of CVD will be measured in fasted serum samples - TNF-α, IL-1β, IL-6, IL-8, MCP-1, and GMCSF.

From *ex vivo* work isolating monocytes from peripheral blood mononuclear cells (PBMCs), production of TNF-α will be measured.

Secondary assessment of the impact of whole grains on the metabolism will also take place by monitoring the following:

**Glycaemic control and lipid profile**

Measuring both fasting and post-prandial levels of glucose, insulin, triglycerides and non-esterified fatty acids, in addition to both total and HDL-cholesterol.
Chapter 2

2 Materials and Methods

All materials and methods detailed within this section describe the standardised methodological procedures used for all observational and interventional studies that form the SLOWCARB Project. Human studies (both intervention and observational) were carried out in the Clinical Investigation Unit (CIU) at the Faculty of Health & Medical Sciences, University of Surrey. Laboratory analysis was carried out in the Nutrition laboratory also within the Faculty of Health and Medical Sciences (FHMS) at the University of Surrey.

2.1 Screening and recruitment

2.1.1 Ethical approval

All studies gained a favourable ethical opinion from the University of Surrey Ethics Committee.

2.1.2 Consent

Prior to the drawing of blood from a participant - whether venepuncture or cannulation - informed and written consent from the participant was required – see Appendix A.1. Consent was required for all procedures, including screening sessions and the intervention studies.

2.1.3 Participant recruitment

For all five studies, participants from the staff and student population of the University of Surrey were recruited via posters and global emails on-campus. Advertising in the local media was also utilised to recruit from the local population.

All participants were invited to attend a formal screening session to be assessed for suitability for taking part in their chosen study (specific recruitment criteria detailed in latter chapters).

Each study had specific age, gender, and anthropometric inclusion criteria. However, participants for all studies were also required to self-report that they met the basic lifestyle criteria as set out below, via a set of standard questionnaires:

- **Healthy**: No current or previous medical conditions that may impact on compliance or interfere with the digestion of the intervention products such as diabetes, gastrointestinal disorders (Crohn's disease, Coeliac disease, Irritable Bowel Syndrome), liver disease,
depression, psychological disorders, eating disorders, drug or alcohol abuse. No history of cardiovascular disease (i.e. heart attack, angina, stroke). Not currently taking medication or nutritional supplements within the preceding 6 months. Information was sought using a Health & Lifestyle Questionnaire (see Appendix A.7 for example).

- **Exercise:** Moderate amounts of exercise permitted; no more than 3 x 30 min sessions per week.

- **Stable dietary intake:** Not actively dieting, not consuming high amounts of whole grains (i.e. no more than 3 portions/day), no excessive intakes of single food groups. Intake was assessed by a Food Frequency Questionnaire which was developed specifically for this project (guidance on development from (Cade et al., 2002)) and used in all the intervention studies. The questionnaire was not formally validated but this was not deemed necessary due to its use for screening purposes only. No allergies to ingredients found within the intervention products.

- **Non-smokers, moderate drinkers (within government safety limits).**

Full study details, including the time commitment, aims of the study and the procedures involved, were explained to the prospective participants with time for questions and a cooling-off period before written, informed consent was sought.

Once the study had been completed, participants received financial remuneration for their time and inconvenience.

### 2.1.4 Haemoglobin levels

Prior to commencing the *High Risk Study* participants were required to provide a capillary blood sample via finger prick to determine circulating haemoglobin levels and thus risk of anaemia. A portable haemoglobin monitor (201+ Hb monitor, HemoCue AB, Ängelholm, Sweden) was used, with levels less than 12 g/dl warranting further investigation and possible exclusion from the study. This precaution was deemed necessary due to the two days of post-prandial studies and the associated blood collection within the *High Risk Study* (~260mls in total).

### 2.1.5 Dutch Eating Behaviour Questionnaire

The Dutch Eating Behaviour Questionnaire (DEBQ), developed by van Strien et al. (1986), is constructed using 33 questions that are used to determine the behaviour patterns of an individual associated with eating. Divided into three categories, external, emotional and restricted eating, the subscores for each category would identify an abnormal response to hunger and satiety signals; specifically whether they are susceptible to the influence of their emotions and so feel an urge to eat,
are affected by *external cues* such as the smell of food, or intentionally *restrict* their intake (Van Strien et al., 1986).

This questionnaire was used as part of the screening for the High Risk Study and Sensory Evaluation exclusively, due to the need for sensitive appetite and sensory measurements during the respective studies. Completed questionnaires were scored by the investigator according to a standardised scoring system and any individual with scores outside of the acceptable range was excluded from the particular study regardless of other screening data. See Appendix A.2 for questionnaire.

The maximum possible score per category is 5.0 with scores within the range 0 to 4.0 deemed acceptable for each sub scale. Scores of 4.1-5.0 were deemed at the extreme end of the scale and therefore participants were excluded at the screening phase.

### 2.2 Diet diary and analysis

All participants were requested to record their dietary intake at various time-points within the individual intervention studies. Diaries varied in length between 3 and 7 days (dependent on the requirements of the studies), which were always recorded on consecutive days, including one weekend day. Weighed diaries were deemed impractical therefore participants were requested to use household measures (i.e. teaspoon, slices), stating brand names and providing packets wherever possible. Diet diaries provided pictures of standardised portions of common food items to assist participants. Diaries were analysed using the dietary analysis program ‘WinDiets Research’ (2005 version, written by Dr A. Wise, Robert Gordon University, Aberdeen, Scotland).

Mean daily energy and macronutrient intakes (including fibre), plus the percentage energy intake derived from them were calculated at each time point for all subjects.

### 2.3 Sensory evaluation

A sensory evaluation is undertaken in order to assess, compare and differentiate between the organoleptic qualities of a selection of similar foods. In this case, the experimental breads used within the intervention studies were assessed against the closest comparisons available currently on the market. The tests described below were those deemed necessary in order to give the appropriate subjective and objective data required. Guidance for appropriate methodologies (Hedonic, Visual and Difference tests) was obtained by referring to the British Standard for Sensory Analysis methodology (BS ISO 6658:2005, British Standards 2005).

Study conditions were strictly adhered to including the isolation of participants in private booths. All study materials were kept white, i.e. the plates, cups, trays used for the samples, the participants' areas were kept white and plain. In order to allow the participants to focus on the evaluation questions,
environmental distractions were kept to a minimum. Each evaluation was completed in silence with both a minimum and a maximum time allowable to complete the evaluation. This was to ensure that participants engaged fully with the sensory evaluation, had sufficient time to focus on the questions and maintained concentration.

Fifteen male participants and fifteen female participants took part in the sensory evaluation for the rolls used in the High Risk and Low Risk studies with the summarised results detailed below. Average age for male participants was 44 ± 9.25 yr; with all participants in good health. Eighty seven percent of the male participants regularly included whole grains into their normal diet (60% on a daily basis, 27% several times weekly), with no reported dietary restrictions. Only one participant smoked and the same participant did not undertake regular exercise as opposed to the remainder of the participants.

Female participants reflected their male counterparts, with an average age of 47 ± 11.99 yr (no significant difference found for age between two gender groups – p=0.57), with no serious health concerns reported. Twenty percent of females reported self-imposed dietary restrictions, with only one participant reporting no regular intake of whole grains. Fifty three percent of participants consumed whole grains on a daily basis with the remaining 33% several times a week.

2.3.1 Hedonic test

For the Hedonic test, the participant was simply required to indicate, for each sample tested, which statement of like/dislike they most agreed with. Participants were given the following options: Like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much, dislike extremely. They were also asked to indicate specific reasons for their choices (see Appendix A.3 for the Hedonic test evaluation sheet).

Using the hedonic scale, when asked to specify to what degree the participants liked or disliked the individual rolls, the results were varied. With an n of 30, 67% of participants 'liked' the whole grain experimental rolls in comparison to 70% 'liking' the equivalent market version of the whole grain rolls. Conversely, for the milled grain rolls, the results were very different; 30% of participants 'liked' the experimental milled grain roll as opposed to 80% of the participants 'liking' the market equivalent. For the refined grain (control) rolls – both the experimental and market versions were 'liked' by 27% of participants.

2.3.2 Difference test

The purpose of the Difference test is to pinpoint, and thus have the opportunity to compare, the individual qualities of the samples, whilst also giving more qualitative comparative data for the samples.
Eight organoleptic qualities were selected for assessment. Participants were asked to rate each sample on the qualities selected, using a scale of one to nine. A score of 'one' represents the extreme of the quality being scored, a score of 'nine' represents the extreme opposite of the quality being assessed; for example a score of 'one' for saltiness indicates a very salty-tasting sample. Whereas a score of 'nine' would indicate a very bland-tasting sample. Whilst completing this section of the sensory evaluation, the participants were requested to focus on one sample and complete all eight questions before moving onto the next sample and then repeating the assessment (see Appendix A.4 for the Difference test evaluation sheet). In this case the difference tests showed that, when comparing the modal responses alone, there were only moderate differences in rated organoleptic properties of the experimental and commercial products. The most variation between experimental and market products can be seen for the control samples (refined grain) –Figure 2.3– with the milled (Figure 2.2) and whole grain (Figure 2.1) rolls appearing comparable on many qualities. From the graphs, overall for all types of roll, the market versions appear to be favoured slightly. However, when comparing the means of the market versus experimental rolls (for each category of roll), via a Wilcoxon Signed Rank Test, many qualities were significantly different (Table 2.1). There was also a higher degree of variability in the opinions of participants for the experimental whole and milled grain rolls due to a wider range of scores.

![Figure 2.1. Difference test - Market vs. Experimental whole grain products.](image)

Graph depicts the mode for each organoleptic quality for all participants, n=30. A score of 8 is the extreme end of the quality, i.e. extremely moist. Zero is the opposite to this extreme, i.e. dry. Key: MWG - Market whole grain product; EWG - Experimental whole grain product.
Chapter 2: Materials and Methods

Figure 2.2. Difference test - Market vs. Experimental milled grain products.
Graph depicts the mode for each organoleptic quality for all participants, n=30. A score of 8 is the extreme end of the quality, i.e. extremely moist. Zero is the opposite to this extreme, i.e. dry. Key: MMG – Market milled grain product; EMG – Experimental milled grain product

Figure 2.3. Difference test - Market vs. Experimental control (refined grain) products.
Graph depicts the mode for each organoleptic quality for all participants, n=30. A score of 8 is the extreme end of the quality, i.e. extremely moist. Zero is the opposite to this extreme, i.e. dry. Key: MCON – Market refined grain product; ECON – Experimental refined grain product
2.3.3 Visual test

The purpose of the Visual Analysis is to gauge the visual acceptability of the samples when they are part of a 'whole' sample. At this point each sample is randomly assigned a letter (in a different order to the Difference and Hedonic tests), ranging from A to F, the participants then have to rank the samples from the pictures provided to assess the extent to which the visual appearance of a food affects its acceptability. See Appendix A.5 for the Visual test evaluation sheet.

When asked to rank the six products from photographs of the whole roll (instead of the blinded sample they had been using for the tasting – see Appendix A.6 for pictures used), the market and experimental rolls were judged by visual preference in the following order – see below:

1. Experimental & market whole grain
2. N/A
3. Market milled grain
4. Experimental milled grain
5. Experimental refined grain (control)
6. Market refined grain (control)

It was therefore concluded from the results that even though the 'market equivalent' rolls had their obvious advantages in terms of taste and texture, the experimental rolls were still able to be comparable in enough respects to reassure that the use of the rolls in the intervention studies would not unduly affect compliance to the study.
<table>
<thead>
<tr>
<th></th>
<th>Whole grain <em>n</em>=30</th>
<th>Milled grain <em>n</em>=30</th>
<th>Control <em>n</em>=30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Market</td>
<td>Mode</td>
<td>Range</td>
</tr>
<tr>
<td>Moistness</td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
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<td>Chewy</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Hard</td>
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<td>6</td>
<td>5</td>
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<td>Brown</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Filling</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>After Taste</td>
<td>5</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.1: Modal results (with range) of Difference test scores for sensory evaluation of High Risk Study rolls.
2.4 Anthropometric measurements

The following set of methodologies described below are the anthropometric measurements taken for all intervention studies.

2.4.1 Height

Height was measured via a wall-mounted Harpenden Stadiometer (Holtain Ltd, Crymych, Dyfed). Participants stood with their shoes off, heels together, maintaining an up-right posture. Measurements were taken to the nearest 0.1cm.

2.4.2 Weight – Body fat percentage – Body Mass Index

Participants’ body mass and composition were measured using a Tanita TBF-300 Body Composition Analyser (Tanita Europe BV, Netherlands) in accordance with the manufacturer’s instructions. Prior to use the machine was set up for each individual participant; 1.5kg was deducted to take into account the weight of clothing, and information on their gender, age and whether they were ‘athletic’ or ‘normal’ build was also entered. Once set, participants were requested to remove socks and shoes and stand on the scales for approximately 15 seconds until the scales had stabilised and recorded a measurement. Each measurement included data on body fat percentage, total body water, fat free mass and estimated basal metabolic rate, with reference ranges for a healthy population.

2.4.3 Waist circumference – Hip circumference – Waist:Hip Ratio

Waist and hip circumferences were measured using a flexible tape measure, to the nearest 0.1cm. Measurements of waist circumference were taken directly around the navel; hip circumference was measured around the widest part of the hips using the same tape measure. Waist:Hip ratio (WHR) was calculated by waist measurement (cm) divided by hip measurement (cm).

2.4.4 Blood pressure

Participants rested in a supine position for 10 minutes in a quiet room. An OMRON 705IT Auto Arm Blood Pressure Monitor (Omron Healthcare, Kyoto, Japan) was then used to take the blood pressure from the non-dominant arm in accordance with the manufacturer’s instructions. This was repeated three times, with the mean systolic and diastolic blood pressure calculated from these readings.
2.5 Blood sampling

2.5.1 Venepuncture

A vacutainer system (Beckton Dickinson Diagnostics, USA) was used for all venepuncture by a trained individual. In accordance with the training provided by Charles Bloe Training (Venepuncture & Cannulation Course 2007) the procedure was as follows:

i) The participant was requested to lie in a comfortable supine position with a pillow placed underneath their non-dominant arm.

ii) A tourniquet was then applied to the arm, sufficient to partially occlude blood-flow to the arm in order to allow easier identification of the veins.

iii) A suitable site was identified and a Sterets Pre-injection swab - Isopropyl alcohol 70% v/v (Medlock Medical Ltd, Oldham, UK) was used to decontaminate the small area of skin.

iv) Once the alcohol had dried, a BD Vacutainer® Conventional Blood Collection Set 21G ‘butterfly’ (Beckton Dickinson Diagnostics, USA) was inserted into the vein with a BD Vacutainer® One Use Holder (Beckton Dickinson Diagnostics, USA) attached to the end to prevent needle-stick injuries. A ‘flashback’ of blood should appear into the attached catheter indicating the correct placement of the needle.

v) Once secured in place, the tourniquet was removed with the participant keeping their arm straight, the specific BD Vacutainer® blood tubes were used to collect the blood volume(s) required.

vi) Once blood collection was completed, the butterfly was removed followed by pressure being applied to the site using sterile gauze for approximately a minute – or until satisfied the site had ceased bleeding. A plaster was then applied and participants were invited to sit up slowly when they felt comfortable to do so.

vii) Refreshments were offered once the procedure had finished.

2.5.2 Cannulation

Cannulation was required for two intervention studies to allow for continuous blood sampling during post-prandial phases. Due to the more specialised nature of cannulation (as opposed to venepuncture), all participants were attended to by any one of three individual researchers, all of whom were highly-trained in the correct procedure of cannulation. All researchers worked in accordance with the training provided by Charles Bloe Training (as previously stated in Section 2.5.3) with the procedure as follows:
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i) The participant was requested to lie in a comfortable supine position with a pillow placed underneath their non-dominant arm.

ii) A tourniquet was then applied to the arm, sufficient to partially occlude blood-flow to the arm in order to allow easier identification of the veins.

iii) Once a suitable site was identified, a Sterets Pre-injection swab (Isopropyl alcohol 70% v/v (Medlock Medical Ltd, Oldham, UK)) was used to decontaminate the small area of skin.

iv) Once the alcohol had dried, a Y-Can 21G with syringe valve (Beldico, Belgium) was inserted into the vein and the inner guidance wire removed. Similar to the venepuncture, a 'flashback' should occur in the cannula to alert the researcher that it is correctly positioned within the vein and patent.

v) A BD Veca C I.V. dressing (Beckton Dickinson Infusion Therapy AB, Helsingborg, Sweden) was applied to cover the cannulated site, maintaining the clean procedure and to reduce the risk of infection.

vi) Blood was then withdrawn using a sterile syringe (BD® Plastipak™, Drogheda, Ireland) and once the appropriate volume had been taken, a 2ml bolus of sterile Macoflex 0.9% Sodium Chloride solution (Macopharma UK Ltd, Twickenham, UK) was flushed into the cannula to prevent it blocking.

2.5.3 Continuous venous blood sampling (post-prandial studies)

Once the cannula has been inserted and flushed with saline (as per Section 2.5.2), it was ready for continuous blood sampling using syringes. Dependent on study requirements, sampling was timed at 5, 15 or 30 minute intervals. At the first blood sampling post-insertion, a separate 2ml sample was withdrawn first, as this initial sample may contain saline not yet circulated into the venous system. If saline remains in the venous chamber, then this would inevitably compromise sampling and results as the blood sample would be diluted. The actual blood sample intended for analysis was then withdrawn and decanted into the appropriate tubes for processing. A 2ml sample of saline was then flushed into the cannula to maintain patency.

All subsequent blood sampling for the remainder of the intervention followed this protocol.

Upon completion of the intervention, the cannula was removed, with pressure and then a spot plaster applied at the site.

2.5.4 Oral Glucose Tolerance Test (OGTT)

The OGTT involves the participants consuming 75g of anhydrous glucose (Alwan, 1994) dissolved in 400ml of water, within the space of 10 minutes. With fasted blood samples already taken prior to consuming the glucose (T-10minutes, T-5 minutes and T0), further blood sampling of 5mls continues every 15 minutes for a further two hours.
2.5.5 Processing of blood samples

For all studies, except the Oral Glucose Tolerance Test (OGTT) within the Cross-Over Study, the following blood collection tubes were used (all supplied by Beckton Dickinson Diagnostics, USA).

- **BD Vacutainer® Ethylenediaminetetraacetic Acid (EDTA) tubes**
  - Collects: Plasma
  - Determinations for: TC, LDL-C, TAGs, NEFA, insulin
  - Preservative: Dipotassium Ethylenediaminetetraacetic Acid (K₂EDTA)
    - For 4ml draw tube – 7.2mg K₂EDTA
    - For 10ml draw tube – 18mg K₂EDTA

- **BD Vacutainer® SST™ tubes**
  - Collects: Serum
  - Determinations for: Inflammatory markers
  - Preservative: For 5ml draw tube – Clot activator/polymer gel

- **BD Vacutainer® Fluoride tubes**
  - Determinations for: Glucose
  - Preservative: For 4ml draw tube – Sodium Fluoride 10mg, Potassium Oxalate 8mg

For the OGTT, alternative blood collection tubes were used due to the quantities of blood collected at each time point. Samples were collected using the following tubes from Teklab (ML) Ltd (Co. Durham, UK).

- **Teklab® Dipotassium EDTA tubes**
  - Determinations for: TC, LDL-C, TAGs, NEFA, insulin
  - Preservative: 1.75mg per 1ml of blood (2.5ml tube)

- **Teklab® Fluoride Oxalate tubes**
  - Determinations for: Glucose
  - Preservative: 2.0mg Sodium Fluoride & 3.0mg Potassium Oxalate per 1ml of blood (2ml tube).

Once samples were decanted from the syringe into the assigned blood collection tubes (lids of tubes removed, vacuum broken for the BD tubes), the samples were processed as per the manufacturer’s instructions. The EDTA and fluoride tubes were centrifuged immediately at 1751 x g (3000rpm) for 10 minutes at 4°C (Labofuge 400R – Kendro Laboratory Products, Germany). The SST tubes were allowed to clot at room temperature for 30 minutes and then centrifuged as per the other samples.
Post-centrifugation, the samples were aliquoted using disposable pasteur pipettes (liquipette - Elkay Laboratory Products (UK) Ltd, Basingstoke, UK) into assigned 2ml Apex® Plus Screw-cap microtubes (Alphalabs, Hampshire, UK) at volumes of approximately 0.5ml per microtube.

The samples were promptly frozen at -20°C for plasma samples and at -80°C for serum samples.

2.6 Measurements of vascular function

2.6.1 Pulse wave velocity

The Pulse Wave Velocity (PWV) sessions ran to a standardised protocol and were implemented as described below for all studies. The protocol takes into account the recommendations of the First International Consensus Conference on the Clinical Applications of Arterial Stiffness (Van Bortel et al., 2002).

Prior to attending the study, the participant was asked to travel to the Clinical Investigation Unit (CIU) in a mode which would require the minimal level of physical exertion possible (i.e. bus or car). Upon arrival, the participant was taken to a quiet room and requested to lie in a supine position undisturbed (researcher not in the room) for 10 minutes to allow their breathing and heart beat to become regular and rested. Once completed, the researcher re-entered the room and from this point on there was minimal verbal communication from the participant to maintain the relaxed state (unless participant experiences discomfort).

Blood pressure was recorded three times on the non-dominant arm of the participant, and a mean blood pressure was then calculated. The route of the arteries was then traced and measured using a tape measure – see Figure 2.4.

As shown in Figure 2.4, starting at the carotid pulse and measuring down to the suprasternal notch (in mm), gave the proximal distance (yellow arrow). From the suprasternal notch, measuring down along the arm to the radial pulse point gave the distal measurement for the radial-carotid PWV readings (green arrow). For the carotid-femoral PWV, the distance between the suprasternal notch to the femoral pulse point was measured as the distal (blue arrow).
Figure 2.4. Mapping of main arteries used for measuring peripheral and central PWV. Green arrow – peripheral PWV, blue arrow – central PWV.

Figure 2.5. Example of initial set-up for a Radial-Carotid PWV recording. Prior to recording the PWV, the relevant data is required – proximal/distal measurements and blood pressure as a minimum.

A 3-lead ECG (electrocardiogram) was attached, followed by the mean blood pressure recording, plus the proximal and distal measurements entered into the PWV computer programme (SphygmoCor 2000 Version 7.1, AtCor Medical Inc. USA) –Figure 2.4.
Once the participant was comfortable (at this point one pillow should be supporting the participant’s neck and shoulders) and lying down with the ECG attached, recording commenced. The pulse points were recorded sequentially, with the data collated at the end into one report (Figure 2.6). For the radial-carotid recording, the radial pulse was assessed first followed by the carotid; for the carotid-femoral recording, the carotid pulse was measured first then femoral pulse. Each measurement involved the pulse point being located (wrist, neck or femoral), the PWV sensor was placed on top of the pulse point and held still for long enough to gauge whether the pulse was registering sufficiently (signal strength is indicated on the screen) to produce a valid result. Once a steady, consistent pattern of pulse waves was achieved and maintained for at least 10 seconds, the recording was saved. The researcher then progressed to the next pulse point; with data collection complete, a report was produced to indicate the PWV result for either the radial-carotid or carotid-femoral ‘route’ as appropriate. Duplicate recordings were always made to improve reproducibility within the intervention studies that used PWV as an outcome.

![Figure 2.6. A report obtained from a Radial-Carotid PWV recording.](image)

### 2.6.2 24-hour ambulatory blood pressure

For continuous blood pressure monitoring during the intervention studies, the ABPM-04 ambulatory blood pressure recorder was used (Meditech Ltd, Budapest, Hungary). This is necessary when compiling 24hr profiles of study participants’ blood pressure patterns for the purposes of monitoring the short-term effects of an intervention (Figure 2.7 gives typical output data available).
Automatic recordings of blood pressure were taken by the equipment half hourly between 06.00hrs and 22.00hrs and hourly between 22.00 and 06.00 (recommended by the British Hypertension Society). If necessary, participants were able to stop a recording during the inflation phase of the cuff, however they could not interfere with when a recording was due to be taken. Monitors were removed for bathing and extreme physical activity, although strenuous activity was discouraged for the 24hr-period of recording to minimise anomalies.

![Figure 2.7](image)

Figure 2.7. A typical download from a 24-hour ambulatory blood pressure monitor. Output contains the raw data displayed over time in both a tabular (a) and graphical (b) form

### 2.7 Laboratory techniques – Plasma and serum samples

#### 2.7.1 Insulin: Radio-Immuno Assay (RIA)

To quantify levels of insulin from all samples collated from the intervention studies, a commercially-available kit was sourced. Obtained from Millipore (Millipore Corporation, Billerica, MA, USA), the Human Insulin Specific RIA Kit (Cat. HI-14K) suitable for 250 tubes was used.
A $^{125}$I-labeled human insulin tracer antigen was incubated with equal amounts of a human insulin antibody plus an unlabeled antigen (intervention samples). Both labelled tracer and antibody had set concentrations, according to the manufacturer’s instructions.

The antibody had binding sites capable of binding to the labelled tracer (50%), and once the unlabeled antigen (sample) was added to the assay, there was competition for binding sites between the labelled and unlabeled antigens. As the concentration of the unlabeled antigens increased, the amount of labelled antigen bound to the antibody decreased.

To analyse samples, free tracer was removed and the radioactivity counted in the antibody-antigen fraction by a Wallac Wizard 1470 Gamma Counter (Perkin Elmer, USA).

For this specific kit, the range of quantitation was 2-200 µU/ml (12-1200pM), subject to the linearity of the standard curve subsequently produced. Precision of the assay was determined by the manufacturer, with inter-assay coefficient of variation (CV) stated as 2.9-6.0% and intra-assay variability as 2.2-4.4%. Quality controls did not fall beyond two standard deviations (SD). The mean inter-assay CV was 8.6%, with mean intra-assay CV of 8.3% for QC1 and 8.7% for QC2.

### 2.7.2 Lipid and glucose profiles: ILab 650

Plasma glucose, total cholesterol, HDL-cholesterol, non-esterified fatty acid (NEFA) and triglycerides were analysed using an ILAB 650 (Instrumentation Laboratory, Milan, Italy). The ILAB uses an enzymatic colorimetric method incorporating a sequence of step-wise reactions specific to the marker as described below.

**Principle of colorimetric method per marker:**

**Non-esterified fatty acid (NEFA) – FA 115 – Randox Laboratories, UK**

\[ \text{NEFA+ATP+CoA} \xrightarrow{\text{Acyl CoA Synthetase}} \text{Acyl CoA+AMP+PPI} \]

\[ \text{Acyl CoA+O}_2 \xrightarrow{\text{Acyl CoA Oxidase}} 2,3\text{-trans-Enoyl-CoA+H}_2\text{O}_2 \]

\[ \text{2H}_2\text{O}_2+\text{TOOS+4-AAP} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} +4\text{H}_2\text{O} \]

Quinoneimine quantified via spectrophotometry (absorbance = 550nm) and measured against the reagent blank. Mean intra-assay quality control data: QC1 CV = 1.6%; QC2 CV = 1.1%
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**Total cholesterol – IL Test™ 0018250540 – Instrumentation Laboratory, UK**

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol + fatty acids}
\]

\[
\text{cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-AA} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4\text{H}_2\text{O}
\]

Production of quinoneimine is proportional to sample total cholesterol concentration; quantified via spectrophotometry (primary wavelength - absorbance = 510nm, blanking wavelength - absorbance = 700nm). Mean intra-assay quality control data: QC1 CV = 0.0%; QC2 CV = 2.7%.

**Triglycerides – IL Test™ 0018255640 – Instrumentation Laboratory, UK**

\[
\text{Triglycerides} \xrightarrow{\text{lipoprotein lipase}} \text{glycerol + fatty acids}
\]

\[
\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{glycerophosphate oxidase}} \text{dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-chlorophenol} + 4\text{-AA} \xrightarrow{\text{glycerophosphate oxidase}} \text{quinoneimine} + \text{H}_2\text{O}_2
\]

Production of quinoneimine is proportional to sample triglyceride concentration; quantified via spectrophotometry (primary wavelength - absorbance = 510nm, blanking wavelength - absorbance = 700nm). Mean intra-assay quality control data: QC1 CV = 3.0%; QC2 CV = 1.7%.

**HDL-Cholesterol (two stage reaction) – CH 2655 – Randox Laboratories, UK**

1. Clearance of chylomicron, VLDL and LDL-cholesterol

\[
\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol + fatty acids}
\]

\[
\text{cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

2. Release of HDL-cholesterol by detergents

\[
\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol + fatty acids}
\]

\[
\text{cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-AA} + \text{HDAOS} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4\text{H}_2\text{O}
\]
Production of quinoneimine is proportional to sample HDL-cholesterol concentration; quantified via spectrophotometry - absorbance = 600nm. Mean intra-assay quality control data: QC1 CV = 0.7%; QC2 CV = 3.1%, QC3 CV = 1.9%

**Glucose – IL Test™ 0018250840**

\[
\beta-D-Glucose + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2
\]

\[
2H_2O_2 + \text{phenol} + 4-AAP \xrightarrow{\text{Peroxidase}} \text{red quinoneimine} + 4H_2O
\]

Production of quinoneimine is proportional to sample Glucose concentration; quantified via spectrophotometry - absorbance = 510nm. Mean intra-assay quality control data: QC1 CV = 1.6%; QC2 CV = 0.6%.

**Key:** 4-AAP = 4-aminoantipyrine,
TOOS = N-ethyl-N-(2hydroxy-3-sulphopropyl)m-toluidine,
HDAOS = N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline.

### 2.8 Cell culture techniques and analysis

#### 2.8.1 Extraction and activation of monocytes from whole blood

During the *Cross-Over Study*, 10ml of whole blood was collected at baseline to enable the extraction of monocytes. The blood was collected in accordance with the method in Section 2.5.3, using a 10ml BD Vacutainer® EDTA tube as described in Section 2.5.4.

The blood was kept at room temperature and immediately transported to the cell culture laboratory within FHMS. All further procedures were carried out at room temperature and within the cell culture hood to minimise contamination.

10ml of Histopaque 1.077g/litre (Sigma-Aldrich, UK) was pipetted into a 25ml centrifuge tube and allowed to equilibrate to room temperature. 10ml of the whole blood was layered carefully on top using a 10ml pipette. The tube was then centrifuged at 400xg for 30 minutes at room temperature.

After centrifugation, the upper layer was removed to within 0.5cm of the opaque interface and discarded. Into a new centrifuge tube, the opaque interface was transferred and mixed (via gentle aspiration) with 10ml of sterile, filtered phosphate-buffered saline (sfPBS). The mixture was then centrifuged at 250xg for 10 minutes.
After centrifugation, the supernatant was discarded and the remaining pellet re-suspended with 5ml sfPBS. The suspension was then centrifuged again at 250xg for 10 minutes. This was then repeated.

After the final centrifugation, the supernatant was discarded. The pellet was re-suspended with 5ml of cell culture media – RPMI (Roswell Park Memorial Institute Medium, Lonza UK). The RPMI 1640 medium was supplemented with 2.0g/l sodium bicarbonate, 2mM L-glutamine, 50mls heat-inactivated fetal bovine serum, 5.5ml penicillin-streptomycin (100μg/ml). The suspension was then distributed evenly into a six-well plate (Nunclon, Invitrogen, UK) and left to incubate at 37°C for 90 minutes to allow the monocytes to adhere. Post-incubation, the media was removed from all the six wells and discarded. The wells were then washed once with 1ml of sfPBS (room temperature) each.

1ml of fresh RPMI media was then added to each well, followed immediately by 5μl of lipopolysaccharide (LPS) to each well. LPS concentration was 1μg/ml. Lipopolysaccharide obtained via phenol extraction from \textit{E. coli} serotype O55:B55 and sourced from Sigma, catalogue # L 2880 (Sigma, MI, USA.) After 1 minute, the entire media/LPS mixture was removed from two wells and immediately frozen down into four aliquots (A, A', B, B') at -80 °C. The plate (with four remaining wells) was then incubated at 37°C for 48hrs.

At 48hrs, the entire media/LPS mixture was removed from two wells and immediately frozen down into four aliquots (A, A', B, B') at -80 °C. An additional 5μl of LPS was then added to the remaining two wells and incubated at 37°C for a further 24hrs. At 72 hrs post-monocyte extraction, the final two wells of media/LPS were removed and frozen down as described above.

2.8.2 Tumour necrosis factor-α ELISA

The supernatants retrieved from the activation of monocytes (process detailed in Section 2.8.1) were analysed for levels of secreted tumour necrosis factor-α (TNF-α) using a validated in-house sandwich Enzyme-Linked Immunosorbent Assay (sELISA) according to the following protocol:

96-well plates (Maxisorb, Nunc, Invitrogen, UK) were coated with 0.25μg/ml mouse anti-human TNF-α (BD-Pharmingen MAb1 551220), in 0.1M Na₂HPO₄ - pH9.0 at 50μl/well and incubated overnight at 4°C. The first three wells (if triplicate samples used) were coated with buffer alone.

Recombinant human TNF-α standards were made up in PBS-BSA (2%) and diluted initially to 20ng/ml followed by a 1/3 serial dilutions until 10pg/ml. Standards were plated in triplicate, 50μl/well.
Supernatents were diluted from 1/3 to 1/10 and added in triplicate, 50μl/well and the plates were incubated overnight at 4°C.

The plates were washed five times with PBS-T-BSA (0.2%) and biotinylated mouse anti-human-TNF-α (BD Pharmingen, MAb11 554511 diluted in 2% PBS-T-BSA) was added at 1μg/ml, and plated at 50μl/well and incubated for 1 hr at room temperature. The plates were then washed five times using PBS-T-BSA (0.2%).

Avidin-HRP (Dako, Denmark, diluted in PBS-T-BSA (2%)) was added at 2μg/ml, plated at 50μl/well and incubated for 30 minutes at room temperature. The plate were then washed three times with PBS-T, followed by three washes with PBS and three washes with 0.05M citrate phosphate buffer pH 5.0 (Sigma, UK).

Colour was developed using 0.1mg/ml tetramethylbenzidine dihydrochloride (TMB) in 0.05M citrate phosphate buffer (pH5.0) with 2μl of 30% H₂O₂ and plated at 50μl/well. Colour was allowed to develop for at least 20 minutes, and the reaction was stopped with 12.5μl/well of 2M H₂SO₄.

The plate was read at 450nm using a BioTek® ELx800 absorbance microplate reader (BioTek, USA).

In-house evaluation found a coefficient of variation of between 5-10% for both intra and inter-assay results.

2.8.3 Inflammatory markers: Luminex®

To quantify levels of a series of inflammatory markers, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α and MCP-1, commercially available multiplex bead immunoassays were used to analyse samples from the High Risk Study and the Cross-Over Study. The Extracellular Protein Buffer Reagent Kit (Cat.# LHB0001) and the following marker-specific kits were obtained from Invitrogen (Invitrogen Corporation, Camarillo, CA, USA); MCP-1, Human MCP-1 Antibody Bead Kit (Cat.# LHC1011); Cytokines, Human Ultrasensitive Cytokine Ten-Plex Antibody Bead Kit (Cat.# LHC6004).

The principle of the procedures followed that of a four-stage, solid-phase protein assay. Briefly, beads that have been encoded for a specific spectral range and which are also conjugated with an antibody that is analyte-specific, were incubated with the appropriate samples. For multiple markers to be analysed, the appropriate representative beads were present in each well of the assay microplate. Once incubated, with the analyte immobilised and bound to the capture antibody, detector antibodies were applied. The final step was to expose the protein complex to fluorescence via the use of Streptavidin-R-Phycoerythrin.
The microplate was then ready for analysis using a Luminex® 100™ or 200™ machine. Samples were analysed both for the spectrum of the bead but also concentration of fluorescence. The data then translates into quantities of the inflammatory markers being investigated.

Variation within the assays are quoted by the manufacturer as 5.8% for both intra and inter-assay for the MCP-1 assay. For the ten-plex assay, the inter-assay coefficient of variation ranged from 4.4%-8.6% depending on the marker.

2.9 Statistical analyses

Statistical analysis was carried out using SPSS Version 16 (SPSS Inc., Chicago, USA) with statistical significance assumed at p<0.05 unless stated otherwise. Normality of distribution was assessed for all variables, in all studies, via the Kolmogorov-Smirnov test. A significant result from this test indicated non-normally distributed data, in which case non-parametric statistics were employed. In addition, if n <10, non-parametric tests were felt to be justified and appropriate. It was not deemed necessary to transform any data prior to analysis. Where relevant, the specific statistical analysis is detailed within the methods section of each chapter.
Chapter 3

3 Pulse Wave Velocity Validation

3.1 Introduction

3.1.1 Background to Pulse Wave Velocity

Pulse Wave Velocity (PWV) is used as an indicator of arterial stiffness, which in turn is a strong predictor of CVD risk for both healthy and ‘unhealthy’ individuals (Safar et al., 2002c, Mule et al., 2006, Laurent et al., 2001, McEniery et al., 2006).

Arterial stiffness is an important index for assessing the CVD risk of an individual, especially as research has shown that many factors can affect this, including age (Cameron et al. 2003) and the metabolic syndrome (Mule et al. 2006).

Mule et al. (2006) found that subjects with hypertension and metabolic syndrome had a significantly higher age-adjusted PWV than those without metabolic syndrome. Safar et al. (2006) also concluded that metabolic syndrome had a significant effect on PWV and that this could be used as a predictor of CVD risk. PWV has been positively linked to high sensitivity-CRP (Kim et al., 2007), body fat repartition (Czernichow et al., 2005), triglyceride levels, HDL Cholesterol and fasting glucose (Tomiyama et al., 2005).

Aside from the aforementioned associations, PWV has been cited as a strong independent predictor of CVD risk by a growing number of research groups (Blacher et al., 1999, Willum-Hansen et al., 2006). Laurent et al. (2001) recorded Carotid-Femoral PWV (C-F PWV) as part of a longitudinal study assessing CVD risk in individuals with essential hypertension, with mortality due to CVD (in addition to all-cause) as a main outcome. Over the course of the study, 43% of deaths were CVD related and when this was taken into account with PWV in a univariate model of logistic regression, it was found that PWV was significantly related to both CVD-related and all-cause mortality. Furthermore, within a multivariate log regression model, PWV was found to again be significantly associated with mortality (both all-cause and CVD-related) independent of age, diabetes and history of CVD-related illness (Laurent et al. 2001).

The evidence base appears to support the use of PWV within a global framework of assessing CVD risk. Specifically for this programme of research, a measure of arterial stiffness is an extremely useful
Chapter 3: Pulse Wave Velocity Validation

tool in assessing the influences on health and endothelial function. The outcome of this study (i.e. the reproducibility of PWV) will be used to validate the results of future dietary intervention trials that will be implementing PWV as a primary outcome.

3.1.2 Current validation evidence

Within the current literature, there is only a small body of work attempting to set a reference range for PWV – many studies using PWV as an outcome have different cut-offs to differentiate between stages of CVD. For example, Van Popele et al. (2000) used 14m/s as their cut-off to define ‘stiff’ and ‘non-stiff’ populations whereas Blacher et al. (1999) states that 13m/s is the most appropriate cut-off when detecting the degree of atherosclerosis present. Khoshdel et al. (2006), recognising the discrepancies between research groups, undertook a review in order to estimate a reference interval (RI) for PWV that also took into account age, however the author does state that further work is required with the RI to test it against long-term prospective studies in order to be assured that it accurately reflects CVD risk.

Furthermore, there is also limited specific data on the reproducibility of PWV. Despite varying objectives and participant numbers across previous studies, the level of reproducibility appears to be similar. A leading article by Liang et al. (1998) compared a range of methods for detecting arterial stiffness (including PWV) on 50 healthy participants (20M, 30F), aged 20-70yrs. Each participant attended twice, with the visits separated by 2-3 weeks. For the entire subject group, the PWV results were not significantly different between visit 1 and visit 2. The intra-individual CV was 3.2% and both visits were strongly correlated (r=0.93). Of the eight separate measurements of the vasculature tested in this paper, PWV had the most favourable outcome in terms of reproducibility, alongside the techniques of detecting intima-medial thickness (via ultrasound) and augmentation index (via pulse wave analysis, a similar technique to PWV).

Woodman et al. (2005) took a different approach to PWV validation by comparing 15 men with established coronary artery disease (CAD) with 15 healthy men over a range of different techniques designed to assess vascular health (including C-F PWV and R-C PWV, stroke volume/pulse pressure, stiffness index plus systemic, large and small artery compliance). Participants attended under controlled conditions (i.e. overnight fast, same time of day) for two visits, one week apart. The results are starkly different to those of Liang et al. (1998); CVs for the CAD group for R-C PWV were 10.7% and for C-F PWV 7.1%. Healthy subjects showed even more variability, with an R-C PWV CV of 10.9% and a C-F PWV CV of 7.4%. For all 30 subjects, the overall CV for R-C PWV was 12.3% and the overall CV for C-F PWV was 7.6%.

The differences shown by the two studies above present challenges when interpreting data from intervention studies such as those in the SLOWCARB programme, as a consensus as to the expected
reproducibility of this method does not appear to have been reached, even though it is stated to be the gold standard in assessing arterial stiffness (Stehouwer et al. 2008). Due to the in-built quality controls and indicators within the PWV recording software, the error apparently found between results should not be heavily influenced by the operator. Therefore the issue of reproducibility should be focussing on ascertaining how much an individual participant varies in arterial stiffness over time, as well as collating data to understand fluctuations within a population group.

At present, reproducibility of PWV appears to have been investigated only at a ‘local level’, meeting the needs of the individual researchers (such as validating new equipment). Therefore, further research is required to provide meaningful data that may be extrapolated to other intervention studies.

3.1.3 Justification for study
PWV is a widely used technique for establishing an individual’s risk of cardiovascular disease (Safar et al., 2002b). Studies planned within the SLOWCARB project and other nutrition-intervention studies employ this technique as a means of evaluating the effects that whole grains and other fermentable carbohydrates may be having on the function of the endothelium, both acutely and in the longer term.

The current literature shows that accurate readings are attainable from PWV, however, it is desirable to internally validate the technique to define the reproducibility of PWV for the SLOWCARB and future studies. Therefore this is an exploratory validation study designed to test the reproducibility of PWV in a variety of controlled conditions in order to reproduce typical study conditions and normal metabolic processes (fed vs. fasted, sedentary vs. active). This should allow for the natural variation in arterial stiffness within the study population to be assessed, allowing the findings from the intervention studies to be interpreted appropriately.

3.1.4 Aims and objectives
The aim of this study was to assess the impact of typical study conditions on the reproducibility of Pulse Wave Velocity, justifying its use as a robust and appropriate tool for assessing arterial stiffness of individuals identified as high or low risk of CVD.

The purpose of the study design was two-fold: firstly to establish the reproducibility of PWV (both peripheral and central) in a group of men at varying levels of cardiovascular disease risk; secondly to investigate (in the same group of men) the impact of metabolic/physical states on PWV, specifically: fasted versus post-prandial; pre and post-exercise.

The main study objectives were to:

- Evaluate the intra-individual reproducibility of PWV both within and between sessions (6 sessions over a time frame of 6 weeks).
- Explore the level of inter-individual variation in both peripheral and central PWV.
• Explore the relationships between PWV and known risk factors that can influence arterial stiffness, i.e. age and blood pressure.
• Evaluate the effects of acute, moderate-intensity exercise on PWV and blood pressure.
• Evaluate the effects of the early post-prandial phase on PWV and blood pressure.

3.1.5 Hypothesis
It was expected that intra-individual variability would not exceed a coefficient of variation (CV%) of greater than 10% per participant for both within and between the sessions (over time).

When comparing the rested state to post-exercise, and the fasted state to post-prandial, it was expected that there would be significant differences between the mean results for systolic and diastolic blood pressure but not for R-C PWV and C-F PWV.

3.2 Study design and methodology
3.2.1 Study design
The study was an exploratory validation study. Participants were recruited to closely reflect the criteria used for the SLOWCARB nutrition intervention studies (High Risk Study (Chapter 4), Low Risk Study (Chapter 5)).

Participants attended six sessions held at regular intervals over a period of between 4 and 6 weeks. Each session involved the initial recording of repetitive PWV – six repetitions of RC-PWV and then a further six of C-F-PWV. The participants then consumed either a standardised breakfast or undertook their allocated activity (see Section 3.2.3.2 below), followed by duplicate recordings of both RC-PWV and C-F-PWV.

3.2.2 Participant selection and recruitment
As this validation study was exploratory in nature, with no similar study design evident in the literature, a power calculation was not possible at this stage.

Prior to commencing, the study received a favourable ethical opinion from the University of Surrey Ethics Committee – EC/2007/75/FHMS.

To participate in the PWV validation study, participants were required to be age, gender and weight matched to the two target study groups that were planned to be used within the other research projects comprising the SLOWCARB Project.
Chapter 3: Pulse Wave Velocity Validation

Group 1 – High CVD risk

<table>
<thead>
<tr>
<th>Group 2 – Low CVD risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender: Male</td>
</tr>
<tr>
<td>Age: 31-55yrs</td>
</tr>
<tr>
<td>BMI: 26-35kg/m²</td>
</tr>
<tr>
<td>Waist circumference: &gt;37inches</td>
</tr>
</tbody>
</table>

The participants were recruited from the University of Surrey campus via global email and posters. Information regarding the prospective participants was obtained via the Health & Lifestyle questionnaire detailed in Appendix A.7, with a standard exclusion criteria applied as found in Chapter 2.1.3. Prospective participants were also required to undertake a self-assessment of exercise tolerance.

Prior to attending the screening session (at the CIU, Faculty of Health and Medical Sciences) prospective participants received written information detailing the study and a consent form for the study. Informed consent was obtained after the details of the study were explained to them (with an opportunity for queries to be answered). Participants were then requested to complete the Health & Lifestyle and food frequency questionnaires described in Sections 2.1.3.

A sitting blood pressure and heart rate measurement was then recorded along with weight and body mass index. If the participant met the inclusion criteria (dependent on their age group) they were admitted onto the study with an initial study session arranged.

3.2.3 PWV protocol

The methodology of a PWV session follows a standardised protocol as described in Section 2.6.1.

3.2.3.1. The PWV Session preparation:

Participants were requested to consume only minimal alcohol or caffeine for the 24hrs before their study sessions. They also avoided vigorous exercise and consumed a standardised evening meal (cheese and tomato pasta meal, followed by fruit yoghurt – see Section 3.2.4). On the morning of the PWV session, participants were requested not to walk/cycle at a fast, strenuous pace to the Clinical Investigation Unit.

Over the course of a maximum of 6 weeks, participants completed 6 sessions (1 per week) in a randomised order of 3 exercise and 3 breakfast sessions.
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3.2.3.2. Exercise sessions:

i) Early morning - Participants attended the CIU in a fasted state on the morning of the study session having followed the instructions stated above. The preparation of the participant for the PWV study session was completed as described in Section 2.6.1.

For peripheral PWV, the radial pulse was measured first, followed by the carotid to complete one full peripheral PWV recording. For the central PWV, the carotid pulse was measured first, followed by the femoral pulse to complete one full central PWV recording. For first stage of each study session, six peripheral PWV and six central PWV recordings were completed. Once the first 12 sets of data were obtained, the ECG leads were detached. Blood pressure on the non-dominant arm was then taken in triplicate, followed by the participant being asked to embark on the exercise task as follows.

ii) Exercise task - Complete 100 steps using a standard aerobics step in the following manner:

- One basic step consisted of stepping first one foot then the other on top of the step, and then stepping the first foot and then the other back to the floor.
- The participant was expected to complete approximately 25-30 steps per minute minimum; therefore 5 minutes was allowed for completion of the required number of steps. This was to ensure that the heart rate was raised (within safe levels) sufficiently to induce changes in blood pressure and therefore any resulting fluctuations in PWV that can be assumed to occur naturally as a consequence of a change in activity level.

Once the exercise task was completed, the participant took up a supine position, where blood pressure was taken again in triplicate, the ECG leads re-connected and the PWV repeated in duplicate (peripheral then central). Once completed the participant was offered refreshments and allowed to leave the Unit.

3.2.3.3. Breakfast sessions:

i) Early morning - As per the ‘Exercise session’, participants followed the initial protocol in an identical manner to provide the preliminary 12 sets of PWV data.

ii) Breakfast - They were then offered a standardised breakfast of which they had to consume all the food and drink items provided (see Section 4.2.4 for details of dietary items used).

Once the participants had finished breakfast, they were free to leave or stay within the unit (whichever most convenient), but they were required to return to the unit within 1 hour of consuming breakfast. Participants were permitted to undertake only light-sedentary tasks during the 1 hour period. Once they had returned to the unit, the session was completed by a triplicate recording of blood pressure followed by duplicate recordings of radial-carotid and carotid-femoral PWV. The participant was then free to leave the unit.
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3.2.4 Dietary items

The evening prior to each study session, participants were required to consume a cheese and tomato pasta meal with a fruit yoghurt for dessert. To aide standardisation of the process, for the first visit, each participant was provided with 1kg of cooked pasta (mixed with tomato-based pasta sauce, vegetable oil and cheddar cheese) and requested to eat the pasta at one sitting until comfortably full. The next day (first session) the participant returned the left-over pasta for weighing. The amount of pasta that the participant had eaten the previous night was then calculated.

For example, a participant returning 300g leftovers was assumed to have eaten 700g pasta (cooked weight) and this was subsequently set as the amount to be eaten by the participant before each session. For the next 5 sessions, the participant would be provided the same amount of pasta as they had eaten on their first visit and expected to eat all of it. The tomato and cheese recipe was chosen due to its homogeneity and relatively high acceptability with respect to dietary restrictions that participants may have (e.g. vegetarian). Table 3.1 provides the ingredients and nutritional breakdown for both the pasta meal and fruit yoghurt consumed by the participants prior to each study session.

Table 3.1. Ingredients, quantities and nutritional breakdown of the pre-PWV session standard meal – uncooked values

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g)</th>
<th>Energy (kJ)</th>
<th>Protein (g)</th>
<th>CHO (g)</th>
<th>Fat (g)</th>
<th>Fibre (g)</th>
<th>Na (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ragu Original Pasta Sauce</td>
<td>500</td>
<td>950</td>
<td>8</td>
<td>29</td>
<td>9</td>
<td>0.5</td>
<td>2.25</td>
</tr>
<tr>
<td>Tesco Fusilli Pasta (dry weight)</td>
<td>400</td>
<td>6020</td>
<td>50</td>
<td>292</td>
<td>5.6</td>
<td>10.4</td>
<td>trace</td>
</tr>
<tr>
<td>Tesco English Mild Cheddar</td>
<td>100</td>
<td>1620</td>
<td>25.5</td>
<td>0.1</td>
<td>32</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Tesco Pure Vegetable Oil</td>
<td>30</td>
<td>1110</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1030</strong></td>
<td><strong>9700</strong></td>
<td><strong>83.5</strong></td>
<td><strong>321.1</strong></td>
<td><strong>76.6</strong></td>
<td><strong>10.9</strong></td>
<td><strong>13.85</strong></td>
</tr>
<tr>
<td>Tesco finest fruit yoghurt*</td>
<td>150</td>
<td>795</td>
<td>5.1</td>
<td>19.2</td>
<td>10.0</td>
<td>1.8</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note: Once cooked, the total weight would come to ~2500g, therefore participants were consuming ~28-40% of the total values given for the macronutrient content.

For the breakfast sessions (Section 3.2.3.3), participants were required to consume two 50g croissants with 10g of polyunsaturated margarine, 30g of strawberry jam and 250mls of orange juice. This provided 4170kJ (988 kcals), 176.4g of carbohydrate providing 67.6% of energy (138.4g sugars) and 29.2g of fat providing 25.9% of energy (SFA 11.7g, 10.3% energy; MUFA 7.5g, 6.6% energy; PUFA 5.7g, 5% energy).

3.2.5 Statistical analysis

Detailed statistical analysis for this study focused on the intra and inter-individual variability of PWV.

Data was assessed for normality prior to full analysis using the One-sample Kolmogorov-Smirnov goodness-of-fit test. Any significant changes in PWV recordings within-participant (i.e. over the course of the six visits) were calculated using one-way repeated measures ANOVA and between-participant differences detected via one-way ANOVA.
Paired t-tests (or the non-parametric equivalent if appropriate) were used to compare means for fasted/rested versus fed/active PWV recordings.

An independent samples t-test was used to detect any significant differences between the two groups of participants (divided by CVD risk category) for baseline anthropometrics and blood pressure.

### 3.3 Results

#### 3.3.1 Anthropometrics

Participants were recruited to represent a cross-section of men at different levels of CVD risk and to also reflect those involved in the SLOWCARB interventions (see Sections 4, 5 & 6). In Table 3.2 the data is split into ‘high’ and ‘low’ CVD risk groups, both groups clearly match those found in the intervention studies (typical recruitment criteria found in Section 3.2.2.). Eight male participants, aged 23-50yrs and with a body mass index ranging from 20.4 - 32.8kg/m² completed the study and these were age and gender-matched for the SLOWCARB intervention studies (see Chapters 4, 5 and 6).

The full cohort of five young men completed the study to represent the low risk CVD group. However for the high CVD risk group, one participant withdrew from the study before recording any results and another participant withdrew at the half-way point due to difficulties in meeting the necessary time commitments. The data from the participants who withdrew has not been included in the final analysis.

The ‘Risk’ groups were significantly different from each other for age ($p=0.04$) and weight ($p=0.03$). There were no other significant differences between the groups for anthropometric measures at baseline.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>High Risk ($n=3$)</th>
<th>Low Risk ($n=5$)</th>
<th>Total ($n=8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>39.3 ± 9.7*</td>
<td>25.8 ± 2.7*</td>
<td>30.9 ± 9.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>95.4 ± 21.6^</td>
<td>79.8 ± 7.5^</td>
<td>85.7 ± 15.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.5 ± 6.2</td>
<td>23.8 ± 2.5</td>
<td>25.1 ± 4.0</td>
</tr>
<tr>
<td>BF (%)</td>
<td>27.1 ± 10.3</td>
<td>16.3 ± 3.9</td>
<td>20.4 ± 8.4</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>103.6 ± 18.2</td>
<td>89.3 ± 6.2</td>
<td>94.6 ± 13.1</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>125.7±8.1</td>
<td>124.8±5.9</td>
<td>125.1±6.2</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71.0±8.2</td>
<td>70.8±10.4</td>
<td>70.9±9.0</td>
</tr>
</tbody>
</table>

Note: All participants fitted the recruitment criteria dependant on risk category. Significant differences between the two groups was detected for both weight ($p=0.03$) and age ($p=0.04$). Key: BMI – Body mass index, BF – body fat, WC – waist circumference
3.3.2 Exploring the reproducibility of Pulse Wave Velocity via Coefficients of Variation (CV)

PWV was determined by calculating intra and inter-session CVs (Table 3.3)

- Intra-session coefficient of variation (CV) - A CV was calculated for each study session the participant completed. From those six separate study session CVs, a mean CV was calculated.
- Inter-session CV – From each study session (which would produce 6 recordings of R-C PWV and 6 of C-F PWV) a mean PWV result was calculated. The CV was then calculated from the average of the six mean results.

When comparing risk groups, no significant differences were found for either the intra-session CVs, or for the inter-session CVs. This applied to both R-C PWV and C-F PWV, see Table 3.3.

<table>
<thead>
<tr>
<th></th>
<th>INTER-SESSION CV (%)</th>
<th>MEAN INTRA-SESSION CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RC</td>
<td>CF</td>
</tr>
<tr>
<td>HIGH</td>
<td>5.0</td>
<td>4.4</td>
</tr>
<tr>
<td>LOW</td>
<td>5.5</td>
<td>4.5</td>
</tr>
<tr>
<td>GROUP</td>
<td>5.3</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Note: No significant differences were detected when comparing between the high and low risk groups for either inter-session or intra-session CV.

3.3.3 Pulse Wave Velocity (PWV) – the fasted and rested state

Figure 3.1(a) & (b) shows that there were no significant differences detected between groups for the mean results of the six study sessions for R-C PWV and C-F PWV.

For R-C PWV (Figure 3.1 (a)), the high risk group had a mean result of 8.1m/s±0.49SD compared to the low risk group mean result of 7.6m/s±1.26SD. For C-F PWV (Figure 3.1 (b)), the high risk group had a mean result of 6.5m/s±0.18SD compared to the low risk group result of 6.2m/s±1.14SD. The high risk group has consistently higher PWV results both for both R-C and C-F PWV.

Figure 3.2(a) & (b) shows the mean results (±SD) for each study session undertaken, six sessions in total over a maximum of 6 weeks. There were no significant changes over time between study sessions for the whole cohort. As found previously, the high risk group had consistently higher PWV values.

Figure 3.3 shows the individual PWV data for each of the six sessions. Two participants showed significant differences with time for the C-F PWV results (Figure 3.3(a)&(c) $p=0.05$ (F=222.52) and $F=2233.87, p=0.02$ respectively).
3.3.4 The effect of exercise and the post-prandial state on PWV and blood pressure

(i) Exercise

When comparing the mean SBP obtained for the cohort at the fasted/rested state (mean SBP 123.88mmHg±8.27SD) versus the mean SBP post-exercise (142.92mmHg±9.74), there was a significant increase (p<0.001). For the same comparison but for diastolic blood pressure (DBP), no significant difference was detected due to only a small mean increase between the two states (fasted/rested DBP 71.42mmHg±7.63, post-exercise DBP 72.63mmHg±8.21).

(ii) Post-prandial

When comparing SBP for the fasted/rested state against the SBP for the post-prandial phase, there was a significant increase from 123.38mmHg±9.61 (fasted/rested) to 126.13±10.25 (post-prandial) – p=0.05. There was also a significant difference between fasted/rested DBP (69.17mmHg±8.28) and post-prandial DBP (67.00mmHg±9.23) – p=0.03.

However, Figure 3.4(a) &(b) shows that there was no such influence of the post-prandial state and exercise on R-C or C-F PWV. No significant differences were found within the full cohort (or for the two ‘risk’ groups) when comparing between treatments and the fasted/rested state.
Figure 3.1. (a) Mean Radial-Carotid and (b) Mean Carotid-Femoral PWV results for the six study sessions, comparing high vs. low CVD risk groups

Note: No significant differences were detected between the high and low CVD risk groups. HR \( n=3 \), LR \( n=5 \), values represented as mean±SD, each data point is the mean result from six recordings (i.e. one study session). Key: ALL – all participants’ data included \( n=8 \); HR – High risk group; LR – Low risk group.
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Figure 3.2 (a) Mean Radial-Carotid and (b) Mean Carotid-Femoral PWV results per study session compared over time.

Note: No significant differences were detected over time for either the high or low CVD risk groups, or the combined cohort ('ALL'). HR \( n=3 \), LR \( n=5 \), ALL \( n=8 \), values represented as mean±SD, each data point is the mean result from six recordings (i.e. one study session). Key: ALL - all participants' data included; HR - High risk group; LR - Low risk group.
Figure 3.3(a-d). Individual participant mean PWV results (R-C and C-F PWV) per study session compared over time

(The participants represented by graphs (a) and (c) had significant differences over the time-course of their respective C-F PWV study session results – (a) p=0.05, (c) p=0.02. No other significant changes were detected for any other individual's results for both R-C and C-F PWV. Values represented as mean±SD, each data point is the mean result from six recordings (i.e. one study session). Graphs a, g and h are 'high risk' participants; graphs b-f represent those at 'low risk'.)
The participants represented by graphs (a) and (c) had significant differences over the time-course of their respective C-F PWV study session results — (a) p=0.05, (c) p=0.02. No other significant changes were detected for any other individual’s results for both R-C and C-F PWV. Values represented as mean±SD, each data point is the mean result from six recordings (i.e. one study session). Graphs a, g, and h are ‘high risk’ participants; graphs b-f represent those at ‘low risk’.

Figure 3.3(e-h). Individual participant mean PWV results (R-C and C-F PWV) per study session compared over time
Figure 3.4 (a): Mean Radial-Carotid and (b) Mean Carotid-Femoral PWV results comparing fasted/rested to post-exercise and post-prandial.

Note: No significant differences were detected over time for either the high or low CVD risk groups, or the combined cohort ("ALL"). HR n=3, LR n=5, ALL n=8, values represented as means±SD. Key: ALL – all participants’ data included; HR – High risk group; LR – Low risk group; F/R – Fasted and rested state; EX – Post-exercise state; BK – Post-breakfast state (post-prandial).
3.4 Discussion

3.4.1 Key findings

The main aim of this study was to assess the reproducibility of PWV within typical study populations and conditions as found in the SLOWCARB dietary intervention studies (i.e. different levels of CVD ‘risk’).

As PWV was to be used as a primary end point to assess CVD risk in all aspects of the SLOWCARB studies, it was important to firstly determine the reproducibility of this measurement intra-individually via the use of coefficients of variation. The effects of time and whether PWV significantly varies over the six-week intervention period is also a key data-set that could have implications for longer-term dietary intervention studies.

In addition, the data obtained as part of the post-exercise, post-prandial states is especially important as when participants take part in dietary intervention studies they should arrive at the study session fasted and having used the minimal amount of physical exertion (i.e. taken the bus/car instead of brisk walking/cycling). However this is sometimes not always the case and the participants may not report these deviations from the protocol if they occur. Therefore to have data available reflecting three different metabolic states will prove invaluable information when analysing the PWV data retrieved in the SLOWCARB interventions.

Considering these study objectives, the overall study design is one which is novel within the current literature examining PWV reproducibility. As already described in Section 3.1.2, there are very few studies that focus on the validation of PWV and none that appear to take the approach that this study has, which was to clearly define how PWV (both peripherally and centrally) may vary both within the scenario of a study session and over time, in addition to metabolic changes such as being fed or post-exercise.

The indication that PWV can vary from 6.9% (Mean intra-session CV for LR, HR and group C-F PWV) up to a maximum of 10.1% (Mean intra-session CV for Low Risk R-C PWV) within a timeframe of 15 minutes (the approximate time it takes to record the six measures of R-C or C-F PWV) is an extremely important factor to take account of when conducting PWV within the time constraints of a dietary intervention study session. In this sort of scenario, time is limited for each study activity in order to reduce the overall time demands on the subjects; therefore the time allotted to make duplicate recordings of PWV may not be sufficient to capture the ‘true’ PWV. A better scenario may include increasing the number of repetitive PWV recordings within a study-session environment to ensure maximal data is recorded so to capture an accurate ‘mean’, whilst also ensuring that errors do not begin to cloud the data by collecting too many recordings that risk increasing the spread out from the mean result. With this in mind, at present it is not possible to say what the ideal number of recordings...
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is for PWV within an intervention study that would ensure the most accurate result. There is currently
justification for completing duplicate or triplicate recordings as many scientific recordings within
intervention studies are repeated this way for purposes of ensuring accuracy (i.e. blood pressure, waist
circumference etc.) However, extending work in the area of PWV reproducibility is required to try and
determine whether the error seen within the intra-session PWV is random, operator-dependant or
simply a naturally-occurring variance in PWV. It is also very important that given the current level of
deviation from the mean for the intra-session PWV results, there could also be an effect on inter-
session CVs which are crucial for determining aspects of future studies such as power calculations.

It would be expected that if the variability within a session is as described at 9.3% for R-C PWV and
6.9% for C-F PWV (n=8) then this variation within the data would increase once factoring in the
effects of time. However, the opposite is true for this dataset. Interestingly, despite the variability in
PWV recordings within one session, the mean result appears to be very accurate when comparing to
the other validation sessions results for each participant, giving a mean group inter-session CV of
5.3% for R-C PWV and 4.5% for C-F PWV.

The inter-session CV for C-F PWV in this study (4.5%, n=8) was considerably smaller than that of a
comparable C-F PWV result presented by Woodman et al. (2005) who determined that a cohort of 30
participants at differing risk of CVD (similar to the cohort in this study - i.e. mixed population of men
with differing levels of CVD risk) had a CV of 7.6%. Woodman et al. (2005) also reports a CV of
12.3% for R-C PWV which is very different to the level of reproducibility found in this study of 5.3%
CV for inter-session R-C PWV. As previously discussed (Section 3.1.2), it is difficult to
comprehensively compare data between the validation studies currently available for PWV due to the
differences in study design. The work by Woodman et al. (2005) is the closest available in terms of
matching the targeted population and measuring PWV at separate points in time. However, for the
current study, PWV is measured repeatedly over multiple sessions, whereas Woodman’s group
perform one measurement at two separate time points using a piece of un-named equipment with
apparently in-house built software. Comparing that to the equipment used for our study (a
SphygmoCor system) which is a validated (according to manufacturer) system, there becomes a
concern that the data presented by Woodman et al. (2005) may in fact hold little value, when they have
not appeared to use equipment that is in common use nor stated that any preliminary validatory work
had been completed.

It could be argued that one measurement one week apart does not constitute validation in the sense of
measuring variability over the short and medium-term. Instead this paper (Woodman et al., 2005)
shows a stand-alone observation alongside a number of other techniques for assessing arterial
stiffness. As a consequence the validation aspect has been clouded by the focus on comparing the
potential abilities of the range of methodologies. The validation study presented in this chapter has a
more robust methodology, singularly focussing on PWV and the elements that may play a part in affecting it on a day-to-day basis (such as fasted vs. fed state and activity levels). The study is not able to assess the pathological influences on PWV relating to the endothelium for example. Factors such as nitric oxide levels, presence of atherosclerosis or quantifying intima media thickness etc are not easily assessed within a standard clinical study setting.

The data obtained from the post-prandial and post-exercise study are very interesting as they show that there are no differences in PWV when the participant is either fasted or post-prandial, or when rested or post-exercise. This is re-assuring as it suggests that PWV is not influenced by either short bursts of exercise or food intake and could therefore lead to more flexibility in the use of PWV within clinical studies. At present, the current recommendations by the 'First International Consensus Conference on the Clinical Applications of Arterial Stiffness' (Van Bortel et al., 2002), recommend a stringent level of standardisation for both the conditions in which the PWV is recorded but also the preparation the participant must undertake (i.e. prior to study session - limiting exercise, fasted, refraining from alcohol). All recommendations are agreed within the committee as having a specific purpose and reasoning behind them. However, if data were to continue to be produced which shows that within the post-prandial phase and immediately post-moderate exercise there is no significant difference in PWV, the recommendations could be reconsidered. However, on balance, maintaining a standardised approach to clinical studies still remains important, especially if other outcome markers are being assessed.

It is also important to establish that even though PWV is not apparently sensitive to the metabolic changes induced by food or exercise, blood pressure is. It was shown that there were significant increases in systolic blood pressure (both post-prandial and post-exercise) and a significant decrease for diastolic blood pressure in the post-prandial phase. Aside from the inevitable changes in pulse pressure, the marked difference in blood pressure depending on whether the participant is fasted and rested, or not, could have serious consequences for the overall results of an intervention study. By not having the participant fasted and rested at the point of taking blood pressure for the purposes of PWV, there is no true baseline to compare against and each individual participant’s response to either food or exercise pre-recording could be extremely variable and therefore lead to false positives or negatives in the results. This could have severe consequences for drawing comparisons and determining the effects of an intervention.

Thus it is crucial for future studies that it is understood how the differences between the responses to the exercise and breakfast for blood pressure and arterial stiffness occurred. Blood pressure is regulated by the sympathetic nervous system (SNS) with the main function of the SNS therefore is to adapt the vasculature in response to an acute physiological stimuli such as exercise or diet, whilst
maintaining blood pressure within acceptable limits so as not to cause excessive hypo or hypertension (Francischetti and Genelhu, 2007).

Arterial stiffness is a process indicative of long-term vascular aging and/or of an ongoing atherosclerotic disease process. Therefore acute changes were not necessarily expected to be found in this instance but the point of the exercise test was to force a change in blood pressure to then see how this variability in blood pressure could potentially affect PWV. As the results show for this study, no significant differences between fasted and fed, or rested and post-exercise PWV results were found, even though there were significant changes in blood pressure. A study completed by Ahuja et al. (2009) found that after a cohort of 35 men and women (aged 21-80yrs) consumed a breakfast meal, there was a significant reduction in augmentation pressure and index (measures of wave reflection, a surrogate marker of arterial stiffness) as well as brachial diastolic and central blood pressure (both systolic and diastolic). The authors conclude that this response from the augmentation index in particular may be due to a relaxation in the smooth muscle in the splanchnic bed of the artery which could then have the consequence of causing the relaxation of smooth muscle throughout the rest of the circulation (Ahuja et al., 2009). Although arterial stiffness itself was not measured via PWV, it does indicate to some extent a possible mechanistic pathway between the effects of exercise on the structural function of the artery. This finding is then corroborated by Munir et al. (2008) who found that after their cohort of 25 participants completed 12 minutes of exercise, there were significant changes in blood pressure and augmentation index but not pulse wave velocity. Their reasoning behind this finding was that exercise dilates the muscular arteries, which coincided with clear changes in the pulse wave form, similar to those found after the use of nitrogen-based vasodilators such as GTN (Munir et al., 2008).

The studies described above, in combination with the data obtained within this study shows that there is still much work to be done in understanding the exact mechanism separating the response of blood pressure and PWV to physiological stimuli. New avenues of research need to be created to determine whether it is purely a muscular response within the artery or if there is a molecular aspect, such as the response by nitric oxide at the endothelium and how and why exercise/food may influence this.

3.4.2 Study design and limitations

Whilst it is noted that the cohort of participants is relatively small compared to other studies (Liang et al., 1998, Woodman et al., 2005) the volume of data produced from each participant is unrivalled in the current literature and therefore extremely valuable. This study employed a design which, if implemented in further validation studies as a standard, would enable a robust comparison across population groups and different techniques of measuring arterial function, thus enabling clear validation information to be derived.
Chapter 3: Pulse Wave Velocity Validation

Calculating the appropriate power for a validation study of this type can present difficulties due to a lack of similar studies available in the literature. In this instance, the main objective of the study was to gauge the fluctuation in PWV under different conditions from a representative sample of participants that reflected those who would be recruited in the future intervention studies. Therefore it was deemed that in the initial stages, ten participants would be sufficient, especially due to the multiple sessions of recordings, totaling 36 R-C PWV and 36 C-F PWV readings for each participant, not including those taken after the activity or post-prandial stages. It is unfortunate that two participants were required to withdraw from the study and due to the time constraints it was not possible to recruit new participants.

A further limiting factor to consider was the differences in repetitions of PWV within the study design. For the fasted/rested phase of the study session, six repetitions were made for both the peripheral and central PWV. When post-exercise or post-prandial, a duplicate recording was made. The justification for this was that the first phase of the study session was purely to assess reproducibility within the cohort, the second stage was not. The purpose of the second phase was to make a recording of how the exercise or breakfast had affected PWV; therefore the scientific standard of a duplicate recording should be sufficient to provide a valid indication of any possible effect as would happen within a regular intervention study protocol. The issue is the validity of the comparison between the fasted/rested states and the post-exercise/post-prandial state when there is far more data available for the former compared to the latter. However, the difficulty remains that to increase the number of recordings of PWV post-exercise or post-prandially, would risk making the mean results inaccurate as the level of recovery (especially for the exercise) for each participant would differ from person-to-person. Therefore completing a rapid duplicate whilst in the early stages of recovery was deemed the most suitable method in this instance.

All participants were recruited as per the criteria set and followed strict standardisation regarding dietary intake and activity levels for the 24hrs prior to each study session, with a standard protocol for completing the PWV followed rigorously each time.

If the study were to be repeated, it is recommended to build on the data already collected by recruiting more males in order to gain an even more accurate insight into the fluctuation that may be found within a time-course of PWV recordings. It may also be advantageous for the wider field to extend the inclusion criteria for participants in order to diversify the population groups from whom the PWV data is collected. By expanding the database to include participants at different levels of risk defined by factors such as age, gender, ethnic origin and health status would provide an invaluable resource for future research.
For this study, most participants preferred to attend on a specific week day each week to complete the study, therefore a certain degree of standardisation was set. However, a few participants completed the study on a shorter basis (i.e. four or five weeks) which was not a deviation of the study protocol. However, for future studies it may be beneficial to ensure that all participants attend study sessions within a consistent time-frame for standardisation purposes.

3.4.3 Conclusion

Overall, it is clear that within each session, the variability of PWV is low and within acceptable limits (i.e. matching or lower than the current CVs reported in the literature, as well as confirming the study hypothesis – see Section 3.4.1) to confirm reproducibility intra-individually - this is applicable to both the High and Low Risk groups, in addition to considering the group as a whole.

The consistently low variability for the inter-session data is also very encouraging and as the study is the only one in the literature to repetitively record PWV at such a volume and over an extended time-period, it is certainly encouraging. As other studies provide far less data yet make strong statements on the reproducibility of PWV (Liang et al. 1998, Woodman et al. 2005). With the extensive PWV data collection from this study, it could be considered that this study offers the best data to reflect real changes in PWV.

The lack of significant differences between fasted/rested states and the post-prandial or post-exercise state is an interesting finding and warrants further investigation. However, by allowing PWV to be recorded during the acute post-prandial phase or immediately post-exercise, it could be detrimental to the overall standardisation of the measurement, certainly in terms of the research environment. Yet in the clinical environment this may be useful as the data obtained in this study did show no significant differences across the different metabolic states. By not being restricted to taking PWV measurements at particular times of day (i.e. early morning, pre-breakfast), this could allow more flexibility and increase the use of this vital piece of equipment within the healthcare system that enables a recording of arterial stiffness that is predictive of cardiovascular health.

In conclusion this study suggests that both intra and inter-session variability is within acceptable ranges as it matches or betters the current validation data available in the literature. Importantly the mean results for both the high and low CVD risk groups, as well as the full cohort demonstrated CVs for both the intra and inter-session results as less than 10%.
Chapter 4

4 An investigation into the effects of a diet rich in whole grain on arterial stiffness and inflammatory markers in subjects at an increased risk of Cardiovascular Disease: The High Risk Study

4.1 Introduction

4.1.1 Whole grains and health: current perspective
As previously noted (Section 1.1.2), the latest data from the British Heart Foundation ‘Heart Stats’ 2008 Report shows that Coronary Heart Disease (CHD) is still the biggest killer in the UK, despite radical advances in both prevention and treatment. With 94,000 deaths in 2006 for those under 75yrs, not including the estimated 851,000 people who have had a heart attack and survived (Allender et al., 2008), reducing the risk of CHD (as well as related CVD events) becomes an essential public health strategy to reduce the social and economic burden of the disease in the UK. Even though mortality rates for CHD have reduced by 40% over the last ten years for those under 75yrs of age (Allender et al., 2008), there remains scope within nutrition research to explore the benefits of dietary modification to improve health outcomes. Logistically CHD is difficult to research in terms of short-term interventional study designs due to impracticalities assessing the impact of the intervention on the coronary arteries. For CVD, non-invasive, validated indicators are available (i.e. PWV) which enable meaningful research related to the health of the vasculature. Although drugs could seem to be the most influential tool in modifying CVD risk, there is sufficient evidence within the current literature to support an effective role of diet in lowering the risk of CVD development and the rate of CVD progression (Esposito et al., 2004).
In terms of specific dietary components both the increased consumption of oily fish (or equivalent supplementation) and the use of food products with added stanols or sterols have been proven to assist in maintaining the vascular health of the population, especially those at risk of CVD (Baker et al., 2009).

Whole grains represent an interesting and relatively new line of enquiry with ongoing investigations into their potential impact on CVD risk. Initial research appears to show that increasing whole grains in the diet may be influencing cardiovascular health. For example, two recent meta-analyses using data from a number of large prospective cohort trials demonstrate a beneficial effect. From an analysis of 12 studies a 26% reduction in CVD risk was calculated when whole grain foods are regularly consumed (Anderson et al., 2000). Likewise, Mellen et al. (2008) have also found a reduction in risk, with an odds ratio of 0.8 for increased whole grain consumption within the population groups studied – see Figure 4.1.

![Figure 4.1. Odds ratios of incident cardiovascular disease, comparing high versus low whole grain intake - ©Mellen et al. (2008)](Nutr Metab Cardiovasc Dis 18, 283-90)

Linking CVD risk to the amount of whole grains in the diet is not a novel concept with long-term prospective cohort studies such as the Health Professionals Follow-Up Study (Fung et al., 2002), Iowa Women’s Health Study (Jacobs et al., 1998), Nurses’ Health Study (Liu et al., 2000) and the Insulin Resistance Atherosclerosis Study (Mellen et al., 2007) all investigating this relationship. On a unilateral basis, these studies conclude that whole grain intake is inversely associated with CVD or other related disease processes such as ischaemic stroke (Liu et al. 2000), atherosclerosis (Mellen et al. 2007) and type 2 diabetes (Fung et al. 2002).

However when this epidemiological data has been compared against intervention studies looking at whole grain consumption and the resultant effects on CVD risk factors, or indeed any health outcomes, the conclusions are less conclusive. For a number of the intervention studies looking at increased whole grain consumption within the diet, reductions have been found in both blood pressure...
Chapter 4: The High Risk Study

(Behall et al., 2006) and fasting insulin (Pereira et al., 2002), as well as there being reports of improvements in HDL-cholesterol and insulin sensitivity (Jang et al., 2001). However, there have also been studies where no significant improvements in the study outcomes have been detected after the inclusion of whole grains in the diet (Hallfrisch et al., 2003, Andersson et al., 2007). A large intervention study looking at increased whole grain consumption and the potential effects on markers of CVD risk was completed by Brownlee et al. (2010) and published at the time of writing this thesis. However, given the status of the study (it is the largest and most powerful study to date) it is worth noting that they too found no effect on anthropometric, vascular, inflammatory, lipid or glycaemic markers (Brownlee et al., 2010).

This discrepancy between the outcomes of the intervention studies may be due in part to the heterogeneous nature of some of the study designs employed, making comparisons difficult. Indeed, whole grains have many sources such as wheat, rye, oats and barley – all these sources have differing levels of protein, fat, starch, soluble fibre and total dietary fibre. In addition, there is little information on the quantities of whole grains included in the interventions (from whatever source) making the design of future robust intervention studies difficult.

An example of this can be found in the study of Pereira et al. (2002), who found an improvement in insulin sensitivity after an intervention in overweight males and females. Participants were required to swap refined grain foods within a set meal plan for whole grain alternatives within a crossover design of two 6-week intervention periods and a 6-9 week wash-out phase. Even though a positive outcome was obtained, the study lacked information for fellow researchers as to the amount of whole grain consumed by the participants, therefore missing an opportunity to establish the effective dose of whole grains necessary to induce metabolic changes. Swapping refined grain foods for whole grain is a methodology common within the literature (see Brownlee et al., 2010, Andersson et al. 2007, Behall et al. 2006) however, as already mentioned, this approach sometimes does not produce a clear indication of the amount of whole grains that participants are actually consuming, thus limiting the extrapolation of the findings into robust public health recommendations.

As a response to this problem, a direct system of delivering whole grains into the diet without unduly disturbing the routine or dietary habits of the participant may be the way forward in producing concise and clear information from intervention studies. By developing a methodology that enables a consistent amount of whole grains to be consumed by the participant, several of the problems inherent in previous studies may be solved, especially in terms of controlling the intervention (portion sizes) by the researcher. It is also a simpler concept for participants to understand, thus hopefully leading to a greater chance of retaining the full study cohort. Therefore the current study endeavours to use such a methodology in order to assess the systemic effects of whole grains on CVD risk factors and thus the
Chapter 4: The High Risk Study

High Risk Study is the first study of its kind to deliver a targeted amount of whole grain in such a manner.

4.1.2 Justification for study
Assessing the global effects of whole grains within the body is important as it appears that many studies tend towards reporting on a relatively narrow remit, with no studies having specifically focussed on targeting vascular function. For example Pereira et al. (2002) focused on insulin sensitivity whilst Behall et al. (2006) reported only effects on blood pressure.

The gaps in the literature relate in particular to the effect of whole grains on arterial stiffness and endothelial function (namely the associated inflammatory process), which are important, yet relatively recent, additions to the battery of risk markers associated with CVD. There is no reported data using techniques to measure these outcomes, and so the High Risk Study was developed to address this knowledge gap, and assess whether the more traditional methods of assessment are missing crucial steps in the mechanism of progressive CVD. By identifying arterial stiffness via Pulse Wave Velocity and circulating inflammatory markers, it is hypothesised that findings may be extrapolated as to the atherosclerotic activity that is occurring at the endothelium and the cascade effect within the body.

Currently there are no formal dietary recommendations for whole grain consumption within the UK; with only the US Department of Agriculture recommending 3 portions of wholegrain foods per day (providing 48g whole grain) (USDA, 2005). With little comparable intervention data available and no tenable evidence-base for a quantitative dietary recommendation, it was important to design a robust dietary intervention study that had the opportunity to detect any possible relationship between whole grain intake and cardiovascular disease risk.

There are currently no intervention studies that directly compare whole grains versus refined grain using the specific system of delivery described above (i.e. using bread rolls with exact amounts of the intervention instead of using a system exchanging whole foods), or any comparing the structural properties of the grain itself (i.e. whole grains versus milled grains) in relation to cardiovascular disease. Finally there are no studies using Pulse Wave Velocity to investigate the effects of whole grains on markers of vascular health.

Therefore this study aims to determine whether a diet rich in whole grains will beneficially affect markers of cardiovascular disease, including vascular function, comparing to a diet high in an equivalent amount of milled whole grain or refined grain. If beneficial effects are revealed the data accumulated will show if it is the amount or the structure of the grain, or a combination of both, in the diet that is the determining factor for reducing cardiovascular disease risk.
4.1.3 Aims and objectives

The overall aim of this study was to investigate the effects of the addition of 48g of whole grain to the diet (in an intact form) versus an equivalent amount of milled whole grain and a control (refined grain) on markers of cardiovascular disease in men and women identified as being at high risk of developing CVD.

The main study objectives are to investigate the effects of the different grains on both fasting and post-prandial blood samples, specifically analysing changes to the following parameters:

- Pulse Wave Velocity, as a measure of arterial stiffness; and blood pressure as a whole body measure of endothelial dysfunction.
- Insulin resistance and glucose tolerance, as measured over the full time course of the post-prandial phase.
- Fasting lipid profile, including total and HDL cholesterol, triglycerides and non-esterified fatty acids (NEFA).
- Inflammatory markers: IL-1β, IL-8, GM-CSF and MCP-1, as measures of systemic and localised inflammation.
- Anthropometrics and static blood pressure.

4.1.4 Hypothesis

Increasing the amount of whole grains (whether intact or milled) in the diet will have a positive influence on CVD risk factors when compared to refined grain in those population groups at risk of developing CVD. Based on evidence from the current literature it is expected that this will be mediated via an improvement in insulin sensitivity and a reduction in systemic inflammation.

4.2 Methodology

4.2.1 Study design

The study was a 3-way, parallel, controlled, randomised dietary intervention study in men aged 30-55yrs and post-menopausal (both surgically or hormonal-related menopause) women aged 30-70yrs. Participants consumed either two whole grain, milled grain or refined (control) grain rolls per day on top of their habitual diet for 8 weeks. The whole grain was sourced from wheat, with the intact and milled grain rolls containing 48g of whole grain per two rolls – see Figure 4.3. The participants attended two study sessions; one at the beginning (Day 0), and one at the end (Day 56) of the intervention – see Figure 4.2.
4.2.2 Participant selection

A power calculation based on the predicted change in post-prandial insulin area under the curve (AUC) was calculated using data from a study by Frost et al. (1998). A sample size estimate of 17 participants per group would have a minimum of 80% power to detect a clinically significant drop in insulin (20% of AUC – metabolically significant) between the groups assuming that the common standard deviation in serum insulin is 20%. The estimate of the standard deviation was based on published data for subjects who took part in a study of similar design conducted by Frost et al. (1998). To include drop-outs (15%) the target sample size was 20 per group.

Prior to commencing, the study received a favourable ethical opinion from the University of Surrey Ethics Committee – EC/2006/89/SBMS.

The rolls used in the High Risk Study were whole grain, milled grain and refined grain (control). Both the whole and milled grain rolls contained identical amounts of whole grain (48g per two rolls) however the milled grain contained the grains crushed.
Participants suitable for inclusion:

- Adult males (aged between 30-55 years) and post-menopausal females (30-70 yrs)
- Overweight (body mass index 25-35 kg/m²)
- Waist circumference greater than 94 cm (37 inches) for men and 80 cm (31.5 inches) for women

A standard exclusion criteria was applied during the recruitment phase and is described in Section 2.1.3. The participants were recruited from the University of Surrey campus via global email and posters, in addition to publicising the study to the local population by poster and newspaper advertisement. Local businesses and public departments were also contacted.

Prior to attending the screening session (at the CIU, Faculty of Health and Medical Sciences) prospective participants received written information detailing the study and a consent form for the study.

4.2.3 Randomisation

Participants were randomised into one of the three groups via the web-based randomisation software available at www.randomization.com. However groups were stratified for gender to prevent clustering within any particular group.

4.2.4 Study protocol

Before commencing the study, prospective participants were invited for screening. Informed consent was obtained after the details of the study were explained to them (with an opportunity for queries to be answered) and before any screening activities were started. Participants were then requested to complete the Health & Lifestyle and food frequency questionnaires found in Appendix A.8.

The screening process involved the participant attending the Clinical Investigation Unit, in the Faculty of Health and Medical Sciences (University of Surrey) for one early morning visit for a small finger-prick blood sample which was used to test for anaemia (as described in Section 2.1.4). The presence of anaemia excluded the participant from the study for health reasons. Height, weight, waist circumference and blood pressure were also recorded. Information regarding the prospective participants was obtained via the Health & Lifestyle questionnaire. Individuals not meeting the criteria stated above were unable to take any further part in the study. Once all screening activities had been completed and the participant was deemed suitable to participate, the participant was then randomly assigned to an intervention group.

The week prior to attending the first study session, participants were requested to complete a 7-day diet diary. For the evening before the study session a standardised meal (see Appendix A.9 for choices) was consumed and was followed by a 12-hour fast (overnight). The range of ready meals and desserts on offer were chosen to provide (when combined – 1 ready meal, 1 dessert per person)
approximately the same macronutrient profile of \(\approx 700\text{kcal}, 35\text{g fat}, 60\text{g carbohydrate}, 30\text{g protein}, 8\text{g fibre}, 1\text{g salt}\). It was also advised that participants refrained from strenuous exercise or consuming alcohol the day before the study.

4.2.4.1. Study session – Day 0

On the first day (Day 0) participants attended the Clinical Investigation Unit (CIU) based at the Faculty of Health and Medical Sciences at the University of Surrey, in the early morning. After confirming with the participant that they were in a fasted state, happy to proceed and understood the activities of the session, anthropometric measurements were taken – height, weight, body fat percentage, body mass index, waist circumference, hip circumference (see Section 2.4.1-3). They were then invited to rest in a quiet room with dimmed lighting for ten minutes in preparation for the PWV measurements. After the resting phase, triplicate recordings of static blood pressure were taken (see Section 2.4.4) and the process of recording PWV commenced (see Section 2.6.1).

Immediately after the PWV recordings, the subjects were cannulated (see Section 2.4.2). Cannulation was necessary to allow multiple blood samples to be safely taken throughout the study session. A fasting blood sample (30ml) was taken at baseline.

For every blood sample drawn throughout the study, the blood was processed into SST, EDTA and fluoride oxalate tubes (see Section 2.4.4) to provide the appropriate amount of serum and plasma aliquots required for analysis.

Once the fasted baseline blood sample had been drawn, the participant was then requested to consume a standard breakfast test meal that did not contain either whole grains or refined grains. A warmed chocolate drink was considered the most appropriate as it was balanced in macronutrients and palatable. The drink contained 59.4g total carbohydrate, 21.4g total fat providing 505.8kcal. The participant was requested to drink the entire drink within 15 minutes; half hourly blood samples (10ml) were then taken for three hours post intake. Once the final blood sample had been taken, the metabolic study was completed and the participant was free to leave.

The following day the participants would commence the consumption of their assigned rolls for the next 8 weeks. Participants were encouraged to incorporate the rolls into their habitual diet (for example by adapting their lunch by substituting their routine bread for the study rolls) as opposed to consuming them in addition to their habitual diet. There were no restrictions on what study rolls could be consumed with and the rolls could be warmed before eating, if preferred. The products were manufactured and supplied by Holgran Ltd.
4.2.4.2. Participant follow-up and completion of study

For the duration of the 8 week study, participants were contacted regularly to discuss any issues with compliance that they may be experiencing plus the arrangements for delivery or collection of the rolls. Throughout the intervention period, participants were supplied with the correct amount of the bread rolls to ensure consistent intake, which provided 2 servings per day (48g of the active ingredient). When participants collected the study rolls or they were delivered to them by the researcher, the packaging for the previous batch of rolls was checked for any rolls not eaten. No spare rolls were found for any participants during or post-intervention, on occasion no packaging was available. Upon discussion with the participants regarding compliance, all strongly denied disposing of any rolls that had not been consumed. No formal data collection was possible to record the number of study rolls returned, as the participants indicated that all rolls were consumed during the intervention period. However, at the beginning of the eighth week (Day 49), participants commenced the final 7-day diet diary which assisted in the monitoring of roll consumption and thus gave an indication of compliance (see Section 4.3.6 for diet diary analysis). On the last day of the study (post-intervention assessment day), the participants returned to the CIU for the final study session identical to ‘Day 0’.

4.2.5 Statistical analysis

Detailed statistical analysis was undertaken to focus on the effects of the intervention over time. Baseline blood, PWV and anthropometric results were compared with post-intervention results via pairwise comparisons. The post-prandial phase data was also compared between pre and post-intervention to detect any changes in parameters of glucose and insulin sensitivity. The study was under-powered to perform a MANOVA.

Prior to commencing all analysis, data was tested for normality using the Kolmogorov-Smirnov test. If not normally distributed, non-parametric statistical tests were used, graphically represented using the median and interquartile range.

4.2.6 Study responsibilities and data sharing

This intervention study was performed in collaboration with fellow researchers Miss Caroline Bodinham (CB) and Miss Nicola Muirhead (NM); who each had their own individual, specific objectives and retrieved samples and data to reflect that. Responsibility for the running of study sessions and the tasks involved were divided equally between the three co-investigators. The exception to this was the PWV which was performed solely by the author due to the need to minimise inter-operator bias. Laboratory tasks were equally divided for the analysis of samples for the entire study cohort, with data made available to the co-investigators. Tasks were completed as follows: Insulin analysis – LT and CB; Lipid profile – NM; Inflammatory markers – LT and NM (CB did not require this data). All data analysis and inferences thereof are the authors’ own work.
4.3 Results

For this study, fifteen men and six women were recruited, with a mean age of 42.4yrs±9.3SD for men and 60.5yrs±7.1SD for women (Table 4.1).

<table>
<thead>
<tr>
<th>Study outcome</th>
<th>Sex</th>
<th>n</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Male</td>
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<td>Weight (kg)</td>
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<td></td>
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<td>71.1±8.0</td>
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<td></td>
<td>All</td>
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<td>89.8±17.2</td>
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<td>BMI (kg/m²)</td>
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<td>Waist Circumference (cm)</td>
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<td></td>
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<td>Systolic BP (mmHg)</td>
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</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>Male</td>
<td>14</td>
<td>5.3±0.3</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>4.5±0.3</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>20</td>
<td>5.1±0.5</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>Male</td>
<td>14</td>
<td>4.3±0.9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>5.5±1.3</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>20</td>
<td>4.7±1.1</td>
</tr>
</tbody>
</table>

Note: Descriptive statistics show gender differences between participants at baseline. There was a significant difference between genders for age (p<0.001) and waist circumference (WC) of p<0.001.

This age difference was significant between genders (t=-4.79, p<0.001) and a significant gender difference in baseline waist measurements was also seen (Mean results - M:107.1cm±7.5SD, F:90.8cm±5.5SD; t=5.50, p<0.001) however this was to be expected given the necessary differences in the recruitment criteria. The further criteria of body mass index (Mean results - M:29.9kg/m²±2.9SD F:26.5kg/m²±2.3SD, t=2.91, p=0.01) were also well matched to the recruitment criteria within each gender group.

Drop-out rate was low with only one participant (female) not completing the study due to health reasons unrelated to the study. As the study was in the early stages, a further female was recruited to maintain participant numbers.
4.3.1 Anthropometrics

4.3.1.1. Baseline characteristics

Subject baseline characteristics are shown in Table 4.2 dependant on their assigned intervention group. When compared at baseline, no significant differences were detected between the three intervention groups except for diastolic blood pressure (DBP). Under further examination of the DBP results to determine whether the difference is influential to future results, a Mann Whitney U test initially showed a statistically significant difference in diastolic blood pressure between the whole grain (Median:74mmHg,12IR) and milled grain group (82mmHg,16IR, \( p=0.04 \)), with a large effect size from the milled grain group (\( r=0.58 \)). However after post-hoc testing (Bonferroni correction), it was found that there was no significant effect overall. After controlling for the pre-intervention results, it was found that there was no significant difference between groups for post-intervention diastolic BP.

Overall, when assessing the baseline anthropometric data, it appears that although the participants were all overweight with relatively large waist circumferences and high body fat percentage, they had blood pressures within the healthy range.

4.3.1.2. Pre- versus post-intervention (absolute measurements)

When comparing pre versus post-intervention (Table 4.2), no significant changes were found within the control group. For the whole grain group, diastolic blood pressure significantly increased from 71.86mmHg to 79.57mmHg (\( Z=-2.37, p=0.018 \)). For the milled grain group, there was a significant change in waist circumference which reduced from 103.6cm to 101.8cm (\( Z=-1.99, p=0.05 \)). However, this is still above the desirable range for a healthy waist circumference (<94cm).

4.3.1.3. Percentage change over intervention

The percentage change between pre and post-intervention was calculated for all outcomes for the study (Tables 4.2-4). For diastolic blood pressure (DBP), a significant difference between groups was detected (\( F=6.48, p<0.01 \)), the effect size (calculated using eta squared) was large, 0.42. Post-hoc testing determined that DBP for WG group (+10.9% change) was significantly different from the MG group (-4.1% change, \( p=0.03 \)) and CON group (-6.2% change, \( p=0.01 \)). MG vs. CON was not significant. Percentage change in pulse pressure (an index using blood pressure data) was also significantly different between groups: \( F=5.42, p=0.014 \), with an effect size of 0.38. Post hoc tests showed this was due to the significant difference between WG (-9.6% change) and CON (+11.3% change) \( p=0.016 \). Neither MG vs. CON nor WG vs. MG were significant.

Percentage changes in no other anthropometric outcomes or other non-invasive parameters (including PWV), were found to be significantly different between groups.
<table>
<thead>
<tr>
<th>Intervention</th>
<th>Whole grain (n=55, F2)</th>
<th>Milled grain (n=55, F2)</th>
<th>Control (n=55, F2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>%Δ</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>45.86 ± 12.19</td>
<td>51.43 ± 4.83</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.49 ± 13.71</td>
<td>86.77 ± 13.38</td>
<td>0.39</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.34 ± 3.08</td>
<td>28.49 ± 3.09</td>
<td>0.51</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>101.74 ± 8.41</td>
<td>101.43 ± 7.66</td>
<td>-0.22</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>29.56 ± 8.19</td>
<td>29.54 ± 8.12</td>
<td>0.09</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120.86 ± 10.48</td>
<td>123.57 ± 13.15</td>
<td>2.13</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71.86 ± 5.58*</td>
<td>79.57 ± 6.53*</td>
<td>10.92*</td>
</tr>
</tbody>
</table>

Note: A Kruskal-Wallis test detected no significant differences at baseline between the three intervention groups, except for diastolic BP between the whole and milled grain group. The effect was detected to be from the milled grain group, with a median of 82mmHg compared to 74mmHg for the whole grain group, however the effect was not significant after Bonferroni correction. * A Wilcoxon Signed Ranks test detected a significant increase in diastolic BP pre vs post intervention (p<0.018) for the whole grain group. # A Wilcoxon Signed Ranks test detected a significant decrease in waist circumference pre vs post intervention (p<0.05) for the milled grain group. No significant changes were revealed for the control group. A one-way between-groups ANOVA was performed to compare percentage change (pre vs post-intervention) between the three intervention groups for all anthropometric outcomes. Key: BMI - body mass index; WC - waist circumference; BP - blood pressure; M - male; F - female; %Δ - percentage change (pre vs post).
4.3.2 Pulse Wave Velocity (PWV)

Vascular health was monitored via the use of Pulse Wave Velocity as well as the blood pressure-related indices Mean Arterial Pressure (MAP) and Pulse Pressure (PP).

Due to PWV being the primary outcome for this study, the data was analysed for goodness-of-fit to check that the study data was not significantly different from a normal distribution. A One-Sample Kolmogorov-Smirnov (K-S) Goodness-of-Fit Test was implemented and all data collected for both radial-carotid and carotid-femoral PWV, at pre and post-intervention, were found to be non-significant. Therefore it can be assumed that the study data is from a normal distribution (data presented in Table 4.3); however data was analysed using non-parametric tests due to small sample sizes in each intervention group (n<10).

As shown in Table 4.3, at baseline, there were no significance differences between the three groups for either radial-carotid (R-C) or carotid-femoral (C-F) PWV. Median results with the interquartile range (IR) were as follows: Whole grain group – 7.3m/s, 3.6IR for R-C PWV and 7.8m/s, 1.1IR for C-F PWV; Milled grain group – 8.2m/s, 2.7IR for R-C PWV and 7.8m/s, 2.4IR for C-F PWV; Control group – 7.8m/s, 2.3IR for R-C PWV and 6.9m/s, 1.3IR for C-F PWV.

<table>
<thead>
<tr>
<th>PWV Route</th>
<th>Intervention group</th>
<th>Pre</th>
<th>Post</th>
<th>% Δ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-C</td>
<td>Whole grain</td>
<td>7.3m/s (3.6)</td>
<td>7.7m/s (0.4)</td>
<td>-15.2</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>8.2m/s (2.7)</td>
<td>8.1m/s (2.7)</td>
<td>-1.8</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.8m/s (2.3)</td>
<td>7.7m/s (2.9)</td>
<td>1.9</td>
</tr>
<tr>
<td>C-F</td>
<td>Whole grain</td>
<td>7.8m/s (1.1)</td>
<td>7.4m/s (1.5)</td>
<td>-7.8</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>7.8m/s (2.4)</td>
<td>8.9m/s (2.8)</td>
<td>-5.3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6.9m/s (1.3)</td>
<td>6.3m/s (2.3)</td>
<td>-10.5</td>
</tr>
</tbody>
</table>

Note: A Kruskal-Wallis test detected no significant differences at baseline between the three intervention groups. A Wilcoxon Signed Ranks test detected no significant changes in either R-C or C-F PWV for any intervention group over time. A further Kruskal-Wallis detected no significant changes when comparing percentage change between groups for both R-C and C-F PWV. * Denotes median of the percentage change from each intervention. Key: K-S Z – Kolmogorov-Smirnov Z (K-S test result); Sig. – significance of K-S Z result.

Post-intervention, there were reductions in C-F PWV for both the whole grain (pre: 7.8m/s, 1.1IR, post: 7.4m/s, 1.5IR) and control groups (pre: 6.9m/s, 1.3IR, post: 6.3m/s, 2.3IR) however they were not significant. Conversely, the C-F PWV results for the milled grain group showed an increase (pre: 7.8m/s, 2.4IR, post: 8.9m/s, 2.8IR) but this was also not significant.
A Wilcoxon Signed Ranks test indicated no significant change in R-C PWV over time for any of the intervention groups.

- outlier of 1.5 box lengths from the edge of box;
- extreme outlier, more than 3 box lengths from edge of box.

Pre: wg—n=7, mg—n=7, control—n=5
Post: wg—n=7, mg—n=7, control—n=5

Key: wg – whole grain group; mg – milled grain group; control – control group

Figure 4.4 Radial-Carotid (peripheral) PWV before and after an 8-week intervention comparing whole grain, milled grain and control

A Wilcoxon Signed Ranks test indicated no significant change in CF PWV over time for any of the intervention groups.

- extreme outlier, more than 3 box lengths from edge of box.

Pre: wg—n=4, mg—n=4, control—n=4
Post: wg—n=5, mg—n=6, control—n=5

Key: wg – whole grain group; mg – milled grain group; control – control group

Figure 4.5. Carotid-Femoral (central) PWV before and after an 8-week intervention comparing whole grain, milled grain and control
The Mean Arterial Pressure (MAP) and Pulse Pressure (PP) data (Figure 4.6 and Figure 4.7 respectively, and Table 4.4) showed significant changes within the whole grain group. For MAP, the median values show a significant increase from 90.0 (7.7IR) pre-intervention to 96.0 (18.6IR) post-intervention (Z=-2.20, p=0.03).

Pulse pressure reduced from 44.0 (22.0IR) to 40.0 (10.0IR), Z= -2.13, p=0.03. The milled grain group experienced small reductions (not significant) in MAP from 96.7 (10.0IR) pre-intervention to 92.7 (13.0IR) and in PP from 51.0 (11IR) to 43.0 (12IR). For the control group MAP reduced from 93.3 (6.0IR) to 91.3 (12.6IR) and PP increased from 51.0 (20IR) to 58.0 (20IR), however neither result for the control group was significant.

When comparing the percentage change over time and between the three intervention groups (using a one-way, between groups, ANOVA) a significant change was detected for both MAP (p=0.03) and PP (p=0.01).

As shown in Table 4.4, for MAP there is a marked difference comparing how each group responded to their respective intervention. The whole grain group experienced an increase in MAP by 6.8%, as opposed to a reduction of 4.5% for the milled grain group and reduction of 3.2% for the control group. Overall, this gives a significant result of F=4.11 and p=0.03; a large effect (0.31) was detected using eta squared. Using post-hoc testing (Tukey HSD), the mean score for the whole grain (6.8±4.5) was significantly different compared to the milled grain group (-4.5±9.3), p=0.04.

Table 4.4: Mean arterial pressure and pulse pressure data from the three intervention groups of the High Risk Study – Pre versus post intervention and percentage change

<table>
<thead>
<tr>
<th>PWV Route</th>
<th>Intervention group</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>Whole grain</td>
<td>90.0 (7.7)*</td>
<td>96.0 (18.6)*</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>96.7 (10.0)</td>
<td>92.7 (13.0)</td>
<td>-4.5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>93.3 (6.0)</td>
<td>91.3 (12.6)</td>
<td>-3.2</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>Whole grain</td>
<td>44.0 (22.0)^</td>
<td>40.0 (10.0)^</td>
<td>-9.6</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>51.0 (11.0)</td>
<td>43.0 (12.0)</td>
<td>-5.5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>51.0 (20.0)</td>
<td>58.0 (20.0)</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Table 4.4: A Kruskal-Wallis test detected no significant differences at baseline between the three intervention groups. A Wilcoxon Signed Ranks test detected a significant difference for the whole grain group for MAP (p=0.03) and PP (p=0.03), no other significant changes were detected for any other intervention group. A further Kruskal-Wallis detected a significant difference when comparing the percentage change between groups for MAP (p=0.03) and PP (p=0.01).

For PP, the whole grain group experienced a reduction of 9.6% over time, with the milled grain group reducing PP by 5.5% and the control group showing an increase of 11.3%. Overall this gives a significant result of F=5.42 and p=0.01; a large effect (0.38) was detected using eta squared. Using post-hoc testing (Tukey HSD), the mean score for the whole grain (-9.6±9.9) was significantly different compared to the control group (11.3±16.0), p=0.02.
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A Wilcoxon Signed Ranks test indicated a significant increase in MAP for wg group \((p=0.03)\), no other changes were detected.

\* - outlier of 1.5 box lengths from the edge of box;

Pre/Post: wg-\(n=7\), mg-\(n=7\), control-\(n=7\)

Key: MAP - mean arterial pressure; wg - whole grain group; mg - milled grain group; control - control group

Figure 4.6. Mean arterial pressure (MAP) before and after an 8-week intervention comparing whole grain, milled grain and control

A Wilcoxon Signed Ranks test indicated a significant reduction in PP for wg group \((p=0.03)\), no other changes were detected.

\* - outlier of 1.5 box lengths from the edge of box;

Pre/Post: wg-\(n=7\), mg-\(n=7\), control-\(n=7\)

Key: PP - pulse pressure; wg - whole grain group; mg - milled grain group; control - control group

Figure 4.7. Pulse pressure (PP) before and after an 8-week intervention comparing whole grain, milled grain and control
4.3.3 Glucose and insulin profiles

Glucose and insulin profiles were analysed at both the fasting and post-prandial stages, as shown in Table 4.5, no significant differences were detected between the groups for HOMA-IR, HOMA-%\(\beta\), glucose or insulin at baseline.

The mean fasting levels of insulin were all found to be high (i.e. >60pmol/l), with no significant reduction over the course of the intervention detected for any group. Fasting insulin levels increased post-intervention for both the whole and milled grain group by 8.2% and 8.9% respectively however this was found to not be significant; all groups remained normoglycaemic at pre and post-intervention with no significant changes.

The data obtained for glucose area under the curve (AUC) as part of the post-prandial studies are shown in Figure 4.8, no significant difference was detected when comparing pre versus post-intervention within the wholegrain group; likewise for the milled grain group and control group there was no significant effect of the intervention over time.

There was also no significant difference between the groups either at baseline or post-intervention for the glucose AUC.

Post-prandial glucose curves are plotted pre vs. post intervention for each intervention group as found in Figure 4.11, with separate comparisons between the interventions groups at pre and post intervention found in Figure 4.10. For the glucose results at the pre-intervention post-prandial phase (Figure 4.10), there was no significant interaction between the intervention groups and time. However as would be expected, there was a significant effect for time (Wilks Lambda 0.05, F=28.37, p<0.001, partial eta squared = 0.95). The main effect of comparing post-prandial phases between intervention groups indicated no significant differences. Therefore at pre-intervention, there was no significant differences between each groups' glycaemic response to the standard meal (see Figure 4.10(a)).

Post-intervention (Figure 4.10(b)), there is again no significant interaction between the intervention groups and time; there was a significant effect for time (Wilks Lambda 0.04, F=37.28, p<0.001, partial eta squared = 0.96). However, no significant difference was detected when comparing between the intervention groups at post-intervention. Each intervention group's post-prandial results for glucose at pre and post-intervention can be found in Figure 4.11.

Across the three groups at the pre-intervention study, show that for both glucose and insulin, no significant effect was detected (see Figure 4.10 for glucose and Figure 4.12 for insulin).

For insulin AUC (Figure 4.9) there were no significant differences between the groups at baseline. The wholegrain intervention again did not show any significant changes over time, the milled grain group and control group also followed the pattern of no significant effects on the post-prandial response to
the standard meal. However, for post-intervention, there was a significant difference between groups, $F=4.21, p=0.04$.

Comparing the insulinaemic responses between the three intervention groups (see Figure 4.12) it was found that there were no significant differences at pre-intervention (Figure 4.12(a)) nor at post-intervention (Figure 4.12(b)). For pre-intervention, the interaction between the intervention groups and time, no significant differences were found. For the effects of time, there was a significant difference of $p=0.002$ (Wilks lambda 0.14, $F=9.32$, eta squared = 0.86). Yet overall, when comparing the three types of intervention, there was no significant differences detected.

The pre-intervention results differ dramatically from the comparisons between the post-prandial insulin curves for the intervention groups. There is both a significant interaction between the intervention groups and time (Wilks Lambda 0.09, $F=3.54$, $p=0.008$, eta squared = 0.70), and also a significant effect of time (Wilks Lambda 0.09, $F=3.54$, $p=0.008$, eta squared = 0.91). However, when comparing directly between intervention groups, no significant difference was detected.

Each intervention group’s post-prandial results for insulin at pre and post-intervention can be found in Figure 4.13.
Table 4.5. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) and β-Cell function (HOMA-%B)

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Whole grain n=6</th>
<th>Milled grain n=7</th>
<th>Control n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.22 ± 1.96</td>
<td>3.52 ± 2.44</td>
<td>3.88 ± 1.91</td>
</tr>
<tr>
<td>HOMA-%β</td>
<td>161.06 ± 103.9</td>
<td>165.11 ± 108.50</td>
<td>205.31 ± 108.0</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>85.55 ± 52.98</td>
<td>92.53 ± 63.64</td>
<td>103.41 ± 49.89</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.05 ± 0.59</td>
<td>5.04 ± 0.50</td>
<td>4.85 ± 0.74</td>
</tr>
</tbody>
</table>

Note: A Wilcoxon Signed Rank Test found no significant differences were detected between the groups at baseline, nor when comparing pre vs. post-intervention. Key: HOMA – Homeostasis Assessment Model; IR – Insulin resistance; %β – β-cell function (insulin sensitivity).
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Figure 4.8. Mean Glucose Area Under Curve (AUC) representing the post-prandial response to the standard meal before and after an 8-week intervention comparing whole grain, milled grain and control.

Figure 4.9. Mean Insulin Area Under Curve (AUC) representing the post-prandial response to the standard meal before and after an 8-week intervention comparing whole grain, milled grain and control.
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Figure 4.10. Pre and post-intervention post-prandial glucose curves comparing the three intervention groups.

After the standard meal, over the post-prandial phase of 3 hours, there is no significant variation in glucose measurements between the intervention groups at any time point. Pre (a): WG n=5, MG n=6, CON n=6; post (b): WG n=6, MG n=5, CON n=6. ▲/■/● - values indicate mean result per intervention group, error bars = SD. Key: WG - whole grain group; MG - milled grain group; CON - control group.
Figure 4.11. Pre and post-intervention post-prandial glucose curves per intervention group.

After the standard meal, over the post-prandial phase of 3 hours, there is no significant variation in glucose measurements within any of the intervention groups at any time point. Pre vs post intervention: a) \( p=0.77 \), b) \( p=0.87 \), c) \( p=0.41 \). Pre: WG \( n=5 \), MG \( n=6 \), CON \( n=6 \); post: WG \( n=6 \), MG \( n=5 \), CON \( n=6 \). *+/−/■ values indicate mean result per intervention group, error bars = SD. Key: WG - whole grain group; MG - milled grain group; CON - control group.
After the standard meal, over the post-prandial phase of 3 hours, there is no significant variation in insulin measurements between the intervention groups at any time point. Pre (a): WG $n=5$, MG $n=6$, CON $n=6$; post (b): WG $n=6$, MG $n=6$, CON $n=5$. • values indicate mean result per intervention group, error bars = SD. Key: WG – whole grain group; MG – milled grain group; CON – control group.
Figure 4.13. Pre and post-intervention post-prandial insulin curves per intervention group.

After the standard meal, over the post-prandial phase of 3 hours, there is no significant variation in insulin measurements within any of the intervention groups at any time point. Pre vs post intervention: a) $p=0.13$, b) $p=0.93$, c) $p=0.80$. Pre: WG $n=5$, MG $n=6$, CON $n=6$; post: WG $n=6$, MG $n=6$, CON $n=5$. A */#/# - values indicate mean result per intervention group, error bars = SD. Key: WG - whole grain group; MG - milled grain group; CON - control group.
4.3.4 Lipid profile

As shown in Table 4.6, at baseline, no significant differences were found between the intervention groups for any of the fasting lipids analysed or for the respective AUC results (from the post-prandial phase).

Table 4.6 shows that the markers included in the profile: total cholesterol (TC), HDL-cholesterol (HDL), triglycerides (TAG) and non-esterified fatty acids (NEFA). No significant changes were detected when comparing within each group for pre to post-intervention fasted samples. Unlike the other constituents of the lipid profile and despite not being significant, for the TAG results, all groups experienced a uniform increase in serum concentration post-intervention – WG: 12.47%, MG: 22.22%, CON: 16.86%. The area under the curve (AUC) was calculated for NEFA and TAG during the postprandial study days, with no significant changes detected for any of the intervention groups, as shown in Figure 4.14 and Figure 4.15.
Table 4.6. Lipid profile per intervention group – pre vs. post-intervention

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Whole grain n=6</th>
<th>Milled grain n=7</th>
<th>Control n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>%Δ</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>%Δ</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>1.02 ± 0.40</td>
<td>1.34 ± 0.47</td>
<td>12.47</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>3.81 ± 0.97</td>
<td>4.28 ± 0.90</td>
<td>10.70</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td>0.54 ± 0.12</td>
<td>0.48 ± 0.15</td>
<td>-4.89</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.01 ± 0.23</td>
<td>1.10 ± 0.26</td>
<td>9.31</td>
</tr>
</tbody>
</table>

Note: Wilcoxon Signed Rank Test detected no significant changes pre vs post-intervention within any groups for the lipid profile.

Key: TAG – triglycerides; TC – total cholesterol; NEFA – non-esterified fatty acids; HDL – High density lipoprotein cholesterol.
Mean TAG AUC with 95%CI. Paired samples t-test indicated no significant changes within the groups pre vs post-intervention

Pre: WG—n=5, MG—n=5, CON—n=6
Post: WG—n=6, MG—n=5, CON—n=5

Key:
WG - whole grain group,
MG - milled grain group,
CON - Control group.

Figure 4.14. Mean Triglyceride (TAG) Area Under Curve (AUC) representing the post-prandial response to the standard meal before and after an 8-week intervention comparing whole grain, milled grain and control.

Mean NEFA AUC with 95%CI. Paired samples t-test indicated no significant changes within the groups pre vs post-intervention

Pre: WG—n=5, MG—n=6, CON—n=6
Post: WG—n=6, MG—n=5, CON—n=5

Key:
WG - whole grain group,
MG - milled grain group,
CON - Control group.

Figure 4.15. Mean Non-Esterified Fatty Acid (NEFA) Area Under Curve (AUC) representing the post-prandial response to the standard meal before and after an 8-week intervention comparing whole grain, milled grain and control.
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Figure 4.16. Pre and post-intervention post-prandial TAG curves comparing the three intervention groups.

After the standard meal, over the post-prandial phase of 3 hours, there is no significant variation in TAG measurements between the intervention groups at any time point (pre-intervention \( p=0.54 \); post-intervention, \( p=0.23 \)). Pre: WG \( n=5 \), MG \( n=5 \), CON \( n=6 \); post: WG \( n=6 \), MG \( n=5 \), CON \( n=5 \). ▲/■/ ■ - values indicate mean result per intervention group, error bars = SD. Key: WG - whole grain group; MG - milled grain group; CON - control group.

Figure 4.17. Pre and post-intervention post-prandial NEFA curves comparing the three intervention groups.

After the standard meal, over the post-prandial phase of 3 hours, there is no significant variation in NEFA measurements between the intervention groups at any time point (pre-intervention \( p=0.48 \); post-intervention, \( p=0.67 \)). Pre: WG \( n=5 \), MG \( n=6 \), CON \( n=6 \); post: WG \( n=6 \), MG \( n=5 \), CON \( n=5 \). ▲/■/ ■ - values indicate mean result per intervention group, error bars = SD. Key: WG - whole grain group; MG - milled grain group; CON - control group.
4.3.5 Inflammatory markers

The fasted serum samples taken pre and post-intervention were analysed for a series of inflammatory markers; IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Monocyte Chemoattractant Protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and Granulocyte Macrophage Colony-Stimulating Factor (GMCSF).

Many of the serum samples proved to have undetectable levels of certain markers according to the multiplex bead immunoassay kit that was used; only IL-1β, IL-8, MCP-1 and GMCSF produced sufficient data to be analysed. As it was not possible to draw full comparisons across the three intervention groups for the other markers, the remaining data is not presented.

Figure 4.19 shows that there were no significant changes in IL-8 concentration within and between intervention groups. IL-8 increased by a mean percentage of 18.6% for the whole grain group when comparing pre versus post-intervention; this was not however significant. The control group also increased by an average of 36.2% post-intervention which was also not significant. Even though in contrast, the milled grain group showed a reduction of 3.6% over time, this too was non-significant. When comparing the percentage change between the three intervention groups, there was no significant change.

Figure 4.20 shows that none of the intervention groups experienced a significant change in MCP-1 when comparing pre to post-intervention (data presented as median with interquartile range). The whole grain group reduced 10.1% from a median of 2468pg/ml (868IR) pre-intervention to 2232pg/ml (315) post-intervention. The milled grain group reduced by 7.7%, from 2316pg/ml (1287) pre-intervention to 2256pg/ml (1491) post-intervention. The control group followed the trend and also reduced over time by 5.0% from 1754pg/ml (619) to 1561pg/ml (1649) post-intervention. When comparing the percentage changes across the groups, no significant result was found either.

As shown in Figure 4.21, GMCSF also did not show any significant differences for any of the intervention groups when comparing pre versus post-intervention. The whole grain group had an increase of 9.0% from a median level of 6.79pg/ml (86.53) pre-intervention to 6.89pg/ml (82.79). The milled grain group experienced a much larger increase in levels over time (21.8%) from 0.62pg/ml (3.14) pre-intervention to 2.25pg/ml (6.47) post-intervention. And in contrast, the control group had a large reduction in levels of 49.7% (pre-intervention: 8.31pg/ml, post-intervention: 1.91pg/ml); however this result is based on n=2, therefore it is not possible to calculate the interquartile range. There was no significant difference between groups for percentage change.
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Figure 4.18. Pre and post-intervention IL-1β levels comparing the three intervention groups.

Pre vs. post-intervention the WG group had significantly increased IL-1β levels ($p=0.04$), as did the CON group ($p=0.03$), MG had no significant difference. Pre: WG $n=6$, MG $n=7$, CON $n=7$; post: WG $n=6$, MG $n=7$, CON $n=7$. ▲/■ - values indicate median result per intervention group, error bars = interquartile range. Key: WG - whole grain group; MG - milled grain group; CON - control group.

Figure 4.19. Pre and post-intervention IL-8 levels comparing the three intervention groups.

Pre vs. post-intervention no group had significantly increased IL-8 levels over time. Pre: WG $n=6$, MG $n=7$, CON $n=7$; post: WG $n=6$, MG $n=7$, CON $n=7$. ▲/■ - values indicate median result per intervention group, error bars = interquartile range. Key: WG - whole grain group; MG - milled grain group; CON - control group.
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Figure 4.20. Pre and post-intervention MCP-1 levels comparing the three intervention groups.

Pre vs. post-intervention no group had significantly increased MCP-1 levels over time. Pre: WG n=6, MG n=7, CON n=7; post: WG n=6, MG n=7, CON n=7. ♦/•/■ - values indicate median result per intervention group, error bars = interquartile range. Key: WG - whole grain group; MG - milled grain group; CON - control group.

Figure 4.21. Pre and post-intervention GM CSF levels comparing the three intervention groups.

Pre vs. post-intervention the no group had significantly increased GM CSF levels over time. Pre: WG n=6, MG n=6, CON n=2; post: WG n=6, MG n=4, CON n=2. ▲/■/♦ - values indicate median result per intervention group, error bars = interquartile range. Key: WG - whole grain group; MG - milled grain group; CON - control group.
4.3.6 Dietary analysis

Participants completed 7-day diet diaries at baseline and in the final week of the study (referred to as ‘end-of-intervention’) as a means to record compliance and monitor any changes in dietary patterns and intake.

At baseline, energy (KJ) and the main macro nutrients (fat, protein, carbohydrate (CHO)), in addition to Dietary Fibre (DF) and Sodium, showed no significant differences at baseline. The data shown in Table 4.7 depicts the mean (± SD) for each nutrient pre versus post-intervention per group. The values for post-intervention (for all groups) include the two study rolls consumed per day which was confirmed via diet diary.

When comparing baseline versus end-of-intervention, neither the milled or control group showed any significant changes in dietary intake for the main macronutrients, despite the participants consuming the study rolls. However, for the whole grain group, the addition of the rolls appears to have significantly altered the participants' normal dietary intake. Energy increased by 17.5% (\(Z=-2.20, \ p=0.03\)) with a similar increase in protein of 18.2% - \(Z=-2.20, \ p=0.03\). Carbohydrate increased by 31.2% (\(Z=-2.37, \ p=0.02\)). Although not significant, the amount of fat eaten on average per day lowered by 4.7% as did dietary fibre by 7.8%; however the consumption of sodium increased by 13.3% from 2922mg to 3311.86mg, which is equivalent to 8.3g of salt per day.
Table 4.7. Diet diary analysis (average daily intake) baseline versus end-of-intervention for all intervention groups

<table>
<thead>
<tr>
<th></th>
<th>Whole grain n=7</th>
<th></th>
<th>Milled grain n=5</th>
<th></th>
<th>Control n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td><strong>Energy (KJ)</strong></td>
<td>8444.29±1953.86</td>
<td>9920.00±1764.94</td>
<td>11478.20±2060.68</td>
<td>10191.40±1197.67</td>
<td>-11.21</td>
</tr>
<tr>
<td><strong>Fat (g/d)</strong></td>
<td>75.67±26.56</td>
<td>72.11±16.41</td>
<td>110.94±18.44</td>
<td>84.90±22.20</td>
<td>-23.47</td>
</tr>
<tr>
<td>% energy from fat</td>
<td>33.2</td>
<td>26.9</td>
<td>-</td>
<td>35.8</td>
<td>30.8</td>
</tr>
<tr>
<td><strong>Prot (g/d)</strong></td>
<td>74.83±11.95</td>
<td>88.43±10.39</td>
<td>99.40±31.98</td>
<td>87.04±15.62</td>
<td>-12.43</td>
</tr>
<tr>
<td>% energy from prot</td>
<td>15.1</td>
<td>15.2</td>
<td>-</td>
<td>14.7</td>
<td>14.5</td>
</tr>
<tr>
<td><strong>CHO (g/d)</strong></td>
<td>249.76±61.03</td>
<td>327.79±72.40</td>
<td>331.88±75.84</td>
<td>307.70±39.79</td>
<td>-7.29</td>
</tr>
<tr>
<td>% energy from CHO</td>
<td>47.3</td>
<td>52.9</td>
<td>-</td>
<td>46.3</td>
<td>48.3</td>
</tr>
<tr>
<td><strong>Sodium (mg/d)</strong></td>
<td>2922.00±741.25</td>
<td>3311.86±833.17</td>
<td>3744.40±1624.19</td>
<td>2862.40±530.03</td>
<td>-23.56</td>
</tr>
</tbody>
</table>

Note: No significant differences were detected at baseline between groups for any major nutrient. Neither milled nor control group indicated significant changes in dietary intake between the two dietary records. Whole grain showed significant increases in energy, protein and carbohydrate. Values represent mean±standard deviation. Key: KJ = kilojoules; CHO = carbohydrate; DF = dietary fibre.
4.4 Discussion

4.4.1 Key findings

The aim of this study was to establish whether increasing the consumption of whole grain in the diet would positively influence key markers of the atherosclerotic process within those individuals at increased risk of CVD.

Overall, this study has demonstrated that incorporating the current recommended amount of whole grain (48g, equivalent of 3-4 one ounce portions of whole grain foods per day, (USDA 2005)) into the diets of free-living adults results in little observed benefit to whole body and clinical cardiovascular risk factors.

(i) Vascular markers

No effect was found on pulse wave velocity for any of the intervention groups in this study, however this study is the only one in the literature to specifically investigate the possible relationship between whole grain consumption and arterial stiffness. The closest comparison in terms of the objective to establish the effects of carbohydrate on arterial stiffness is a study by Philippou et al. (2009). In a parallel study design, 38 men aged 35-65 years, with at least one CVD risk factor (i.e. raised blood pressure or total cholesterol:HDL-cholesterol ratio or high BMI or high waist circumference) were randomly assigned to consume either a high glycaemic index (GI) or low GI diet for six months. Participants were also expected to follow a healthy diet and were supported in doing so by repeated dietary consultations (Philippou et al., 2009). After 6 months of the intervention, overall there was a significant effect within the low GI group on central PWV, LDL-cholesterol and triglycerides that was not found within the high GI group. Both groups had lower fasting insulin levels, HOMA-IR, total cholesterol and 24 hour blood pressures post-intervention, but the low GI had the greater improvements for those outcomes (Philippou et al. 2009). The important similarity between this study and the High Risk Study is that both studies included whole grains in the participants’ daily intake in some way. Albeit for the Philippou study whole grain intake was not a priority as the diet just had to be low GI, however whole grains would have been contained in such foods as seeded breads and porridge (foods mentioned as part of the staple diet within the study methodology). Whereas the High Risk Study used the direct delivery system of bread rolls containing set amounts of grain alongside the participants’ habitual diets. Another similarity was the whole-body approach, with both studies observing the glycaemic and insulinaemic profiles, lipid profiles and vascular function in an attempt to elucidate a mechanism of how the intervention affects the metabolism which in turn affects CVD risk.

However, the main and crucial difference between these two studies is the difference in intervention periods, with the Philippou study being conducted for six months (with interim measurements at 3 months) and the High Risk Study being conducted over 8 weeks. This obvious difference between
intervention periods coupled with the reductions in PWV and lipid profile in the low GI group for Philippou where none was found for the High Risk Study seems to indicate that there is a real need for long-term intensive intervention studies where decisive changes can be made to the diets of the participants. Only then can we be absolutely sure that there is no causal relationship between the increase in whole grains and reduction in arterial stiffness. It is not sustainable within the current literature to rely on proxy studies that do not directly answer the pertinent questions surrounding the effects of nutrition on arterial stiffness. Especially when in this instance, the study by Philippou involves a 'healthy eating' diet in addition to the alterations to GI so any fluctuations in metabolic markers may purely be associated to the reduction in energy within the diet as opposed to specific food groups.

Despite no recorded effect on PWV, contrary to the hypothesis for this study, the intact whole grain intervention appears to be linked to a deleterious response in diastolic blood pressure (DBP) and consequently mean arterial pressure (MAP) as both had significantly increased at the end of the intervention phase. This indicates that there may be increasing systemic vascular resistance (SVR) or central venous pressure (CVP), or a combination of both; it could also be due to an increase in cardiac output (CO). These three factors that combine to form the index of MAP (Safar et al., 2003) could all possibly have been influenced by the intervention of a whole grain diet.

Even though the increases in DBP and MAP were statistically significant, clinically the mean post-intervention result for the participants consuming whole grains (79.57mmHg [SD 6.53]) did not meet the criteria for a diagnosis of hypertension. However, because the systolic blood pressure (SBP) in the whole grain group also increased slightly (by 2mmHg) over the course of the intervention there was a significant overall reduction in pulse pressure (PP) for the whole grain group. Pulse pressure is inversely linked to aortic compliance as it is a measure of the pressure created by blood ejection from the left ventricle during the systole phase (Blacher et al., 2000); therefore this result of reduced PP in combination with the reduction in C-F PWV (from median result of 7.8m/s pre-intervention to 7.4m/s post-intervention) is possibly indicative of improved vascular compliance. However this cluster of results from the whole grain group centering around blood pressure do not support each other and are contradictory as they do not follow the basic physiology of blood flow and pressure. With no conclusive shift in blood pressure and its associated indices if MAP and PP either positively or negatively from this study (irrespective of the intervention group), it is difficult to ascertain why these results occurred. It could be argued that the most important issue to consider is the significant increase in diastolic blood pressure which is both concerning and intriguing. If future studies were to also find an inverse association between the consumption of whole grains and diastolic blood pressure then this would obviously raise many questions as to the reasoning behind the promotion of whole grains as a health food. The other point to remember is that systolic blood pressure did increase, thus also
affecting MAP and PP but it was not a significant increase in systolic blood pressure. Therefore are the MAP and PP results truly indicative of a definitive systemic change in vascular function? Should PP even be considered given that it has been influenced by a statistically non-significant result that has now ended in data that does not conform to the established understanding of the physiology and biomechanics of blood flow? Comparing to other data within the literature where the effects of increased whole grain consumption has been measured on blood pressure (Behall et al, 2006, Brownlee et al. 2010) no such results have been found as was for the High Risk Study. However, the field of whole grain research is still relatively young and there are many questions still to be answered.

When comparing the outcome of this study to those in the current literature that also focussed on whole grain dietary interventions and the effects on blood pressure, there are marked differences in the responses of the respective cohorts. Behall et al. 2006 conducted a study looking at increased consumption of whole grains in the form of a diet enriched with barley versus a diet high in brown rice and whole wheat. The results from the study showed that as whole grain consumption increased, both diets were associated with reductions in mean arterial pressure, systolic and diastolic blood pressure. However a very similar study conducted by Hallfrisch et al. (2003) showed no such consistency in their results with no overall significant effect from whole grains detected.

Therefore the current picture of if and how exactly whole grains affect the vasculature remains to be elucidated. Especially given the fact that there are no intervention studies within the current literature that use pulse wave velocity as a primary outcome within a dietary intervention study involving whole grains or other fibre derivatives apart from the High Risk Study. Therefore the only conclusions that can be made as to how whole grains affect vascular function are using the information from studies measuring blood pressure as a marker for blood flow and possibly indicating the structural function of the arteries by indicating the prevalence of hypertension and whether there is a response to the increased consumption of whole grains. The alternative is to look at the literature detailing the effects of whole grains on flow mediated dilatation (a marker for endothelial function) however there are also no studies within the literature examining this relationship. This serious gap in knowledge via the limited range of research impedes any attempt at determining the key pathways whole grains may use to elucidate an effect and there is a clear need for further research exploring the relationship between dietary alterations and the possible consequential effects on vascular function. It is particularly important for more information to become available as to the effects of whole grains on arterial stiffness and also endothelial function.

Although whole grain studies have overall failed to provide robust evidence of an effect on blood pressure, other dietary studies have had positive effects. For example, within the Dietary Approaches to Stop Hypertension (DASH) study, a study was set-up to focus on how different levels of sodium may affect blood pressure whilst continuing with either the DASH diet or a control diet (Sacks et al. 2003).
Participants were required to consume either a ‘combination’ diet otherwise known as the DASH diet (i.e. low fat, high fruit/vegetable) or a control diet which followed the typical intake in the US (37% energy from fat, 48% energy from carbohydrate and low levels of fibre at 9g). Within each diet the participants ate foods that had high, moderate or low sodium contents. After every participant (n=412) consumed the high sodium control diet for two weeks, participants were randomly assigned to either the DASH or control diet to continue for the rest of the study. In a cross-over design, participants would then consume their particular diet at the three levels of sodium intake for 30 days at a time in a randomised order (Sacks et al., 2001). Post-intervention, when comparing the results for blood pressure, there were significant, consecutive reductions in both systolic and diastolic blood pressure as the participants consumed progressively lower levels of sodium for both dietary patterns. Overall, comparing the high sodium to the low sodium DASH diet resulted in a 3mmHg reduction in systolic blood pressure and 1.6mmHg reduction in diastolic blood pressure. The control diet also experienced reductions in blood pressure comparing from the high to the low sodium diets of 6.7mmHg in systolic blood pressure and 3.5mmHg in diastolic blood pressure (Sacks et al. 2001).

Therefore it is clearly possible for dietary changes to induce significant reductions in blood pressure, however it has not been confirmed either via the High Risk Study or other studies that whole grains may too have a capacity to lower blood pressure in a consistent fashion.

(ii) Inflammatory markers

Inflammatory markers have long been associated with the development of CVD risk mainly via the acceleration of endothelial dysfunction. And with the inflammatory markers being measured as part of the High Risk Study being involved in the innate immune response, changes could be expected to occur within 24 hours of beginning the dietary intervention. But due to the cascade of cytokines that are involved in the process currently linked to atherosclerosis, combined with how long the endothelial cells may take to change their behaviour in response to the cytokines, it could be much longer before an influence of the intervention is detected.

For this study, a range of inflammatory markers were measured in an attempt to ascertain whether whole grains may have capabilities to dampen the inflammatory response. In the case of IL-1β, for both the whole grain and control group, there were significant increases post-intervention.

IL-1β is a cytokine whose main activity is based within atherosclerotic lesions and it is capable of driving the inflammatory response alongside TNF-α very effectively (Hansson 2001). Produced alongside TNF-α by activated macrophages within the atheroma, the release of IL-1β initiates smooth muscle cell proliferation which then results in the production of IL-6 (Hansson 2001), but when combined with TNF-α, the effects can be even more far-reaching. In a co-stimulatory mechanism, IL-1β and TNF-α are known to be linked to the activation of macrophages and release of matrix
metalloproteinase-9 (Saren et al., 1996), in addition they also have the capability to inhibit lipoprotein lipase thus affecting lipid uptake (Beutler and Cerami, 1985). Therefore the increase in IL-1β in both the whole grain and control group is an interesting result. It is clear that any increase in this cytokine could be detrimental due to the potential consequences as explained. However, in the case of this study, no other inflammatory markers linked to the activity of IL-1β (such as IL-6, TNF-α or MCP-1) significantly increased. What is also interesting is that two of these key inflammatory markers involved with IL-1β activity were not even circulating systemically within a detectable range. As found for both pre and post-intervention, when analysing samples for inflammatory markers using a multiplex bead immunoassay, IL-6 and TNF-α were found to be far below detectable levels. This could be indicating that despite the intervention of whole grains in the diet, on balance there is no co-ordinated inflammatory response ongoing within the study cohort at present and that even though the cohort is at risk of CVD via the traditional classification system (i.e. high blood pressure, large waist circumference etc) the inflammatory response has not yet been initiated. The expected systemic effects of an inflammatory cascade has also not occurred, such as a concomitant increase in the lipid profile alongside the rise in IL-1β. Since it has been shown within previous literature that IL-1β is linked to the inhibition of lipoprotein lipase, it would have been expected that there would also be a response such as raised LDL-cholesterol for example. However this did not happen and the key factor in this pathway could be the lack of TNF-α and IL-6 response.

This leads to a very confused picture as to the exact role whole grains have to play in the inflammatory response linked to CVD. All intervention groups appeared to have had a stimulatory effect on IL-1β and dampened MCP-1 secretion in similar proportions. Currently within the literature there are no references to the effects of whole grains on MCP-1, nor IL-1 which makes comparisons and conclusions difficult to draw. Especially as in the case of the High Risk Study there were reductions of MCP-1 (although not significant) in all intervention groups (10.1% WG; 7.7% MG; 5.0% CON) thus confusing the picture as to whether the intervention would really be expected to have any sort of significant effect specifically to whole grains when MCP-1 appears to be sensitive to any change in diet, even when as noted in Table 4.7, there were no significant to changes to the diet of the milled and control groups even with the addition of the study rolls (the whole grain group did experience significant increases in a number of macronutrients).

The evidence for the effects of dietary fibres on IL-1β, IL-6 and other inflammatory markers is similarly as scarce as for MCP-1. In terms of human data, there are few intervention studies looking at the effects on inflammatory markers, and of those that do there appears to generally be little response from the inflammatory markers to the dietary intervention. Andersson et al. (2007) showed that despite an inclusion in the diet of 112g of whole grain for six weeks, no significant changes in inflammatory markers were detected. Both IL-6 and C-RP were measured to define the level of inflammatory
response, a marker of lipid peroxidation (8-Iso-prostaglandin F(2a)) was also measured but there was no response systemically to the dietary changes. In a similar study by Brownlee et al. (2010) comparing the effects of whole grain to refined grain on inflammatory markers (in addition to other CVD risk factors), there was no significant effect on inflammatory markers, as there also were no effects on whole body markers such as blood pressure and anthropometrics, nor were there changes in insulin sensitivity and lipid levels (Brownlee et al. 2010). Therefore currently it appears that the intervention data does not support the observations made from cross-sectional studies which seem to find evidence for a dampening in the inflammatory response with increasing levels of whole grain intake (Qi et al., 2006, Lutsey et al., 2007, Masters et al.). However, whole grains contain relatively high levels of antioxidants, and antioxidants from other foods have been found to decrease inflammatory markers (Castilla et al., 2008). Therefore more research could be conducted into the bioavailability of these antioxidants contained within the whole grains to see what factors could be altered to enable them to become effective within the body. The High Risk Study has already attempted to address the issue of bioavailability by using isocaloric bread rolls matched in content of whole grains but with one variant of roll with the grains intact and the other with the grains crushed. However since no significant differences were found between the two types of roll, this may not be the issue and therefore other possibilities could be explored. For example, considering the individual’s diet alongside the consumption of the rolls is important as is the actual matrix of the bread rolls. The addition of additives or preservatives may be required to perhaps maintain the integrity and function of the antioxidant elements of the whole grains within the bread rolls.

(iii) Insulin, glucose and the lipid profile

For the High Risk Study, no effect was found on fasting insulin, glucose or lipid levels, nor were there any effects on the post-prandial glycaemic and insulinaemic response to a test meal at the post-intervention point. These results are in contrast to the current literature who appear to show that on the whole, the increased consumption of whole grains have a beneficial effect on insulin, glucose and lipids overall. As found by Rave et al. (2007) who investigated the effects of a whole grain product (derived from double-fermented wheat) compared to a meal replacement product on body weight, insulin sensitivity and lipids in a group of 31 overweight subjects (BMI 33.9±2.7kg/m². The subjects consumed either the whole grain or meal replacement product in place of two meals per day, for four weeks. This was then followed by a 2-week wash-out and then 4-weeks consuming the other product. The results of the study are interesting and appear to initially show that the whole grain product had a beneficial effect on body weight, BMI, waist:hip ratio, fasting glucose/insulin and LDL-cholesterol (Rave et al., 2007). However, the reductions in these parameters are mirrored by the same reductions for the meal replacement product. After correction for weight loss the improvements in fasting glucose/insulin and HOMA-IR with the whole grain product just reached significance when compared
to the meal replacement. The meal replacement only showed significant reductions in total cholesterol and LDL-cholesterol (Rave et al., 2007). It must also be pointed out that when comparing the two products, the whole grain product had twice the amount of fat (12.0g/100g), 3g/100g less fibre and 20.3g/100g less total carbohydrate than the meal replacement product, with participants expected to consume 200g of the products per day, regardless of which intervention they were consuming. The meal replacement also has 9.0g of inulin per 100g, whereas the whole grain product has none. These compositional differences therefore make it difficult to draw conclusions as to the effect of whole grains per se or to extrapolate the data to wider public health recommendations.

Two other research groups found apparently positive effects of whole grains on insulin sensitivity which were Jang et al. 2001 and Pereira et al. 2002. Jang et al. (2001) conducted a parallel, study for 20 weeks (including an initial 4 week run-in control diet) on 76 men with coronary artery disease, looking into the effects of a whole grain and legume powder which totaled 70g consumed per day in place of refined carbohydrate at breakfast versus a control diet. Comparing the wholegrain to the control results, there was a significant reduction in fasting glucose (p<0.001) and HOMA-IR (p<0.001) in addition to an increase in HOMA-β% (p=0.003). Similarly for Pereira et al. (2002), comparing a whole grain intervention to refined grain, there was a significant reduction in fasting insulin (p<0.01) and HOMA-IR (p<0.01) and an indication of increased insulin sensitivity (p<0.05) from the euglycaemic hyperinsulinaemic clamp. For this study, 12 overweight participants were included (6 male, 6 female) and they were randomised within a cross-over study design to consume either a pre-designed diet rich in whole grains or refined grains. The intervention periods were for 6 weeks each (6-9 week wash-out) as opposed to the far lengthier 16 weeks employed for the study conducted by Jang et al. (2001). However, essentially the same outcome was achieved with demonstrable improvements in insulin sensitivity after the increased consumption of whole grains compared to a refined grain diet.

A later study by Andersson et al. (2007) however found that for their cross-over intervention study design, no significant effects were detected after the consumption of whole grains within the diet (mean consumption 112g/day) compared to refined grains. This is clearly at odds with the results of the two previous studies, but echoes the results of the High Risk Study, especially given that their participants consumed far less whole grains on a daily basis than those participating in Andersson et al’s study - Jang et al. consumed 70g mixed powder per day, equivalent to 46.6g whole grain/day; Pereira et al. consumed an additional 10.2g of dietary fibre, 8.9g insoluble fibre and 1g of soluble fibre than the refined grain group.

The Andersson study recruited 30 overweight participants (22 females, 8 males) consuming a strict quota of specified whole or refined grain products. Using the same time-frame as Pereira et al. (2002) but arguably more whole grain on a daily basis, the results from the ‘Andersson’ trial are not
supportive of the apparent relationship between increased consumption of whole grains and improved insulin sensitivity. Given the lack of effect from whole grains for both the Andersson study and the High Risk Study, also not forgetting the Brownlee trial (Brownlee et al. 2010), it appears that some dispute may begin to occur as to the consistency of the effect of whole grains. Three robustly designed studies are now questioning the potency of whole grains in their ability to improve insulin sensitivity and lipid profiles. Although this is only a small collection of studies, the growing discrepancies between the overall findings of whole grain dietary intervention studies is a cause for concern and warrants careful consideration in terms of the chosen study design for any future research.

(iv) Anthropometrics

Clearly there was minimal effect on vascular or inflammatory markers over time from the consumption of whole grains. It is interesting that there were also no effects on the anthropometric markers for the study apart from a significant reduction in waist circumference within the milled grain group. A positive dietary energy balance can lead to an increase in visceral adiposity and has the potential to create the right conditions (via the production of adipokines and ensuing insulin resistance) to eventually influence the induction of atherosclerosis. Therefore if no significant weight loss is found in intervention studies such as this study, especially around the visceral area (indicated by waist circumference) then there may be no further effects which could cause a reduction in the production of inflammatory markers and thus blood pressure or even arterial stiffness.

However this theoretical model is difficult to support when the milled grain group showed a significant reduction in waist circumference by 1.8cm, yet there was no concurrent weight loss. By the milled grain intervention not inducing whole-body weight loss as well, it is difficult to determine whether the reduction in waist circumference is truly a loss of adiposity (for which you would expect a corresponding reduction in weight) or whether this reduction represents a degree of error. Whether it be on the side of the researcher or if the participants themselves affected the result by factors such as changing their posture during the measurement for example, it is not possible to verify which factor had the greater influence.

(v) Overall assessment of CVD risk factors

The most recent and high-profile study completed to assess the effects of whole grain consumption on a wide range of CVD risk factors was the WHOLEheart study (Brownlee et al., 2010). Conducted with 266 participants who were overweight and low consumers of whole grains (<1.5 servings p/d), a parallel study design was used with participants randomly assigned to one of three diets for 16 weeks: Control (maintain normal diet), consume 60g/day of whole grains or consume 60g of whole grains for 8 weeks followed by 8 weeks of 120g. Despite clear evidence of good compliance from the participants, it was found that neither of the whole grain interventions induced any significant changes
to the study outcomes (which were anthropometrics, blood pressure, lipid profile, insulin sensitivity and inflammatory markers). When compared against the mean of the control group, the differences in outcome markers post-intervention for the intervention groups was minimal, fluctuating from +2.24 to -3.11%. The authors note that the study was robust in design and execution however healthy participants were chosen, and so they were at no specific risk of CVD. This study provides evidence for a lack of beneficial effect of whole grains within the general population however it does not provide any information on the potential benefits to those already at increased risk of disease, such as those individuals with hyperlipidaemia, glucose intolerance or inflammatory process for whom greater risk mediation by wholegrain may be predicted. Therefore more work needs to be done in order to fill this gap in the literature and to be aware of the impact of whole grains within the wider population as opposed to specific population groups which studies tend to target.

Katcher et al. (2008) undertook a randomised, parallel study in overweight participants (25 male, 25 female) to compare the effects of diets rich in refined and whole grains. Obtaining all their grain-based foods from either whole grain (~5 portions/d) or refined grain, whilst also adhering to a hypocaloric diet and advice concerning daily portions of fruit and vegetables etc to include in the diet, the dietary intervention lasted for 12 weeks. When comparing the two intervention groups, the whole grain group experienced a significant reduction in body fat % (p=0.03) and C-RP (p=0.01). However as the overall diet that was being consumed was hypocaloric, both groups experienced significant reductions from baseline in weight and waist circumference, Plasminogen Activation Inhibitor-1, as well as total, LDL and HDL cholesterol (p<0.05). Even though there were significant improvements in the lipid profiles, the authors note that there were no significant changes in LDL particle size. There were also no significant reductions in TNF-α nor IL-6 for either group. In terms of insulin resistance, the area under the curve for insulin from the OGTT indicated a significant reduction for the whole grain group (p=0.04, time effect) however further analysis indicated there was no significant group x time interaction (Katcher et al., 2008).

This study presents an interesting picture in terms of the potential effects of whole grains, with reduction in body fat and C-RP possibly indicating a potential mechanism for whole grains on the loss or at least reduction in size of adipocytes and the potential amelioration of the inflammatory markers that adipocytes (in the visceral adipose tissue) are known to produce. By reducing key adipokines, this could in turn reduce CRP which is in itself an acute phase protein, an indicator of generalised systemic inflammation and predictor of CVD risk. However, the authors do not appear to have analysed a range of adipokines, instead (in addition to CRP) focusing on TNF-α and IL-6 that, granted, are produced from adipocytes and the macrophages that are infiltrated within them. However other adipokines such as adiponectin or leptin could have been having an effect at a higher level within the inflammatory marker cascade that is reported to be linked with adipocytes and CVD risk. There were also critical
differences between the groups at baseline – systolic blood pressure and the level of LDL-III subclass particles were significantly higher in the refined grain group compared to the whole grain. Given that the purpose of the study was to examine the mechanism via which whole grains exert their effect on CVD risk, it is vital that differences at baseline are eliminated as far as possible and if they do occur, that they are statistically controlled for. The authors do not state whether they have controlled for these baseline differences, therefore making it difficult to make confident inferences from the data.

Comparing the studies by Brownlee et al. (2010) and Katcher et al. (2008) with the High Risk Study, it is clear that as comprehensive studies looking at a wide range of CVD risk factors, there is very little in the way of correlation. Brownlee et al. find no significant effect for any of their study outcomes yet Katcher et al. finds that increased whole grains in the diet results in reductions in body fat and C-RP. The High Risk Study on the other hand, found that the effects of whole grains centred around increases in diastolic blood pressure and the related indices of MAP and PP along with an increase in IL-1_β and reduction in waist circumference (milled grain group). The High Risk Study clearly does not corroborate the evidence of Brownlee et al. or Katcher et al. as there is not only no effect from whole grain in general (or even milled grain) but there is actually a negative effect on blood pressure. Since the field of research within whole grains is so narrow as it is still relatively young, it is difficult to say essentially which study depicts the true effect of whole grains on CVD risk. So as already recommended, further research on a much wider scale is required to establish what causal relationship there may be (if any) between whole grains and cardiovascular health. The lack of correlation in the results and even the relative lack of effect is puzzling as all the risk factors for CVD that were measured are thought to be inter-linked (as shown in Figure 1.6, Section 1.5) and therefore it would be rational to think that if one CVD risk factor were to be affected by the consumption of whole grains, then other factors would follow in some way. Katcher et al. appear to support the pathway of the link between obesity and CVD as suggested within this thesis (see Figure 1.1, Section 1.2.1). Their study showed that by reducing the amount of calories within the diet as well as increasing the amount of wholegrain, then both body composition and the inflammatory response is affected. This could indicate a pathway that may be affected by whole grains via the production of adipokines which results in the production of cytokines being increased which in turn increases endothelial dysfunction and eventually results in CVD. However, the High Risk Study results from the whole grain intervention group do not support this and instead could suggest a pathway linking blood flow with inflammatory markers, possibly supported by the (non-significant) 10-12% increases in total cholesterol and triglycerides.

It is hoped that as the field of research into the effects of whole grain on CVD health widens and gains more data, then the true effects may be found as at this moment in time the picture is very confused, with no consistent results found throughout the current available literature.
(vi) Dietary analysis

The adequate completion of diet diaries by the study participants for the High Risk Study are important for two reasons: to assess compliance to the dietary modification required for the study and to assess the impact of the dietary modification on the habitual diet of the participants.

Compliance to the consumption of the study rolls overall was recorded moderately by the participants, with those clearly noting the consumption of the rolls also recording good amounts of detail of the types of food the rolls were consumed with. Overall, approximately 60% of participants recorded that they consumed the bread rolls in the quantities expected. The rest of the diaries either recorded no mention of the intervention rolls or made vague and poorly detailed references to them. Therefore in these instances no rolls could be entered into the analysis for those particular participants as they may have been referring to a different food item.

Assessing the diaries of those participants who did note consumption of the study rolls, dietary patterns did seem to change to a degree when comparing pre to post-intervention. The main differences were at breakfast and lunch, where staple items such as toast (breakfast) and bread or bread rolls (lunch) were substituted with the study rolls. Many participants did choose energy dense accompaniments to consume the rolls with such as butter, jam, soft cheese etc., however there was no real indication that the amounts of these food items used in conjunction with the rolls were any more than if the participants had been consuming them with their habitual bread or other baked goods. Other habitual items in the diet did not obviously differ between the pre and post-intervention diets for example, the amount and type of fruit eaten, the number and type of snacks or the quantities eaten at the evening meal. Overall, the consumption of the rolls appeared to have very little impact on the evening meal with only a few participants (~5) detailing consumption with this meal.

Despite consumption of the study rolls being reported in the diet diaries, the actual analysis shows dietary fibre intake decreases across all three intervention groups post-intervention. This could be indicating that despite the qualitative review of the diet diaries, the habitual diets of the participants could have been unduly altered by the introduction of the study rolls. The amount of dietary fibre per study roll is 4.5g for the whole grain rolls, 5.2g for the milled grain roll and 2.9g for the control. Therefore when referring to Table 4.7, it is clear that the amount of dietary fibre consumed matches the amount that would have been provided by the rolls, but there is very little excess within any intervention group for further fibre consumption within the rest of the diet. It must be noted that the results from Table 4.7 are mean results, with relatively large standard deviations indicating a wide spread of results. There is also the number of poor records from participants as to whether they consumed the rolls or not, however it is certainly concerning that the dietary fibre consumption post-intervention is so low.
This instance has highlighted a recurring issue of the practical implementation of diet diaries within dietary intervention studies. They are open to large sources of error as they rely on very accurate recollection of dietary intake by the participant as well as equally accurate interpretation by the diary assessor. It is often impractical for the diary assessor to refer to the participant during the analysis of the diary however the data recording at source could be improved and would be an important component of dietary research to be reviewed. For the *High Risk Study*, the lack of detail within the diet diaries is an issue and makes extrapolation of results and inferences regarding the clinical outcomes difficult. Due to the lack of clinical significance for the large majority of study outcomes, it could possibly be assumed that compliance may have been poor and therefore led to no effect from the intervention but due to the accuracy of the diaries, this cannot be confirmed and most certainly cannot be assumed.

For the macronutrients fat, protein and carbohydrate, no significant effects were noted for either the milled or control group despite a 23% reduction in both fat and salt (84.9g and 7.1g respectively post-intervention) for the milled grain group. For both groups, percentage energy from fat remained at approximately 30-35%, for protein at 14-15% and for carbohydrate 44-48%. However the whole grain group demonstrated significant increases in protein (+18%) and carbohydrate (+31%) which appears to have contributed to an increase in energy intake of 17%. In addition sodium intake increased by 13% to the equivalent of consuming 8.3g of salt per day.

Overall the three intervention groups remain within recommended guidelines for the macronutrients (i.e. no more than 35% energy from fat) however in terms of healthy eating, no groups consume over 18g of dietary fibre per day and the amounts of sodium consumed are alarming, with the control group consuming an average of 8.8g pre-intervention and 8.3g post-intervention and these amounts being evident for the other two intervention groups.

Overall, on a qualitative basis the diet diaries returned by the study participants give a fair generalised idea of the habitual dietary patterns and intake of the study cohort. However, they do not all clearly represent the consumption of the study rolls and so when assessing the dietary data, these accuracy issues make detailed analysis and inferences difficult. For future use, greater efforts to familiarise participants with the diet diaries method and enhancing accessibility and ease of use will work to improve the consistency and accuracy required to ensure that diet diaries support dietary intervention studies in the results they produce.

### 4.4.2 Study design and limitations

The possible advantage that Pereira et al. (2002) and Andersson et al. (2007) had over the current study is in the use of a cross over study design. This can be seen as the more robust approach as the
participants may act as their own control. For a parallel design such as the High Risk Study, it is imperative that the recruiting criteria are meticulously followed and that participants are as metabolically similar as possible, as well as matching groups for age and gender to prevent clustering and thus a potential source of error. The three intervention groups were very well matched for anthropometrics and baseline glucose, insulin and lipid profile results (Section 4.3). No significant differences were detected between the groups at baseline, with the exception of diastolic blood pressure. The latter baseline difference was addressed via an analysis of covariance (ANCOVA) controlling for pre-intervention diastolic BP (DBP) across the intervention groups. This indicated that even though there was a significant difference at baseline for DBP, there was no effect detected for the related vascular outcomes of MAP, R-C PWV and C-F PWV, but not for PP. After adjusting for pre-intervention DBP, there was a weak significant difference between intervention groups on post-intervention PP ($F=3.79, p=0.04$, partial eta squared 0.31). This is possibly to be expected since PP is an index very closely attributed to blood pressure; therefore issues at baseline could theoretically be further translated through to the post-intervention results.

Taking this into account, on balance it can be concluded that the 21 participants recruited were satisfactorily matched and therefore the parallel design should not limit the conclusions that have been drawn from the intervention due to the minimal impact the differences at baseline had in reality, with only PP being affected.

As an additional benefit, the parallel study design ensures a reduced burden to the participant in terms of their involvement in the study due to the relatively simple study design. By requesting the participants to only attend two study sessions and consume one type of intervention roll for eight weeks, the more simplistic study design and reduced subject burden fosters good participant compliance. In contrast, cross-over study designs can be a complicated concept for participants to understand; as well as increasing the time commitment of the participants through more study visits. There is also the issue that participants may not fully ‘wash-out’ between intervention stages thus creating a situation where the participants may not go back to their pre-intervention state before starting a new stage which then continues to influence and potentially give false positive results.

The study was designed to enable a clear perspective of the practicalities of using the experimental rolls, as well as providing initial data to use as evidence for powering future, larger studies. Initial recruiting targets were three intervention groups with 12 participants each (Section 4.2.2 - power calculation). However, the final number of participants recruited to the study was 21; with seven participants per intervention group. The recruiting issues for the study were not fully anticipated and it proved difficult to recruit participants who not only fitted the inclusion criteria but who were also prepared to make sizeable changes to their daily diet for the intervention time-frame. Had the difficulty of recruiting this specific study population been predicted it may have supported the
arguments for a cross-over design, with the smaller sample sizes this generally involves. However this would not necessarily have improved overall recruitment success, given the greater burden (see above) of this type of study.

The 8-week time-frame for this dietary intervention was substantially longer than has been reported by most other investigators. The current trend for length of intervention for whole grain interventions appears to be 5-6 weeks. Behall et al., (2006), Hallfrisch et al., (2003) and Pereira et al., (2002) all found significant changes in their primary outcomes (including insulin sensitivity and blood pressure) using this time-scale; with the exception of Andersson et al., (2007) who found no significant changes over the course of a six week intervention study (in a cross-over design) or for Brownlee et al. (2010) who ran a very strong parallel study for 16 weeks. For the High Risk Study, the main focus was on how the vasculature responded to the dietary intervention by measuring arterial stiffness via pulse wave velocity. It is difficult to pinpoint an amount of time to expect for an intervention to start having an influence, however for blood pressure to change it appeared that >5 weeks should be sufficient (Behall et al. 2006), therefore after this length of time other vascular indicators could theoretically begin to be influenced too (i.e. PWV).

Participant numbers are comparable to the studies previously mentioned by Pereira et al. and Andersson et al., however the cross-over design of their studies warrants fewer participants for reasons already stated above.

4.4.3 Conclusion

The High Risk Study found that in a population group at increased risk of Cardiovascular Disease, the increase in consumption of whole grains had no effect on established markers of CVD risk.

Overall the study was conducted in a robust manner, strictly following study protocols and ensuring participant compliance as carefully as possible. However, the recruiting issues have possibly impaired the results of this study due to the lack of power and so fails to indicate with confidence whether whole grains have the potential to ameliorate CVD risk factors or not. However, as has been found previously, even those studies which recruit high numbers (Brownlee et al. 2010), still found no significant effect overall therefore the High Risk Study should still hold merit in terms of the results it has shown. Especially since there is a lack of correlation between studies for outcomes of CVD risk no matter what size of cohort or study design (such as cross-over or parallel).

Despite these discrepancies, for future studies, it would be beneficial to extend the study in terms of larger participant numbers – although this can only be assured if a reliable database of participants can be established first (i.e. partnerships with local GP surgeries).
Although these initial indicators within the intact whole grain group are suggestive of a beneficial effect on blood flow and arterial stiffness, which may in turn be translated into long term reductions in CVD risk, the lack of statistical and clinical significance leading to equivocal results means that further investigation is essential.
Chapter 5

5 An investigation into the effects of a diet rich in whole grain on arterial stiffness and ambulatory blood pressure in subjects at low risk of Cardiovascular Disease: The Low Risk Study

5.1 Introduction

5.1.1 Current evidence

Traditionally the focus of concerns regarding cardiovascular health has been on middle-aged and older men (40+ years), with increased awareness of the benefits of monitoring the risk factors for CVD in these population groups. Subsequently lifestyle advice is available to those in need, as set out by the Department of Health’s National Service Framework for Coronary Heart Disease (published March 2000 - www.dh.gov.uk).

As seen in Table 5.1, the mortality rate for CVD in men increases exponentially with age; with a very low mortality rate in those men aged under 35 years (n=129) compared to older populations (13.0% in men aged 55-64 yrs, n=6687).

However, with fatty streaks proven to develop in the teenage years (McGill et al., 2000, McMahan et al., 2006) and possibly even younger (Holman et al., 1958). Early intervention to prevent the onset of potential CVD risk factors and delay the development of disease may be a key strategy in reducing CVD-related mortality later in life.

From data collected in the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study (Mcmahan et al. 2006), the extent of the surface area of fatty streaks present in those aged 15-19 years with a healthy lipid profile, were found to involve just under 20% of the surface area of the abdominal aorta lumen and 2% for the right coronary artery. For those in the same age-group with lipid profiles
in excess of the recommended levels, the percentage of surface area of artery lumen were as follows: approximately 32% for the abdominal aorta and around 3.5% for the right coronary artery (McGill et al. 2000).

Table 5.1. CVD Mortality rates in 2007 for men dependent on age

<table>
<thead>
<tr>
<th>Age group (yrs)</th>
<th>CVD-related deaths in men for 2007 (n)</th>
<th>Percentage of total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 35</td>
<td>129</td>
<td>0.25</td>
</tr>
<tr>
<td>35-44</td>
<td>783</td>
<td>1.5</td>
</tr>
<tr>
<td>45-54</td>
<td>2679</td>
<td>5.2</td>
</tr>
<tr>
<td>55-64</td>
<td>6687</td>
<td>13.0</td>
</tr>
<tr>
<td>65-74</td>
<td>11335</td>
<td>22.1</td>
</tr>
<tr>
<td>75+</td>
<td>29760</td>
<td>57.9</td>
</tr>
<tr>
<td>Total</td>
<td>51373</td>
<td></td>
</tr>
</tbody>
</table>


As it has been demonstrated that fatty streaks are present in younger age-groups, investigating the impact of lifestyle changes on the onset and progression of CVD risk in these populations may be of great use in future public health strategy, especially given the policy shift from treatment to prevention.

As previously described in Section 4.1.1, there is a large body of epidemiological data pointing towards increased wholegrain consumption benefiting CVD risk factors. In combination with this work, the Bogalusa Heart Study found that those young people (aged 19-38) who were in the highest quintile for dietary whole grain (as part of a dietary pattern also containing fruits, vegetables, refined grain products, legumes and nuts) had a hazard ratio of 0.64 (>12.1 portions per day) for heart disease risk as opposed to those in the lowest quintile with a HR of 1.00, <6.2 portions per day (Yoo et al., 2004). Although not as specific nor as convincing as the data from the Nurses’ Health Study (Liu et al. 2000) and the Insulin Resistance Atherosclerosis Study (Mellen et al. 2007), this study does raise the possibility that younger people may also benefit from the same healthy eating advice as the older population receive, crucially for the same reason: to reduce the incidence of CVD-related mortality and morbidity.

5.1.2 Justification for study

There is currently little firm evidence to support the CVD risk reduction potential of whole grains in young adults.

Chapter 4 describes the research undertaken with older adults including a summary of the current evidence base, the justification for such research and the resulting analysis of the data from the High Risk Study. In order to extrapolate these results to the wider population and extend the scope of current whole grain research, and specifically given the evidence of earlier cardiovascular impairment (fatty...
streaks) in younger adults, it was felt important to investigate the protective effect of whole grains in a healthy, low risk population. This is especially pertinent given the current focus of public health initiatives within the UK on more proactive approaches such as early obesity prevention strategies like the ‘Change4Life’ campaign; a Department of Health initiative launched January 2009.

5.1.3 Aims and objectives
The overall aim of this study was to investigate the effects of increasing the amount of whole grain in the diet to 48g per day (in an intact form) versus an equivalent amount of milled whole grain and a control (refined grain) on markers of CVD risk in young men (who are assumed to be at low risk of CVD based on clinical parameters).

The main study objectives were to investigate the effects of the different grain supplementation protocols on metabolic and physiological parameters specifically:

- Arterial stiffness, as assessed by Pulse Wave Velocity.
- Diurnal blood pressure profiles and endothelial function as assessed by 24hr ambulatory blood pressure.
- Insulin resistance and glucose tolerance, as assessed by fasted plasma glucose and insulin levels.
- Lipid profile, including total, HDL and LDL cholesterol, triglycerides and non-esterified fatty acids (NEFA).
- Anthropometrics and static blood pressure, as markers of general health

5.1.4 Hypothesis
Increasing the amount of whole grains (both intact and milled) in the diet will beneficially influence CVD risk factors when compared to refined grain in young men at apparently low risk of developing CVD. It is expected that this will occur due to an improvement in insulin sensitivity and a reduction in arterial stiffness, as demonstrated by reductions in HOMA-IR and PWV.

5.2 Study methodology
5.2.1 Study design
The study was a 3-way, parallel, controlled, randomised dietary intervention study in young men aged 21-29yrs. Participants consumed either two whole grain, milled grain or refined (control) grain rolls for 8 weeks. The whole grain was sourced from wheat, with the intact and milled grain rolls containing 48g of whole grain per two rolls – see Fig. 4.3, Section 4.2.2. The participants attended two study sessions; one at the beginning (Day 0), and one at the end (Day 56) of the intervention – see Figure 5.1.
5.2.2 Participant selection

Based on sample size calculations for the previous chapter (4-The High Risk Study) we aimed to recruit the same number of participants (or more if possible, i.e. >7 per intervention group) as the High Risk Study due to the shared aim of studying the effects of whole grains on the risk of CVD over time using a very similar study design.

Prior to commencing, the study received a favourable ethical opinion from the University of Surrey Ethics Committee – EC/2006/89/SBMS.

Participants suitable for inclusion:

- Adult males aged between 21-29 years
- Healthy weight - body mass index 20-25 kg/m²
- Waist circumference less than 94 cm (37 inches)

A standard exclusion criteria was applied during the recruitment phase and is described in Section 2.1.3. The participants were recruited from the University of Surrey campus via global email and posters, in addition to publicising the study to the local population by poster and newspaper advertisement. Local businesses and public departments were also contacted.

5.2.3 Randomisation

Participants were randomised into one of the three groups via the web-based randomisation software available at www.randomization.com.

5.2.4 Study protocol

The week prior to attending the first study session, participants were requested to complete a 3-day diet diary. For the evening before the study session a standardised meal (as per choices for High Risk Study) was consumed and followed by a 12 hour fast (overnight). It was also advised that participants refrained from strenuous exercise or consuming alcohol the day before the study.

5.2.4.1 Study session – Day 0

On the first day (Day 0) participants attended the Clinical Investigation Unit (CIU) based at the Faculty of Health and Medical Sciences at the University of Surrey, in the early morning. After
confirming with the participant that they were in a fasted state, happy to proceed and understood the activities of the session, anthropometric measurements were taken – height, weight, body fat percentage, body mass index, waist circumference, hip circumference (see Section 2.3.1-3). They were then invited to rest in a quiet room with dimmed lighting for ten minutes in preparation for the PWV measurements. After the resting phase, triplicate recordings of static blood pressure were taken (see Section 2.3.4) and the process of recording PWV was commenced (see Section 2.5.2).

Since the participant was in a safe and convenient supine position post-PWV, venepuncture (see Section 2.4.1) was completed straight away, with a single, fasted 30 ml sample obtained. For every blood sample drawn throughout the study, the blood was processed into SST, EDTA and fluoride oxalate tubes (see Section 2.4.4) to provide the appropriate amount of serum and plasma samples to enable the thorough analysis as planned.

Once the fasted baseline blood sample had been drawn, the participant was trained with the ambulatory blood pressure equipment (Section 2.5.1), which they were required to wear for the 24hrs directly after the study session.

Once all activities were completed for the study, the participants were offered refreshments and allowed to leave the research unit. The ambulatory blood pressure monitors were returned the following day; the participants then commenced the study by consuming the bread rolls that they were assigned to for the 8 week duration of the intervention. Participants were encouraged to incorporate the rolls into their habitual diet (for example by adapting their lunch by substituting their routine bread for the study rolls) as opposed to consuming them in addition to their habitual diet. There were no restrictions on what study rolls could be consumed with and the rolls could be warmed before eating, if necessary.

5.2.4.2. Participant follow-up and completion of study

As per the High Risk Study (see Section 4.2.4.2), participants were followed up on a regular basis to maximise compliance and ensure sufficient supplies of the study rolls. Packaging was checked for any uneaten rolls (none reported) and a 3-day diet diary was completed by the participants during the final week of the intervention to assist in the assessment of the overall compliance and level of roll consumption. On the last day of the study (post-intervention assessment day) the participants returned to the CIU for the final study session identical to 'Day 0'.

5.2.5 Statistical analysis

Detailed statistical analysis focussed on the effects of the intervention over time therefore baseline lipid, glucose, insulin, PWV, ambulatory blood pressure and anthropometric results were compared as pre versus post-intervention via pairwise comparison. Prior to commencing all analysis, data was
tested for normality using the Kolmogorov-Smirnov test, with non-parametric statistical tests used as appropriate for non-normally distributed data.

One-way analysis of variance (ANOVA) was also performed at baseline to detect any differences between groups. If significant differences were detected, a one-way analysis of covariance was performed to control for this situation with the appropriate post-hoc testing.

This intervention study was performed in collaboration with Miss Nicola Muirhead (NM); each researcher had their own individual, specific objectives and retrieved samples and data to reflect that. Responsibility for the running of study sessions and the tasks involved were divided equally between the two co-investigators. The exception to this was the PWV which was performed solely by the author due to the need to minimise inter-observer bias; and ambulatory blood pressure. Laboratory tasks were divided equally for the analysis of samples for the entire study cohort, with all data (except PWV and the ambulatory blood pressure) made available to the co-investigators. Tasks were completed as follows: Insulin analysis – LT; Lipid profile – NM. All data analysis and inferences thereof are the authors own work.

5.3 Results

For this study 25 young men were recruited who met the recruitment criteria (Section 5.2.2) with a mean age of 22.8yrs±2.0, a body mass index (BMI) of 23.5kg/m²±3.0 and waist circumference of 85.69cm±7.1 (see Table 5.2). Nine males were recruited to the whole grain group, eight to the milled grain group and eight to the control group. The drop-out rate was 7.4% with two participants not completing the study due to personal reasons. As the study was in the latter stages, further participants were not recruited.

<table>
<thead>
<tr>
<th>Study outcome</th>
<th>n</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>24</td>
<td>22.8±2.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>24</td>
<td>75.7±10.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24</td>
<td>23.5±3.1</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>24</td>
<td>85.4±7.1</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>24</td>
<td>122.3±10.0</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>24</td>
<td>62.5±6.9</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>22</td>
<td>5.1±0.4</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>22</td>
<td>4.0±0.8</td>
</tr>
</tbody>
</table>

Note: Descriptive statistics at baseline for the entire cohort – all participants were male.

5.3.1 Anthropometrics

As shown in Table 5.3, no significant differences were found between the three groups at baseline except for BMI ($p=0.05$). There was a statistically significant difference in BMI between the whole grain (Median: 20.5kg/m², 3.7 IR) and milled grain group (25.0kg/m², 3.5 IR, $p=0.02$), with a large
effect size from the milled grain group ($r=0.55$). This was further investigated using a one-way analysis of covariance (ANCOVA) to determine whether the post-intervention results were compromised. Controlling for the pre-intervention BMI results, it was found that there was no significant difference between groups for post-intervention BMI, 2.6% of the variance in post-intervention BMI was explained by the intervention groups. The ANCOVA was repeated on waist circumference (WC), body fat percentage (BF) and weight to determine whether the baseline difference in BMI had had an effect on the post-intervention results. After controlling for pre-intervention BMI as a covariate, the ANCOVA detected no significant differences between the groups for WC, BF and weight, therefore there was no effect from BMI on the other anthropometric outcomes.

There was no significant difference for the anthropometric markers when comparing pre versus post-intervention within the intervention groups. Nor when comparing between groups for the percentage change; no significant differences were found between the groups for any anthropometric parameters.

### 5.3.2 Pulse Wave Velocity (PWV)

The primary outcome for this study was Pulse Wave Velocity, supported by the blood pressure-related indices Mean Arterial Pressure (MAP) and Pulse Pressure (PP).

#### 5.3.2.1. PWV

Table 5.4 shows that at baseline, there were no significance differences between the three groups for either radial-carotid (R-C), or carotid-femoral (C-F) PWV.

As shown in Figure 5.2 and Figure 5.3 the eight-week intervention appeared to induce no significant changes in R-C PWV (peripheral) or C-F PWV (central) for any intervention group, with the exception of the whole grain group who experienced a significant reduction in Carotid-Femoral PWV (see Table 5.4, grouped median data shown in Figure 5.3). As shown in Figure 5.4, there is a mean reduction in C-F PWV, with Figure 5.5 confirming that the majority of the individual wholegrain participants had a reduction in C-F PWV over time.

Comparing pre to post-intervention results for the milled and control groups, no differences were detected. Median results for pre and post-intervention with the interquartile range (IR) are as presented in Table 5.4. Post-intervention there were increases in R-C PWV for the milled grain group as well as for C-F PWV however both results were not statistically significant. The control group experienced only minor, non-significant fluctuations in C-F PWV and no change for the R-C PWV. When comparing the percentage change over time, there were no significant differences between groups.
Table 5.3. Anthropometric data from the three intervention groups of the Low Risk Study – Pre versus post intervention

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Whole grain n = 9</th>
<th>Milled grain n = 8</th>
<th>Control n = 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Mean ± SD</td>
<td>Post Mean ± SD</td>
<td>Pre Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>22.67 ± 2.06</td>
<td>23.63 ± 2.07</td>
<td>22.00 ± 1.77</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.10 ± 10.22</td>
<td>71.82 ± 10.46</td>
<td>81.76 ± 10.34</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.80 ± 2.67*</td>
<td>22.04 ± 2.69</td>
<td>25.25 ± 2.83*</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>84.80 ± 7.72</td>
<td>84.23 ± 7.04</td>
<td>86.78 ± 8.19</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>13.60 ± 4.24</td>
<td>14.07 ± 4.70</td>
<td>18.05 ± 4.60</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>122.33 ± 10.01</td>
<td>119.44 ± 7.80</td>
<td>118.50 ± 10.47</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>63.89 ± 7.74</td>
<td>62.33 ± 7.40</td>
<td>60.50 ± 5.48</td>
</tr>
</tbody>
</table>

Note: *A Kruskal-Wallis test detected no significant differences at baseline between the three intervention groups, except for BMI between the whole and milled grain group (p=0.05). The effect was detected to be from the milled grain group, with a median of 25.0 kg/m² compared to 20.5 kg/m² for the whole grain group, however the effect was not significant after Bonferroni correction. An ANCOVA was completed, with pre-intervention BMI as a covariate, and showed that the difference in BMI at baseline did not have any effect on post-intervention anthropometric results. No significant changes were detected when comparing each group pre vs post-intervention. A one-way-between-groups ANOVA was performed to compare percentage change (pre vs post-intervention) between the three intervention groups for all anthropometric outcomes – no significant changes detected. Key: BMI – body mass index; WC – waist circumference; BP – blood pressure; %Δ - percentage change (pre vs post).

Table 5.4. Pulse wave velocity data from the three intervention groups of the High Risk Study – Pre versus post intervention and percentage change

<table>
<thead>
<tr>
<th>PWV Route</th>
<th>Intervention group</th>
<th>Pre Median (IR)</th>
<th>Post Median (IR)</th>
<th>Sig.</th>
<th>%Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-C (m/s)</td>
<td>Whole grain</td>
<td>7.0 (1.8)</td>
<td>7.0 (2.5)</td>
<td>0.18</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>7.4 (0.9)</td>
<td>7.7 (1.7)</td>
<td>0.89</td>
<td>-0.6</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.1 (2.7)</td>
<td>7.1 (0.9)</td>
<td>0.87</td>
<td>2.1</td>
</tr>
<tr>
<td>C-F (m/s)</td>
<td>Whole grain</td>
<td>5.8 (1.0)</td>
<td>5.5 (0.9)</td>
<td>0.02</td>
<td>-5.2</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>5.9 (1.0)</td>
<td>6.2 (0.7)</td>
<td>0.62</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5.7 (1.5)</td>
<td>5.8 (1.1)</td>
<td>0.40</td>
<td>-3.9</td>
</tr>
</tbody>
</table>

Note: A Kruskal-Wallis test detected no significant differences at baseline between the three intervention groups. A Wilcoxon Signed ranks test detected no significant changes in either R-C or C-F PWV for any intervention group over time. A further Kruskal-Wallis detected no significant changes when comparing percentage change between groups for both R-C and C-F PWV.
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A Wilcoxon Signed Ranks test indicated no significant change in PWV over time for the peripheral PWV measurement for all intervention groups.

- outlier of 1.5 box lengths from the edge of box.
- extreme outlier, more than 3 box lengths from edge of box.

Pre: wg-9, mg-8, control-8
Post: wg-8, mg-8, control-7

Key: WG - whole grain group; MG - milled grain group; CON - control group

Figure 5.2. Radial-Carotid (peripheral) PWV before and after an 8-week intervention comparing whole grain, milled grain and control in healthy young men.

A Wilcoxon Signed Ranks test indicated a significant change in PWV over time for the central PWV measurement for the whole grain group only - $p=0.02$

- outlier of 1.5 box lengths from the edge of box.

Pre: wg-9, mg-8, control-7
Post: wg-9, mg-8, control-7

Key: WG - whole grain group; MG - milled grain group; CON - control group

Figure 5.3. Carotid-Femoral (central) PWV before and after an 8-week intervention comparing whole grain, milled grain and control in healthy young men.
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Note: A Wilcoxon Signed Ranks test indicated a significant change in C-F PWV over time for the whole grain group \( p = 0.02 \).

Figure 5.4. Carotid-Femoral PWV for the low risk whole grain intervention group – group results

Figure 5.5. Carotid-Femoral PWV for whole-grain intervention group – individual results
5.3.2.2. Pulse pressure

Table 5.5 shows the results for pulse pressure for both pre and post-intervention. There was no significant difference in pulse pressure (PP) comparing between groups at baseline, nor pre vs. post intervention within the groups. The percentage change between the two study visits was also calculated and no significant difference in percentage change was found between the groups (Figure 5.4).

<table>
<thead>
<tr>
<th>PWV Route</th>
<th>Intervention group</th>
<th>Pre-intervention Median (IR)</th>
<th>Post-intervention Median (IR)</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP</td>
<td>Whole grain</td>
<td>83.0 (6.5)</td>
<td>82.3 (6.7)</td>
<td>-2.1</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>81.0 (8.2)</td>
<td>79.2 (9.9)</td>
<td>-0.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>82.8 (7.7)</td>
<td>84.2 (8.8)</td>
<td>1.3</td>
</tr>
<tr>
<td>PP</td>
<td>Whole grain</td>
<td>60.0 (16)</td>
<td>58.0 (21)</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>Milled grain</td>
<td>60.0 (15)</td>
<td>58.0 (14)</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>57.5 (26)</td>
<td>60.0 (21)</td>
<td>-2.3</td>
</tr>
</tbody>
</table>

Table 5.4: For both MAP and PP: a Kruskal-Wallis test detected no significant differences at baseline between the three intervention groups. A Wilcoxon Signed Ranks test detected no significant difference over time for any intervention group. No significant difference was found when comparing the percentage change between groups.

5.3.2.3. Mean arterial pressure

As per PP, Table 5.5 also depicts the data collected for mean arterial pressure (MAP) as a further index of cardiac function. No significant differences were detected at baseline between the groups or over time within groups. There were also no significant differences in percentage change in MAP (pre to post intervention) between the groups (Table 5.5).

5.3.3 Ambulatory blood pressure

Figures 5.6-5.9 show the ambulatory blood pressure recordings (mean±SD), both systolic and diastolic blood pressure are split depending on whether the data was collected during the day or night. For the initial analysis, when comparing between groups at baseline, no significant differences were detected except for the pre-intervention night diastolic blood pressure (NDBP) ($\chi^2 = 5.96$, $p=0.05$). There was a significant difference detected in NDBP between the milled grain (median: 56mmHg, 8IR) and the control group (69mmHg, 16IR, $Z=-2.09$, $p=0.04$), with a large effect size from the control group ($r=0.54$). As for all previous results that have differed at baseline, a one-way analysis of covariance
(ANCOVA) was completed to determine whether the intervention results were compromised by the significant difference at baseline for this variable. Controlling for the baseline differences in pre-intervention 24hr NDBP (as the covariate), there was no significant difference between groups for post-intervention 24hr NDBP.

When comparing pre vs. post-intervention for both day and night, little variance is noted for any intervention group.

As shown in Figure 5.6, for day-time systolic blood pressure (DSBP) no significant changes were detected for any intervention group over time, with minimal shift in results for all groups. The milled grain group did however show an increase in DSBP from a median of 116.9mmHg (8.0SD) pre-intervention to 119.9mmHg (8.3SD) post-intervention that was approaching significance - Z=-1.80, p=0.07. Likewise, as shown in Figure 5.8, for the night-time systolic blood pressure (NSBP) no significant changes were detected for any of the intervention groups, with again minimal shift in values after the intervention.

The whole grain group experienced a reduction in NSBP from a mean value of 112.0mmHg (5.69SD) pre-intervention to 109.9mmHg (7.13SD) at post-intervention (ns). The milled grain group experienced an increase in NSBP from a mean result of 110.0mmHg (6.44SD) to 116.3mmHg (8.46SD) which was approaching significance (Z=-1.87, p=0.06).

For Figure 5.7 which shows the data for the day-time diastolic blood pressure (DDBP), no significant changes were detected for any intervention group over time, with again a minimal shift in results for all groups.

For the night-time diastolic blood pressure (NDBP) shown in Figure 5.9, no significant changes were detected for any of the intervention groups, with another minimal shift in values after the intervention. The whole grain group reduced from a mean score of 59.4mmHg (8.58SD) to 56.8mmHg (6.04SD), a difference that was approaching significance (Z= -1.87, p=0.06).
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Figure 5.6. Pre vs post-intervention 24-hr ambulatory systolic blood pressure – daytime hours.

Figure 5.7. Pre vs post-intervention 24-hr ambulatory diastolic blood pressure – daytime hours.

Note: A Kruskal-Wallis test detected no significant difference between the groups at baseline. No significant change over time was detected via a Wilcoxon signed rank test for any intervention group. The milled grain group showed a trend towards significance $p=0.07$. 
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Note: A Kruskal-Wallis test detected no significant difference between the groups at baseline. No significant change over time was detected via a Wilcoxon signed rank test for any intervention group, the milled grain group showed a trend towards significance $p=0.06$.

Figure 5.8. Pre vs post-intervention 24-hr ambulatory systolic blood pressure - night-time hours.

A Kruskal-Wallis test detected a significant difference between the groups at baseline ($p=0.05$). No significant change over time was detected via a Wilcoxon signed rank test for any intervention group, the whole grain group showed a trend towards significance $p=0.06$.

Figure 5.9. Pre vs post-intervention 24-hr ambulatory diastolic blood pressure - night-time hours.
5.3.4 Lipid profile

Table 5.6 shows results for the lipid profile monitored as part of the *Low Risk Study*—Total cholesterol (TC), HDL-Cholesterol (HDL-C), Triglycerides (TAGs), Non-esterified fatty acid (NEFA). No significant differences between the groups in baseline lipid profiles were detected with the exception of NEFA ($\chi^2 = 6.26, p = 0.04$) which was found to be significantly lower in the milled grain compared to the control group (control: median: 0.52mmol/l, 0.51 IR; milled grain: 0.29mmol/l, 0.12IR) group with a large effect ($r=0.6$).

When investigating the effects of the intervention over time, no significant changes in lipid profiles were detected for the whole and milled grain group. However, the control group were found to have a significant reduction in NEFA ($Z = -2.1, p = 0.04$). Given the significant difference at baseline for NEFA involving the whole and milled grain group, in addition to the significant reduction in the control group, a one-way analysis of covariance (ANCOVA) was performed to control for the baseline data and examine whether this confounds the post-intervention data. It was found that there was no significant difference between groups for NEFA at post-intervention.

5.3.5 Glucose and insulin profiles

As shown in Table 5.7, there was a significant difference between groups at baseline for glucose ($\chi^2 = 6.99, p = 0.03$); the significant difference was found to be between the whole and milled grain groups ($r=0.6, Z = -2.37, p = 0.02$). No significant within-group changes were detected when comparing pre to post-intervention for any intervention group for HOMA-IR, HOMA-%B, glucose and insulin. Following an ANCOVA of the post-intervention glucose results for the intervention groups and controlling for the pre-intervention glucose, no significant differences were detected between the groups. For the whole and milled grain groups, there was minimal variation between the pre and post-intervention results. In contrast, the control group experienced approximately 12% increases in fasting insulin post-intervention (ns) and subsequently increases in the linked index of the homeostasis assessment model for insulin resistance (HOMA-IR) (ns). The HOMA for beta cell function (HOMA-%B) also increased by more than 18% (ns).

5.3.6 Dietary analysis

Participants completed 3-day diet diaries at baseline and in the final week of the study (referred to as ‘end-of-intervention’) as a means of recording compliance and monitoring any changes in dietary patterns and intake.

Table 5.8 shows the pre- and post intervention dietary data for each group. The values for post-intervention (for all groups) include the two study rolls consumed per day which was confirmed via diet diary. Diet diary return by the participants was low (17 out of 25 participants completed both pre and post-intervention diaries); the completion and detail of the food items eaten within the diaries was
extremely poor. No significant differences were found at baseline between the intervention groups. No significant differences between the pre and post-intervention phases for either energy intake or any of the macronutrients were found for the whole and milled grain group. However the control group indicated significant increases in energy, protein, carbohydrate, dietary fibre and sodium post-intervention ($p=0.02$).
Table 5.6. Lipid profile per intervention group – pre vs. post-intervention

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Whole grain n= 9</th>
<th>Milled grain n= 8</th>
<th>Control n= 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>%Δ</td>
</tr>
<tr>
<td>TAGs (mmol/l)</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>%Δ</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>0.91 ± 0.61</td>
<td>0.72 ± 0.24</td>
<td>-20.83</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td>3.76 ± 0.60</td>
<td>3.79 ± 0.67</td>
<td>0.89</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.40 ± 0.13</td>
<td>0.34 ± 0.07</td>
<td>-16.87</td>
</tr>
</tbody>
</table>

Note: *A Kruskal-Wallis test detected a significant between group difference at baseline (p=0.04) for NEFA (milled versus control groups). *A Wilcoxon Signed Rank test calculated a significant reduction in NEFA pre vs. post-intervention (p=0.04) for the control group. For both the raw data and secondary percentage change analysis no significant differences for any other lipid outcomes were detected. Key: TAGs – triglycerides; TC – total cholesterol; NEFA – non-esterified fatty acids; HDL – High density lipoprotein cholesterol.

Table 5.7. Glucose, insulin, HOMA-IR and HOMA-% β profiles per intervention group – pre vs. post intervention

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Whole grain n= 9</th>
<th>Milled grain n= 8</th>
<th>Control n= 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>%Δ</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>%Δ</td>
</tr>
<tr>
<td>HOMA-%β</td>
<td>2.15 ± 0.92</td>
<td>2.12 ± 1.15</td>
<td>-1.29</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>127.68 ± 56.11</td>
<td>116.82 ± 43.18</td>
<td>8.5</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>59.01 ± 21.87</td>
<td>57.74 ± 28.39</td>
<td>-2.15</td>
</tr>
</tbody>
</table>

Note: * A Wilcoxon Signed Rank Test found a significant difference between the whole and milled grain groups at baseline for glucose, p=0.02. When comparing pre vs. post-intervention within groups, no significant changes were found for any of the outcomes, nor when analysing the percentage changes from baseline between groups. Key: HOMA – Homeostasis Assessment Model; IR – Insulin resistance; % β – β-cell function (insulin sensitivity).
Table 5.8. Mean daily nutrient intakes (baseline versus end-of-intervention) for all intervention groups enrolled in the low risk study

<table>
<thead>
<tr>
<th></th>
<th>Whole-Grain n=7</th>
<th>Milled-Grain n=4</th>
<th>Control n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre (Mean±SD)</td>
<td>Post (Mean±SD)</td>
<td>%Δ</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>10246.7±2030.5</td>
<td>11008.2±2144.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Total Fat (g/d)</td>
<td>103.2±26.5</td>
<td>87.1±21.3</td>
<td>-15.6</td>
</tr>
<tr>
<td>% energy from fat</td>
<td>37.3</td>
<td>29.3</td>
<td>-</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>80.3±15.8</td>
<td>91.0±9.7</td>
<td>13.3</td>
</tr>
<tr>
<td>% energy from protein</td>
<td>13.3</td>
<td>14.0</td>
<td>-</td>
</tr>
<tr>
<td>CHO (g/d)</td>
<td>283.7±69.5</td>
<td>337.6±66.9</td>
<td>19.0</td>
</tr>
<tr>
<td>% energy from CHO</td>
<td>44.3</td>
<td>49.1</td>
<td>-</td>
</tr>
<tr>
<td>DF (g/d)</td>
<td>19.1±7.3</td>
<td>25.1±6.0</td>
<td>30.9</td>
</tr>
<tr>
<td>Sodium (mg/d)</td>
<td>3357.0±502.6</td>
<td>3545.5±596.6</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Note: The values for post-intervention (for all groups) include the two study rolls consumed per day which was confirmed via diet diary. There were no significant differences detected when comparing between groups at baseline. Comparing pre to post intervention, no significant differences were detected for either the whole or milled grain groups. The control group had significant increases in energy, protein, CHO, DF and sodium post-intervention. Key: CHO - carbohydrate; DF - dietary fibre.
5.4 Discussion

5.4.1 Key findings

The aim of this study was to determine if a prolonged and consistent period of whole grain consumption could influence CVD risk factors, focussing particularly on arterial stiffness and endothelial function, in young and healthy adult males. Pulse wave velocity (PWV), 24hr ambulatory blood pressure and a series of biochemical markers were used to monitor the physiological effects of the dietary intervention.

The key finding of this study was that the group consuming whole grains (48g per day) for eight weeks, significantly reduced central PWV over the course of the intervention, assessed by carotid-femoral (central) PWV. Although the pre-intervention result for the whole grain group was within the healthy range for PWV, a further reduction demonstrates a beneficial effect of the whole grain intervention. No such reduction in C-F PWV was found in the milled grain or refined grain groups. It is certainly interesting that C-F PWV reduced within the whole grain group when they, like the rest of the cohort, were already considered to be at low risk. Elucidating a mechanism for this result is very difficult as this finding does not coincide with any other significant changes within the whole grain group for any other study outcome or with the High Risk Study (Chapter 4). There are no significant changes in blood pressure nor for MAP or PP, which if they had changes would have been indicative of changes to blood flow and pressure and therefore help to piece together a pathway linking whole grain consumption with arterial stiffness, inflammation and other markers of CVD risk.

In addition, whilst all intervention groups were normoglycaemic, their raised fasted insulin levels and HOMA-IR scores suggest that there was a degree of insulin resistance already occurring in these young men. A possible theory is that the insulin secretion may, even at this young age, already be compensating for low-level insulin resistance, thus starting the metabolic spiral that could lead to metabolic syndrome, diabetes or CVD. In a positive light, insulin levels were reduced (although not significantly so) post-intervention for the whole and milled grain groups and this was in-line with reductions in C-F PWV for the whole grain group. This is suggestive of a possible metabolic change that may benefit from further investigation.

As has previously been described in Section 1.2.1, the development of insulin resistance is linked to excess adiposity around the abdominal region. Obviously within this cohort, the participants are fit and healthy with a normal waist circumference and BMI. Therefore the mechanism between abdominal adiposity and insulin resistance facilitated by inflammatory markers may not hold in this instance. It is difficult to determine what pathway may be linking the reduction in PWV and fasting insulin since the abdominal adiposity is not present. However, a study by Pereira et al. (2002) found similar results of reduction in insulin resistance with no concomitant reduction in weight. They suggested that since
whole grains are high in fibre, the structure of the grain may help to slow gastric emptying and thus lower the overall glycaemic response (Pereira et al. 2002). Over the course of the intervention, as the participants consistently consume the whole grains, Pereira et al. hypothesise that it is the consistent lowered glycaemic response to the whole grains that reduces the need for insulin to such an extent that insulin receptors at the cell-surface are up-regulated. If this mechanism applies to the Low Risk Study then it could tie in with the role that insulin resistance has in arterial stiffness. It has been reported within the literature that long-term exposure to hyperinsulinaemia or even impaired glucose regulation in general is linked to a dramatic remodelling within the lumen of the resistance arteries (Rizzoni et al., 2001). The study by Rizzoni et al. (2001) on a cohort diagnosed with non-insulin dependent diabetes, hypertension or a combination of the two, in addition to a normotensive group found that there was a significant correlation between fasting insulin and the extent of vascular remodelling (i.e. media-to-lumen ratio) within the arteries of the participants. In addition to this evidence, it has also been found that the presence of insulin resistance is linked with vascular growth factors that are implicated in the structural alteration of the arterial walls (mainly via smooth muscle cells) (Nickenig and Bohm, 1998). Therefore the results from the Low Risk Study present an interesting avenue to explore due to the clear and significant reduction in central PWV. In addition there was also a reduction in fasting insulin levels (albeit not significant) within the whole grain group which may be indicating that there is a possibility (based on the evidence present above) that the reduction in insulin could have influenced the level of arterial stiffness by slowing the hypertrophy of vascular smooth muscle cells over the course of the intervention. This is only a hypothesis at this point however, and has raised more questions that can be answered as to whether this mechanism exists and if so, is it likely to be found within a cohort of young and fit males. However the implications of this result cannot be ignored and so it is only prudent to pursue an avenue of research to explore the relationship between whole grain consumption, insulin resistance and arterial stiffness further.

No other effects were found for the cohort in terms of vascular markers (blood pressure, ambulatory blood pressure) or metabolic markers such as the lipid profile and glucose. At both pre-intervention and post-intervention, the mean results per intervention group indicated that the participants were within the healthy ranges for all outcomes. Comparing the results of this study to the evidence within the literature is difficult as very few studies have looked at those population groups at low risk of CVD.

Only one study in the current literature has investigated the effects of whole grains on the health of young men via an intervention study. Enright & Slavin (2010) designed a cross-over study (14 days per leg, no wash-out) to monitor the effects of a whole grain versus refined grain diet on 10 males and 10 females; focussing specifically on antioxidant status as opposed to cardiovascular markers. With a mean age of 27.1 ± 4.0 years and mean BMI of 23.9 ± 3.3 kg/m², the participants in this study and the
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Low Risk Study presented here are very similar in age and body composition. Using a whole food substitution method for their intervention and measuring blood and urine samples at regular intervals, no significant changes for any markers were detected. This is in contrast to the Low Risk Study where a significant reduction in central PWV was detected. This difference in study outcomes could be accounted for by the relatively short duration of the Enright & Slavin trial, whilst the mixed gender sample may have also been ill-advised given the small total sample size. The delivery of whole grains was also very different, with the Low Risk Study using an innovative concept of providing the study participants with the exact amount of whole grain to be eaten each day via ready-prepared bread rolls (see Section 4.2.1) as opposed to whole-food substitution that relies more heavily on the participants’ understanding of portion sizes and appropriate food substitutions, if food is not directly supplied by the researchers.

Aside from this study, the only other relevant comparisons that can be made with the available literature are with the longitudinal epidemiological data derived from studies assessing dietary intakes and CVD risk in adolescents and young adults.

5.4.2 Evidence from epidemiological studies

Firstly, assessing CVD risk in young adults, the ‘Cardiovascular Risk in Young Finns Study’ (Aatola et al., 2010) aimed to establish how risk factors in early life may be influencing PWV in later years. This study found that, based on data recorded at a young age, systolic blood pressure and glucose levels appeared to be predictive of PWV in later life, as too was age and gender. A reduction in CVD risk factors and obesity during childhood translated into a lower PWV in adult life. This study, in addition to the data obtained in the Low Risk Study, helps to build the argument that early intervention to reduce CVD risk factors in the childhood years, if not when as a young adult, could help to reduce the risk of CVD in later life. However, with such hypotheses, care must be taken as this is based on only a relatively small amount of evidence. Yet this is especially pertinent when as McGill et al. (2000) and McMahan et al. (2006) both evidenced that atherosclerosis (mainly in the form of fatty streaks) is present from childhood.

5.4.3 Current intakes of whole grains in young adults

From a dietary aspect, a number of studies have been conducted observing and quantifying the habitual wholegrain intakes of young adults. One particular study by Burgess-Champoux et al. (2010) found that adolescent males (studied in Minnesota, USA), were particularly low consumers of whole grains on a daily basis. Only 11.3% of the male study population consumed >1 serving of whole grains on a daily basis; the majority (third quartile) of 42.2% consumed >0.35-1.0 servings per day, and 11.9% regularly consumed no whole grains. When studied over five years, there was no significant increase in whole grain consumption (Burgess-Champoux et al.). Making the link between this intake data for young adults and health, the Bogalusa Heart Study (Deshmukh-Taskar et al., 2009) showed
that whole grains consumed as part of a 'prudent dietary pattern' were inversely associated with waist circumference, plasma insulin, TAGs and the prevalence of the metabolic syndrome. Finally, the current data regarding adult consumption of whole grains in US young adults points towards an average of only 1 portion of whole grains per day. Therefore, with poor intakes of whole grains apparently running through from childhood to adulthood, further research to pinpoint the mechanism of the apparent benefits whole grains can infer as shown in the Low Risk Study must be undertaken. This is especially important since the findings of Burgess-Champoux et al. (2010) are similar to that from the participants of this study who were also poor consumers of whole grains (low dietary fibre consumption evidenced by 3-day diet diaries). Therefore the significant result found for C-F PWV becomes even more critical and should justify an increase in research to investigate the underlying mechanism for wholegrain-associated CVD risk reduction in young adults.

5.4.4 Comparison of the effects of whole grain between high and low CVD risk population groups

As discussed in Chapter 4.4, the main effects of the whole grain intervention in the older and 'at risk' cohort (the High Risk Study) was found in diastolic blood pressure, mean arterial pressure and pulse pressure. There was also an increase in IL-1β for the whole grain group and a reduction in waist circumference for the milled grain group. In contrast the Low Risk Study found no effect on blood pressure and inflammatory markers were not analysed in this cohort. This difference in the effects that the whole grains exerted between the risk groups possibly indicates that there are at least two pathways to which whole grains may 'use' to reduce the risk of CVD over time. If this is the case, then there needs to be further research to determine if this difference in mechanisms is dependent on non-modifiable or modifiable risk factor differences identified between the groups. For example, there is clearly a large age gap between the two cohorts but gender is not an issue as the high risk cohort were 71% male and the low risk cohort were all male. For modifiable risk factors, there is the difference in weight and waist circumference and activity. The low risk cohort, although not deviating from the inclusion criteria, were noticeably more active in their daily life than the high risk cohort. Overall it was expected that even though there is a clear difference between the risk of CVD for the two groups, that there would be a clear response from the high risk group to the whole grain intervention which would then be mimicked to a lesser degree by the low risk group. Therefore a close assessment to modifiable and non-modifiable risk factors in future research is required to separate out the reasons why the two cohorts apparently responded differently to the whole grain intervention.

The main difference between the study designs for the low and high risk studies is the assessment of inflammatory markers. Given the possible pathway linking insulin concentrations and arterial stiffness, it perhaps would have been beneficial to monitor the markers involved in vascular remodeling (such as matrix metalloproteinases, or vascular endothelial growth factor), or even just a
general marker for systemic inflammation such as CRP may have been informative. However, since this was not deemed necessary at the commencement of the study due to the limited available resources and primary need to focus on the inflammatory process within those at higher risk of CVD, an opportunity for future research has been created to identify whether there is a degree of inflammatory marker involvement at this early stage of possible CVD risk development. Although the inflammatory markers chosen would have to be carefully considered and measured given that even in those at high risk (as in the High Risk Study) were found to have no real evidence of a coordinated inflammatory cascade supposedly driving the risk of CVD even higher.

No studies comparing the CVD risk profile as has been done between the Low and High Risk Studies were identified within the literature. However, the differences in response from the two groups following a near identical study design shows that there is a large gap in the knowledge of how whole grains may affect health across the full diversity of the population.

5.4.5 Study limitations

Dietary analysis

An issue that was noted during the course of this study was the relatively poor return and quality of diet diaries that should have been completed at the time of the study visits (3-day diet diaries). Other than providing a record of consumption of the assigned study foods, the diet diaries also help to monitor, and therefore control for, variability in dietary intake. If variances occur in the habitual dietary intake between the pre and post-intervention phases, this may have repercussions for study outcomes such as weight, BMI, waist circumference, lipid profiles and the glycaemic response. For this study there was 68% return rate which accounted for 17 participants completing the diet diaries, however only four of those were in the milled grain group with seven in the whole grain and six in the control group. As shown in Section 5.3.6, there were no significant differences found between the pre and post-intervention diet diaries that were returned for the whole and milled grain group. Yet the control group demonstrated significant increases in all nutrients reported except for fat.

The main issue noted for the diaries when assessing qualitatively was the apparent propensity for a vast majority of participants to consume large quantities of only a small range of food and drink items. For example, entire pizzas were consumed in one sitting, 2pints of whole milk were drunk on a twice daily basis by one participant and often there would be reports of large quantities of alcohol being consumed (≈15units in one sitting). All participants ate meals at erratic times and frequency with a preference for high fat foods and carbonated drinks. Portion sizes were large and very few participants consumed fruit or vegetables. Compared to the diet diaries retrieved from both the High Risk Study and the Cross-Over Study, the diaries for this particular study are extraordinary and could potentially indicate the habitual diets of the participants as a confounding factor. The lack of routine noted in
dietary intake for almost all diaries makes comparisons pre to post-intervention difficult. This is in addition to the issue of poor detail in their completion.

Despite the concerns regarding the dietary intake of participants and detail in the diaries, when comparing energy intake to estimated energy requirements (including PAL 1.55) the discrepancies are only between 7 and 15% which is an interesting finding and may indicate that there is a degree of accuracy in the diet diaries after all. The clear recording of the consumption of the rolls is also reassuring with all participants who returned diaries, indicating consumption of rolls. Albeit that there was an approximate 50/50 split in those that ate all rolls required and those that ate four out of the six required over the average of the three days. This consistency in the recording of the consumption of study certainly indicates a good level of compliance from the participants.

With the evidence of compliance and apparent low levels in under-reporting of energy intake, there is good reason to assume that the diet diaries presented are a fair representation of the dietary intake of the study participants. However, the actual food choices of the participants are concerning as they have resulted in extremely high levels of salt within the diet (8.8-11.1 g per day) and the fact that the participants have such erratic eating habits compared to the participants within the High Risk Study and the Cross-Over Study could lead to concerns over the validity of allowing such individuals to participate in dietary intervention studies. Consistent habitual intakes of the participants are important in order to allow comparisons to be drawn and indications to be highlighted as to how the dietary modifications from the study have impacted on the lives of the participants. Erratic diets may mask real developments and so makes dietary research difficult. In order to reduce this source of potential error, it may be advisable for potential participants’ diets to be assessed using key indicators, prior to entry to a study in order to reduce the risk of this issue recurring. In addition to the preliminary assessment of habitual diets, for future studies, diet diary return and quality of completion would be one of the top priorities to assist the monitoring of compliance issues which could then be fully accounted for as part of the study analysis.

Power calculation

Another point to consider is that there was no power calculation for this study and therefore the study may be under-powered. However, as a significant result was found for central PWV, it may be possible to calculate an initial sample size based on that. From Liang et al. (1998), they compiled a data-set from a repeatability study to enable a guide for researchers as to the appropriate numbers of participants to recruit based on the study design of their proposed research. They report that in order to detect a 10% variation in PWV within a 2-sided parallel study design, 40 participants are required. Clearly this study did not meet that target of recruitment (Low Risk Study, n=25) however for future reference, this could be a reasonable target for recruitment. It is worth noting that Liang et al. also
suggest to detect a 15% variation in the PWV a group of 18 participants is required and for a 5% variation 156 participants would be required. Presumably they are calculating these numbers of participants on the basis that the smaller the numbers, the larger the influence natural variation within the group may have. Therefore it is still important to ensure adequate participant numbers to ward against any issues of Type 2 error.

Linking in with the possible issues of type 2 error, the final point is the possibility that the results shown within this study are a false positive. It is an unlikely scenario that the increased consumption of whole grains would have a wide-scale effect in reducing CVD risk within a cohort so young and healthy. The finding of a reduction in central PWV insinuates that there are real and lasting changes taking place at the endothelium. Therefore to understand further how and why whole grains apparently reduce arterial stiffness in young men, then there should be a focus on long-term studies (i.e. six months or more) that can periodically monitor CVD risk factors to determine whether the effect is a progressive change towards improved health or more of an acute, temporary change within the arterial structure.

5.4.6 Conclusion
The study presented has important findings regarding the potential effects whole grains may have on lowering the risk of CVD events even within an apparently low risk population (young healthy adult males). However the study cannot tell us whether risk is only reduced acutely or whether these improvements can be sustained longer-term if the dietary changes were made a permanent feature of the participants’ daily diet.

When comparing the effects of whole grains on young men to older men, it appears that there are two different mechanisms engaged. This warrants further investigation and is necessary to determine the true impact of dietary changes on health so as to fully understand the effect on future CVD-related morbidity and mortality. Further research should also be conducted for a much longer period of time in a larger cohort matching that of the Low Risk Study involving whole grains, with specific focus on concomitant dietary consumption and frequent assessments of CVD risk markers (i.e. PWV, BP, lipid profiles) over the intervention period as has already been discussed. This is especially important given the current sample size within the Low Risk Study and the apparent evidence that to confidently calculate any effect on PWV, then increased participant numbers will be required in the future.

Overall this study provides promising preliminary evidence for the benefits of intervening at an early age in terms of the dietary intake of the population. However this has to be rationalised against the potential negatives in terms of actually achieving consistently higher whole grain intakes in the younger population whilst also confirming the mechanistic basis for any effect through further adequately powered studies with a longer period of follow-up.
Chapter 6

Chapter 6

6 An investigation into the effects of a diet rich in inulin and wheat fibre on arterial stiffness and inflammatory markers in subjects at an increased risk of Cardiovascular Disease: The Cross-Over Study

6.1 Introduction

6.1.1 Background

Most intervention studies with whole grains focus on the grains as a whole entity. However, data is becoming available within the literature to suggest beneficial health effects of the individual fibres found within the grain (i.e. inulin, wheat fibre).

For insoluble fibre (wheat fibre), evidence suggests that it may play an important role in improving peripheral insulin sensitivity. For example, Weickert et al. (2006) showed that in a group of individuals who were overweight ($n=17$, mean BMI $30.0\pm2.1\text{kg/m}^2$), and who consumed greater quantities of insoluble fibre (white bread enriched with $31.2\text{g/day}$ of insoluble fibre for 72hrs), whole-body glucose disposal was significantly improved when compared against a control bread. No significant changes were detected within the other study outcomes, including lipid profile, serum magnesium, ghrelin and adiponectin concentrations as well as body weight (Weickert et al., 2006).

Current research into the properties of inulin and their impact on cardiovascular disease risk markers is limited and the findings are equivocal, although lipid profiles seem to have been the main focus for intervention trials within the current literature. Davidson & Maki (1999) designed a cross-over study comparing inulin versus a control (inulin was used as a partial substitute for sugar in foods used for the intervention) in a group of 25 hypercholesterolaemic men and women. Over a six-week intervention period, participants consumed $18\text{g/day}$ of inulin followed by a wash-out phase and then the control
period (or vice versa depending on randomisation). During the control period, significant increases in total and LDL-cholesterol were detected, compared to small reductions in total and LDL-cholesterol during the intervention period. However, when comparing the two stages, the authors state that the net change in total and LDL-cholesterol (“change during inulin minus change during control treatments”) is significant. A net reduction of -14.4±4.3% (p<0.005) was calculated for LDL-Cholesterol; for total cholesterol a net reduction of -8.7±3.3% (p<0.02) was detected. However, it was noted that when comparing at baseline, the participants’ levels of total and LDL-cholesterol were significantly lower prior to commencing the control stage compared to the intervention stage, regardless of which order the participants undertook each stage of the study (Davidson and Maki, 1999).

As Williams & Jackson (2002) point out, there is very little in the way of definitive evidence as to the health benefits of inulin at intakes of approximately 15g/day. This quantity appears to be the best tolerated, any higher and gastrointestinal complaints reported by participants may become prohibitive in the completion of the study (Williams and Jackson, 2002). At the time of publication they noted that of nine dietary intervention studies involving inulin (or oligofructose) included in the review, three detected no changes in lipid levels; three studies detected significant reductions in triglycerides and four studies described moderate reductions in both total and LDL cholesterol (Williams and Jackson, 2002).

Clearly the current body of evidence regarding inulin and indeed wheat fibre and their respective potential activity in lipid lowering is mixed and needs clarification. This can be partly achieved by implementing robust study designs, specifically controlling for confounding factors as much as possible to prevent a scenario such as that found in Davidson & Maki’s work reported above.

6.1.2 Justification for study

As described in Chapters 4 and 5, the potential effects on CVD risk of consuming whole grains has been investigated within two population groups at differing levels of CVD risk. However, these intervention studies were not designed to identify the active component of the whole grain. The question that remains is whether the separate fibres that can be found within a whole grain kernel impart separate effects or whether they must be consumed together as an intact grain to be most effective. Both Inulin (a non-digestible carbohydrate – NDC) and wheat fibre (insoluble fibre) are found within the wheat kernel and evidence is available to suggest that they may exert beneficial effects on cardiovascular health, thus lowering the risk of mortality and morbidity over time.

6.1.3 Aims and objectives

The overall aim of this study is to investigate the effects of inulin or wheat fibre supplementation (15g/day) versus a control, on markers of CVD in men at an increased risk of the disease.
Chapter 6: The Cross-Over Study

The main study objectives are set out below. The purpose is to investigate the effects of the intervention products on both fasting and post-prandial blood samples, specifically analysing changes to the following parameters:

- arterial stiffness, as assessed by Pulse Wave Velocity
- 24 hour ambulatory blood pressure
- Systemic and localised endothelial inflammation as assessed by the measurement of the inflammatory markers: TNF-α, MCP-1, IL-1β, IL-6 and IL-8.
- Insulin resistance and glucose tolerance, as assessed by plasma glucose and insulin measurements taken over the full time course of the post-prandial phase.
- Lipid profile, as assessed by total and HDL cholesterol, triglycerides and non-esterified fatty acids (NEFA).
- Anthropometric data (waist circumference, body mass index, weight and % body fat) will be recorded.

6.1.4 Hypothesis
The inclusion of a non-digestible carbohydrate (NDC) and an insoluble fibre to the regular dietary intakes of participants will both separately reduce CVD risk factors when compared to a control. This will occur due to improved insulin sensitivity (via insoluble fibre) and lipid profile (via NDC), which should lead to reductions in arterial stiffness and circulating levels of inflammatory markers.

6.2 Study design
6.2.1 Study design
The study was a 3-way, cross-over, controlled, randomised dietary intervention study in men aged 30-55yrs. Participants consumed (in a randomised order) bread rolls that contained either wheat fibre or inulin, or the control i.e. refined grain (Figure 6.2). Participants were requested to consume three rolls per day, with each roll containing 5g of the ‘active’ intervention ingredient – either wheat fibre or inulin. The rolls were consumed for 28 days, followed by a 28-day wash out period immediately after. The participants attended four study sessions; one at the beginning (Day 0 - baseline), and then one at the end of each intervention stage (Study Day 1 - Day 28; Study Day 2 - Day 84; Study Day 3 - Day 140) – Figure 6.1. Prior to attending the baseline study session, participants completed a 3-day diet diary; the diaries were then repeated on the final 3 days of each intervention period.
Chapter 6: The Cross-Over Study

Screening

<table>
<thead>
<tr>
<th>Intervention*</th>
<th>Wash-Out</th>
<th>Intervention*</th>
<th>Wash-Out</th>
<th>Intervention*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Study Day 1</td>
<td>Study Day 2</td>
<td>Study Day 3</td>
<td></td>
</tr>
</tbody>
</table>

Key: Intervention* - Participants consumed either Roll A, B or C in a randomised order throughout the study.

Figure 6.1. Study design for the Cross-Over Study

![Roll A - Wheat fibre, Roll B - Inulin, Roll C - Control](image)

Figure 6.2. Bread rolls used as the intervention for the Cross-Over Study

Note: The rolls used in the Cross-Over Study were wheat fibre (Roll A), inulin (Roll B) and control (Roll C). Both the wheat fibre and inulin rolls contained identical amounts of intervention ingredient (5g per rolls). Participants consumed three rolls per day for 28 days per intervention stage.

Prior to commencing, the study was given a favourable ethical opinion by the University of Surrey Ethics Committee – EC/2007/73/FHMS.

6.2.2 Participant selection

The change in insulin and glucose were the primary end-points of the study. Sample size calculations based on a change to insulin were used. A sample size of 10 would have at least 80% power to detect a clinically significant drop in insulin of 20pmol/L (a difference to detect of 25%) between the groups assuming that the common standard deviation is 16pmol/L or 20%. The sample size allowed for 10% drop-out rate. The estimate of the standard deviation was based on published data for subjects who took part in a Resistant Starch Study of similar design conducted by Robertson et al. (2005).

Participants suitable for inclusion:

- Adult males, aged 30-55 years
- Waist circumference >37 inches (94cms)
- Body Mass Index (BMI) in the range of 25-35kg/m²
- Not taking any prescription medicines or supplements within the past 6 months
• not drinking more than 21 units of alcohol per week
• not regularly undertaking vigorous exercise or fitness training (no more than 3 x 30 minute aerobic sessions per week)

The screening process used for this study was identical to that implemented for the High Risk Study (see Section 4.2.4), with the exclusion criteria detailed in Section 2.1.3. In addition, similar to the High Risk and Low Risk Studies, participants were recruited from the local population (via posters and local media advertisements) and from the university community (via email).

6.2.3 Randomisation of participants
Participants were randomly assigned to one of the three intervention groups and to the order in which they would receive the three intervention products using web-based randomisation software available at www.randomization.com.

6.2.4 Study protocol
The week before commencing the study, participants completed a 3-day diet diary to record their dietary intake at baseline. The night before the study, the participants consumed a standard meal – chosen from a set list matched for macronutrient intake, (as per High Risk Study meal choices) – in addition to refraining from strenuous exercise and drinking alcohol or caffeine.

6.2.4.1. Study session – All sessions
All study sessions for the Cross-Over Study followed the same protocol, with study sessions held at the Clinical Investigation Unit (CIU), Faculty of Health and Medical Sciences, University of Surrey. Participants arrived early in the morning (session start times between 7.30am-9am) and in a fasted state (12hrs fasted). Anthropometric measurements were taken first – height, weight, body fat percentage, body mass index, waist circumference, hip circumference (see Sections 2.3.1-3). The participants were then requested to rest in a quiet room with dimmed lighting for ten minutes in preparation for the PWV measurements. After the resting phase, triplicate recordings of static, supine blood pressure were taken (see Section 2.3.4) and the process of recording PWV commenced (see Section 2.5.2).

The participant was then cannulated (see Section 2.4.2) immediately after completion of the PWV and an initial 30ml fasting baseline blood sample taken.

Of this baseline blood, 10ml was immediately processed for separation of monocytes – see Section 2.6.1. The rest of the whole blood was separated into the appropriate blood tubes necessary for the subsequent laboratory analysis (Section 2.4.4).
Chapter 6: The Cross-Over Study

An oral glucose tolerance test was then conducted (see Section 2.5.4). Once the blood sampling had been completed, participants were offered refreshments and they were then fitted with the ambulatory blood pressure monitors to be worn for the next 24 hours (Section 2.5.1).

Prior to leaving the CIU, participants were provided with an allocated quantity of bread rolls for the entire intervention period. Participants were required to consume 3 of their allocated rolls per day and were blinded to the contents of the roll. Participants were encouraged to incorporate the rolls into their habitual diet (for example by adapting their lunch by substituting their routine bread for the study rolls) as opposed to consuming them in addition to their habitual diet. There were no restrictions on what study rolls could be consumed with and the rolls could be warmed before eating, if preferred.

Compliance was monitored via regular telephone or face-to-face contact with participants during the course of the intervention periods. A small number of participants declined to take the full 28 day’s allocation of rolls with them to cover the intervention period. In these cases, participants would instead visit at pre-arranged times to collect more rolls. This opportunity was also used to discuss compliance and any queries the participants may have. As similarly detailed in Section 4.2.4.2, the packaging used for the rolls were requested from all the participants to check for any un-eaten rolls post-intervention. No rolls were found and as per previous studies, the participants denied non-compliance. However, for the final days of each intervention period, participants completed 3-day diet diaries which were used to assess roll consumption and general compliance to the intervention (see Section 6.3.8 for dietary analysis).

At the end of each intervention period participants attended for a study session as described above. The intervention period would then be followed by a wash-out phase of 28 days before the participant rotated on to another intervention stage.

6.2.5 Statistical analysis

The data was explored for normal distribution and descriptive statistics were performed. Detailed statistical analysis focussed on the effects of the interventions (i.e. treatment effect, within-subject, using repeated measures ANOVA or Friedmans dependant on distribution of data) in addition to analysing a time-treatment effect (percentage change from baseline per intervention – analysed with repeated measures or Friedmans).

This intervention study was performed in collaboration with Miss Nicola Muirhead; each researcher had their own individual, specific objectives and retrieved samples and data to reflect that. Responsibility for the running of study sessions and the tasks involved were divided equally between the two co-investigators (including monocyte extraction). The exception was the PWV and ambulatory blood pressure which was performed solely by the author for consistency. All data analysis and inferences thereof are the author’s own work.
Laboratory tasks were equally divided for the analysis of samples for the entire study cohort, with data made available to the co-investigator. Tasks were completed as follows: Insulin analysis – LT; Lipid profile – NM; Inflammatory markers – LT and NM

6.3 Results

Ten overweight males participated in the study. Table 6.1 shows the participants’ anthropometric characteristics. During the course of the study, no participants reported ill-effects (changes in bowel habits were documented but not considered serious by the participants) and no-one withdrew from the study.

Table 6.1. Subject characteristics for participants of the Cross-Over Study

<table>
<thead>
<tr>
<th>Study outcome (n=10)</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>39.8±9.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>101.6±13.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.2±3.0</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>106.5±7.6</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>124.8±9.5</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>71.9±9.9</td>
</tr>
<tr>
<td>Fasting Glucose (mmol/l)</td>
<td>5.5±0.3</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>5.2±0.7</td>
</tr>
</tbody>
</table>

Note: Descriptive statistics at baseline for the entire cohort – all participants were male

6.3.1 Anthropometrics

There were no significant changes in any anthropometric parameters when comparing between intervention stages (Table 6.2). The percentage change between baseline and each individual intervention stage was also calculated and repeated measures ANOVA detected no significant results.

Table 6.2. Participant anthropometric characteristics

<table>
<thead>
<tr>
<th></th>
<th>Baseline n=10</th>
<th>Wheat fibre n=10</th>
<th>Inulin n=10</th>
<th>Control n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>%ΔBS</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>39.8±9.59</td>
<td>101.6±13.4</td>
<td>1.62</td>
<td>101.9±12.15</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>10.6±13.0</td>
<td>103.06±12.23</td>
<td>1.62</td>
<td>101.99±12.15</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.20±3.02</td>
<td>30.70±2.63</td>
<td>1.79</td>
<td>30.39±2.71</td>
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<tr>
<td>WC (cm)</td>
<td>106.45±7.62</td>
<td>106.98±7.90</td>
<td>0.54</td>
<td>105.12±6.28</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>28.54±4.14</td>
<td>28.56±4.21</td>
<td>0.34</td>
<td>29.41±4.48</td>
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<tr>
<td>SBP (mmHg)</td>
<td>125±9.50</td>
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<td>1.77</td>
<td>125±1.62</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>72±9.87</td>
<td>77±8.67</td>
<td>6.84</td>
<td>73±12.18</td>
</tr>
</tbody>
</table>

Note: No significant differences were identified when comparing between intervention stages or for percentage change from baseline (time-treatment effect). Key: BMI – Body Mass Index; WC – Waist circumference; SBP – Systolic blood pressure; DBP – Diastolic blood pressure; %ΔBS – the percentage change between baseline and the intervention.
6.3.2 Pulse Wave Velocity (PWV)
The PWV results are presented in Figure 6.3 (R-C PWV) and Figure 6.4 (C-F PWV). As shown in Figure 6.3, for R-C PWV there were no significant differences between the three intervention stages; wheat fibre (WF), inulin (IN) and control (CON). Likewise, when comparing between the percentage changes from baseline for each intervention, no significant differences were detected.

Figure 6.4 shows the data obtained for C-F PWV. No significant differences were detected between the three intervention stages nor when comparing between the percentage changes from baseline for each intervention.

However, it was observed that for both the R-C and C-F PWV, the inulin intervention did show a reduction in PWV compared to the other intervention stages and from baseline. Yet at both baseline and post-intervention, the mean PWV results (for both R-C and C-F PWV) were below the cut-off proposed to be indicative of CVD (i.e. PWV <12.3m/s (Mattace-Raso et al., 2006)).

Mean arterial pressure (MAP) and pulse pressure (PP) were calculated from the reported blood pressures as supplementary indices and showed no significant differences between the interventions or when taking into account percentage change from baseline (data not shown).

6.3.3 Ambulatory blood pressure
As shown in Figure 6.5(a)-(d), for the daytime ambulatory measurements of systolic blood pressure (SBP) no significant differences were found between the intervention groups when analysing the raw data. When comparing between the wheat fibre, inulin and control interventions for the respective percentage changes from baseline, no significant difference was detected. Daytime diastolic blood pressure (DBP) also showed no significant differences between interventions and no significant differences in the percentage changes from baseline.

Although both SBP and DBP during the night were lower than those taken during the day, the overall pattern of no significant time or treatment effect remained for the night-time data.
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Figure 6.3. Radial-Carotid Pulse Wave Velocity at baseline and post each intervention

Note: Values expressed as mean±SD (n=10). No significant differences between interventions was detected, nor when calculating %change from baseline for each intervention. Key: BS - Baseline; WF - wheat fibre; IN - Inulin; CON - Control

Figure 6.4. Carotid-Femoral Pulse Wave Velocity at baseline and post each intervention

Note: Values expressed as mean±SD (n=10). No significant differences between interventions was detected, nor when calculating %change from baseline for each intervention. Key: BS - Baseline; WF - wheat fibre; IN - Inulin; CON - Control
Figure 6.5. 24-hour ambulatory systolic and diastolic blood pressure at baseline and post each intervention.

Graphs a-d - Repeated measures ANOVA detected no significant changes between interventions nor when comparing percentage change against baseline for each intervention stage. Values expressed as mean±SD (n=6). Graph a) Daytime hours, SBP; b) Daytime hours, DBP; c) Night-time hours, SBP; d) Night-time hours, DBP. Key: SBP - systolic blood pressure; DBP - diastolic blood pressure; BS - Baseline; WF - wheat fibre; IN - Insulin; CON - Control.
### 6.3.4 Inflammatory markers

The inflammatory markers IL-1β (Figure 6.6), IL-6 (Figure 6.7), IL-8 (Figure 6.8) and MCP-1 (Figure 6.9) were chosen to reflect the inflammatory process proposed to be linked to the formation of atherosclerotic plaques (Libby, 2002, Hansson, 2001, Boekholdt et al., 2004). However for IL-1β and IL-6, there appears to have been very little impact on the circulating levels by any of the intervention phases.

For IL-1β (Figure 6.6), no significant differences were detected and there is minimal variation between the intervention phases when comparing the median values (with interquartile range - IR) despite the possibility of an outlier within the data – wheat fibre 10.25pg/ml (4.02IR), inulin 9.58pg/ml (7.04IR) and control 9.77pg/ml (4.15IR).

Similarly for IL-6 (Figure 6.7), no significant differences were found comparing between the interventions - wheat fibre 3.83pg/ml (11.33IR), inulin 2.33pg/ml (19.41IR) and control 2.45pg/ml (11.28IR). There was also no significant difference detected when comparing the percentage change from baseline between interventions for both IL-1β and IL-6. The inflammatory marker data appears to show that the participants are at increased risk of CVD as the reported cut-off to indicate high-risk of CVD is >1.5-3.19pg/ml (Ikonomidis et al., 2005, Ridker et al., 2000, Harris et al., 1999).

The data for IL-8 (Figure 6.8) and MCP-1 (Figure 6.9) suggests greater variability. However, the differences in IL-8 levels between the intervention phases were not significant despite an apparently greater release following the wheat fibre supplementation (Baseline: 11.35pg/ml (12.62IR), post-intervention wheat fibre: 15.29pg/ml (6.26IR), inulin: 12.4pg/ml (11.6IR), control: 10.06 (7.17IR)).

When expressed as percentage changes from baseline, (wheat fibre 25%, inulin 5%, control -5%) there was also no significant difference between groups, possibly due to the evident degree of variability between subjects. Comparing from baseline, MCP-1 (Figure 6.9) was lowered to a small extent by both the wheat fibre and inulin interventions, as well as the control (4%, 11% and 4% respectively) but there was no significant difference between groups. Comparing across the three interventions (using the raw data from each intervention phase), there were no significant differences between them. This was possibly linked to the relatively large interquartile range for both baseline (median 2312pg/ml, 1395IR) and the interventions: inulin (2065pg/ml, 993IR), wheat fibre (2266pg/ml, 1358IR) and the control (2164pg/ml, 591IR).
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Figure 6.6. Interleukin-1β (IL-1β) measured at baseline and post each intervention

Note: Values expressed as median with interquartile range (n=9). No significant differences between interventions was detected, nor when calculating %change from baseline for each intervention. Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.

Figure 6.7. Interleukin-6 (IL-6) measured at baseline and post each intervention

Values expressed as median with interquartile range (n=5). No significant differences between interventions was detected, nor when calculating %change from baseline for each intervention. Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.
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Figure 6.8. Interleukin-8 (IL-8) measured at baseline and post each intervention
Values expressed as median with interquartile range (n=9). No significant differences between interventions was detected, nor when calculating %change from baseline for each intervention. Key: BS - Baseline; WF - wheat fibre; IN - Inulin; CON - Control.

Figure 6.9. Monocyte Chemoattractant Protein-1 (MCP-1) measured at baseline and post each intervention
Values expressed as median with interquartile range (n=10). No significant differences between interventions was detected, nor when calculating %change from baseline for each intervention. Key: BS - Baseline; WF - wheat fibre; IN - Inulin; CON - Control.
6.3.5 *Ex vivo* monocyte production of TNF-α

Figure 6.10 shows the data obtained from the *ex vivo* activation of the monocytes obtained from the participants at each visit. When analysed over the full time-course, no significant differences were found within any intervention despite the sharp increase from ‘T0’ to ‘T48’ (for all interventions). However, when comparing between the ‘T48’ and ‘T72’ time points, no significant differences were found within any of the interventions, except for the control phase; $Z = -2.37, p=0.02$.

Each time point was analysed for differences between the interventions, however no significant changes were detected for T0, T48 or T72. This is despite the clear differentiation of the mean values at ‘T48’ (Figure 6.10) between baseline and control, versus wheat fibre and inulin.

In addition, between ‘T48’ and ‘T72’ the wheat fibre intervention increases further for mean levels of TNF-α, from 1.11ng/ml±0.56SEM to 1.56ng/ml±0.88SEM. This is compared to the inulin intervention which appears to have a more blunted response from 0.92ng/ml±0.37 increasing to 1.09ng/ml±0.65.

![Figure 6.10. Ex vivo production of TNF-α from activated monocytes measured at baseline and post each intervention](image)

**Values expressed as Mean±SEM (T0 m=3; T48 m=9; T72 m=8). No intervention caused a significant difference over time for TNF-α production. No significant difference between the rolls at each time point. Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.**

6.3.6 Glucose and insulin profiles

Table 6.3 shows the fasting glucose and insulin results (in addition to the HOMA-IR and HOMA-% β) for the baseline and post-intervention results. Variation between baseline and post-intervention results ranged from 0.41% for the difference between the baseline and wheat fibre fasting glucose results, to 30.6% difference between baseline and wheat fibre for HOMA-%β. However when comparing the
absolute values between the post-intervention results, these 'between-intervention' differences were not significant for either the treatment or time-treatment effect.

This lack of significance does not reflect the 21.4% increase in HOMA-IR and a 30.6% increase in HOMA-%β for wheat fibre from baseline. There are only modest increases for the inulin and control phases (approximate increases of 5-14% for both HOMA-IR/%β) in comparison. This fluctuation in calculated insulin resistance occurs in concordance with similar increases in fasting insulin for all intervention phases.

Table 6.3. HOMA and fasting glucose & insulin levels at baseline and per intervention.

<table>
<thead>
<tr>
<th></th>
<th>Baseline n=10</th>
<th>Wheat fibre n=10</th>
<th>Inulin n=10</th>
<th>Control n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.40±1.77</td>
<td>3.85±1.37</td>
<td>3.68±1.68</td>
<td>3.63±1.35</td>
</tr>
<tr>
<td>HOMA-%β</td>
<td>120.65±52.16</td>
<td>138.88±48.28</td>
<td>118.92±42.52</td>
<td>113.84±43.56</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>83.91±1.15</td>
<td>95.04±31.95</td>
<td>88.52±37.60</td>
<td>86.37±31.35</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.45±0.31</td>
<td>5.45±0.37</td>
<td>5.53±0.28</td>
<td>5.64±0.37</td>
</tr>
</tbody>
</table>

Note: Repeated measures ANOVA detected no significant differences across the interventions. Values expressed as mean±SD, n=10. No significant differences when comparing percentage change from baseline (time-treatment effect). Key: HOMA - Homeostasis Assessment Model; IR - Insulin resistance; % β - β-cell function (insulin sensitivity); %ABS - the percentage change between baseline and the intervention.

Using the Insulin Sensitivity Index for the Oral Glucose Tolerance Test (Matsuda and DeFronzo, 1999) to estimate whole-body insulin sensitivity, no significant changes in insulin sensitivity between the intervention groups was detected, nor when comparing against the baseline results for each intervention. Considering the fluctuations within the HOMA and fasting insulin data, the ISI-OGTT results show minimal variation between the intervention phases, this index may go some way towards explaining the non-significant result for the HOMA data. For the baseline result, the mean (±SD) result was 3.17±1.51, for the wheat fibre intervention the result was 3.15±1.68, for inulin 3.17±1.79 and the control phase 3.14±1.93.

The OGTT time-course data depict changes between the interventions for both the glucose and insulin results, which are displayed graphically in Figures 6.11 (glucose) and 6.12 (insulin). As shown in Figure 6.11 the OGTT glucose curve for the wheat fibre ('WF') is generally lower than that of the curves for the other intervention phases and baseline data, which is reflected by the lower glucose AUC result in Figure 6.13 compared to the other interventions. The glucose AUC for wheat fibre had a mean result of 868±138, for inulin 930±159 and control 991±200, despite the visual differences, the statistical difference between the interventions was not significant; p=0.08. However, when calculating the difference between the interventions for percentage change from baseline (baseline: 890±153), there is a significant difference between two of the intervention phases (wheat fibre vs control: Wilks' Lambda 0.26, F 7.27, p=0.03).
Figure 6.11. Glucose curves obtained from the Oral Glucose Tolerance Test. (per intervention)
Values expressed as mean±SD (n=10). Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.

Figure 6.12. Insulin curves obtained from the Oral Glucose Tolerance Test. (per intervention)
Values expressed as mean±SD (n=10). Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.
Figure 6.13. Glucose Area Under Curve from the Oral Glucose Tolerance Test

Note: A repeated measures ANOVA detected a small trend towards significance between interventions (p=0.08) and a significant difference when comparing percentage change against baseline for each intervention stage (p=0.03). Values expressed as mean±SD (n=8). Key: BS - Baseline; WF - wheat fibre; IN - Inulin; CON - Control.

Figure 6.14. Insulin Area Under Curve from the Oral Glucose Tolerance Test.

Note: A repeated measures ANOVA detected no significant changes between interventions (p=0.89) nor when comparing percentage change against baseline for each intervention stage (p=0.92). Values expressed as mean±SD (n=7). Key: BS - Baseline; WF - wheat fibre; IN - Inulin; CON - Control.
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Figure 6.15. Triglycerides (TAG) Area Under Curve from the Oral Glucose Tolerance Test

Note: A repeated measures ANOVA detected no significant changes between interventions (p=0.26) nor when comparing percentage change against baseline for each intervention stage (p=0.38). Values expressed as mean±SD (n=7). Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.

Figure 6.16. NEFA Area Under Curve from the Oral Glucose Tolerance Test.

Note: A repeated measures ANOVA detected no significant changes between interventions (p=0.62) nor when comparing percentage change against baseline for each intervention stage (p=0.87). Values expressed as mean±SD (n=7). Key: BS – Baseline; WF – wheat fibre; IN – Inulin; CON – Control.
Although the insulin AUC data (Figure 6.14) appears to mimic the glucose AUC in terms of visual differences between interventions, the error bars indicate a far wider spread of data, therefore the differences between the intervention groups for the insulin AUC was highly non-significant ($p=0.89$).

In a similar scenario, the triglyceride (TAGs Figure 6.15) and NEFA (Figure 6.16) AUC results also appear to show substantial differences between the intervention stages; however they do not support the picture from the glucose and insulin data whereby wheat fibre was associated with the most positive effects. The TAG and NEFA data shows no significant differences in AUC across the intervention phases. When combined with the insulin and glucose data this suggests that acute biochemical responses to a glucose load have not been consistently detected within this intervention study.

### 6.3.7 Lipid profile

At baseline, the participants had healthy lipid profiles. Even though no significant treatment effect was identified for any of the lipid parameters, detrimental effects were identified for triglyceride levels. As demonstrated in Table 6.4, participants experienced relatively large fluctuations in all lipid levels across the interventions.

Although not significant, the changes in triglyceride levels are substantial when assessing percentage changes from baseline. All interventions, when compared against baseline, showed mean increases in triglyceride levels, specifically 14.77% for the control rolls, 29.8% for the inulin rolls and 37.27% for the wheat fibre rolls. When looking at the treatment effect, there were also no significant differences between rolls. Post-intervention, the fasting triglyceride levels were above the recommended level of 1.7 mmol/L (Alberti et al., 2009).

In contrast, total cholesterol showed more moderate fluctuations of $>-5.24$ to $<0.57\%$ (also not significant) across the different intervention rolls with mean decreases in the wheat fibre and control arms compared to a mean increase in the inulin arm. This effect appears to have been largely driven by changes in HDL cholesterol which also showed mean decreases for the wheat fibre and control groups and a mean increase for the inulin group ($ns$). Overall, it appears that despite the increase in total cholesterol (of 0.6%), inulin produced the most favourable lipid response due to it being the only intervention to increase HDL cholesterol. Conversely, the wheat fibre and control interventions have performed differently by inducing reductions in both total cholesterol and HDL cholesterol. Therefore any benefit gained by reducing total cholesterol has likely been lost by the concomitant reduction in HDL cholesterol.
The changes in NEFA were not significantly different across interventions, nor when comparing percentage changes between intervention. Although in this case, relatively large mean increases were seen after the control and inulin interventions (approximately 10%), compared to a mean decrease in the wheat fibre intervention of 1.6%.

<table>
<thead>
<tr>
<th>Table 6.4. Lipid profiles at baseline and per intervention phase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Mean±SD</td>
</tr>
<tr>
<td>TAGs (mmol/l)</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
</tr>
</tbody>
</table>

Note: Repeated measures ANOVA detected no significant differences across the interventions. Values expressed as mean±SD, n=10 (*except for HDL for Inulin stage, sample haemolysed). No significant differences when comparing percentage change from baseline (time-treatment effect). Key: TAGs – Triglycerides; TC – Total cholesterol; NEFA – Non-esterified fatty acid; HDL-C – High-density lipoprotein cholesterol; %ΔBS – the percentage change between baseline and the intervention.

6.3.8 Dietary analysis

Diet diaries were required to be completed by participants prior to commencing the study and then post each intervention phase. There was a 67% return rate, with moderate levels of completion. As shown in Table 6.5, there were five diet diaries completed at baseline, eight after the wheat fibre intervention, six after the inulin and eight after the control intervention. No significant differences were detected when comparing between the intervention phases nor when comparing each intervention from baseline except for dietary fibre. There was a significantly greater amount of dietary fibre consumed during the inulin phase when compared to the baseline (p=0.04). Similarly, significantly more dietary fibre was consumed during the wheat fibre intervention phase when compared to the control phase (p=0.03).

<table>
<thead>
<tr>
<th>Table 6.5. Mean daily macronutrient intakes (baseline versus end-of-intervention) for all intervention phases of the Cross-Over Study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Mean±SD</td>
</tr>
<tr>
<td>Energy (KJ)</td>
</tr>
<tr>
<td>Fat (g/d)</td>
</tr>
<tr>
<td>% energy from fat</td>
</tr>
<tr>
<td>Protein (g/d)</td>
</tr>
<tr>
<td>% energy from protein</td>
</tr>
<tr>
<td>CHO (g/d)</td>
</tr>
<tr>
<td>% energy from CHO</td>
</tr>
<tr>
<td>DF (g/d)</td>
</tr>
<tr>
<td>Sodium (mg/d)</td>
</tr>
</tbody>
</table>

Table 6.4 The values for post-intervention (for all phases) include the three study rolls consumed per day which was assessed via diet diary. There were no significant differences detected comparing between intervention phases except for dietary fibre (p=0.03).
6.4 Discussion

6.4.1 Key findings

Overall, this study has not wholly supported the hypothesis that the consumption of a non-digestible carbohydrate (inulin) or an insoluble fibre (wheat fibre) will beneficially impact on the lipid profile and level of insulin sensitivity and thus reduce CVD risk. The study largely indicated no effect from the intervention phases of either fibre, however some interesting results occurred which warrants further discussion.

(i) Vascular health

Pulse wave velocity is a critical primary measure for this study, indicating the stiffness of the arteries, which in itself is an independent marker of CVD risk.

At baseline, none of the participants were above the cut-off point for PWV recommended by Mattace-Raso et al. (2006) of >12.3m/s indicating CVD risk. There was no significant effect of either of the fibres on arterial stiffness over the course of the intervention periods. This result is in contrast with two studies in the literature examining the effects of fibre on endothelial function. Via the use of flow-mediated dilatation (FMD), it was found that at 4-hours post-prandial (of a mixed high-fibre meal), participants with metabolic syndrome achieved a significant increase from 8.46±4.54% to 11.87±4.42%. When the participants were then measured after consuming a mixed low carbohydrate meal, the FMD reduced significantly from 7.65±4.09% to 5.69±3.43% (Brock et al., 2006). A study examining the longer-term effects of a Mediterranean-style diet (containing whole grains) on 90 participants who had abdominal obesity but neither CVD or T2DM, showed that FMD was also responsive post-intervention (Rallidis et al., 2009). The intervention group (those following the Mediterranean diet) showed a significant increase in FMD (+2.05%) and a significant reduction in diastolic blood pressure (-6.44mmHg) compared to the control group (Rallidis et al., 2009). Although the Rallidis study included whole grains as part of the intervention diet, the study was more focussed on the complete lifestyle change of converting to the Mediterranean-style diet and how this may affect the body as a whole, as opposed to looking at only a single feature of the diet. The Brock study only examines the effects of fibre intake in the very short-term (post-prandial phase) and therefore does not give a clear indication as to how the body may be expected to adapt and respond to the continued intake of fibre at those levels for the long-term. These studies are indicative of the data available within the current literature. They are promising in terms of showing that fibres (whether soluble or insoluble or as intact whole grains) can have beneficial effects on a population group, regardless of their health status and risk of CVD. However, there is still a lack of long-term dietary intervention trials which can back this evidence and show confidently how whole grains and their constituent fibres may be of benefit to health.
Within the wider collective of vascular health markers (PWV, BP, ambulatory BP, MAP and PP), all participants were within the respective healthy ranges at baseline and none of these markers responded significantly to the inclusion of inulin or wheat fibre into the participants' diets. The relatively good baseline levels of vascular health in these study participants may explain the lack of effects seen following the dietary intervention.

This baseline health status is despite a recruitment strategy designed to select those individuals deemed to be at greater CVD risk due to a large waist circumferences and high BMI. This was thought to be sufficient to be indicative of an increased level of CVD risk thanks to the evidence reporting a relationship between obesity and CVD risk (Guh et al., 2009) as well as the current NICE guidelines indicating the cut-off for waist circumference that indicates risk of CVD (NICE, 2006b). Since the participants were still overweight (and therefore still at risk of CVD) it was surprising that no vascular markers were affected by the fibre interventions. A factor to consider may be that vascular function could take longer to respond to a dietary intervention of this kind (i.e. inulin or wheat fibre) as opposed to dietary changes encompassing the whole diet (Rallidis et al., 2009), it is also important to consider whether the length of time an individual is obese affects their vascular function. In effect, the participants may not have been 'unhealthy' enough for long enough to influence vascular health. This leaves an issue as to whether the use of surrogate markers for CVD risk (BMI or waist circumference) is enough to rely on when recruiting for studies such as the Cross-Over Study. An indicative PWV measurement may have been recommended to ensure a cohort with evidence of arterial stiffness.

Evidence is available in the literature that shows in a cohort at risk of CVD (in this case the cohort are hypercholesterolaemic but also normotensive similar to the Cross-Over Study cohort), the consumption of separate fibres (soluble and insoluble) had a positive effect on health, especially on blood pressure (Hallfrisch et al. 2003, Behall et al. 2006).

Hallfrisch et al. (2003) and Behall et al. (2006) both conducted studies assessing the impact of consuming diets containing barley, brown rice and whole wheat. Hallfrisch et al. (2003) recruited 16 normotensive, hypercholesterolaemic men onto the Step 1 diet (American Heart Association recommended dietary pattern) with the addition of brown rice and whole wheat (insoluble fibre), barley (soluble fibre) alone or a combination of all three following a latin square design. It was found that over time the intervention diets did lower systolic and diastolic blood pressure (and therefore mean arterial pressure) when compared against the baseline results. However not all weeks were consistently significantly lower, making it difficult to draw definitive conclusions (Hallfrisch et al., 2003). The reductions in blood pressure are important and may indicate a role for fibre in mediating blood flow which could also affect arterial stiffness, however this study does not have the capability to corroborate such hypotheses.
The study conducted by Behall et al. (2006) appears very similar in terms of the intervention; insoluble fibre vs. soluble fibre vs. a combination of the two. The participants were also very similar with mild hypercholesterolaemia, however for this study seven men, nine pre-menopausal women and nine post-menopausal women were recruited to take part. A two-week run-in diet following the American Heart Association Step 1 diet was followed until the participants were entered into the latin square study design to consume either barley, whole wheat/brown rice or a combination of all for 5 weeks each. The implementation of the dietary interventions resulted in a replacement of the equivalent of 20% energy from carbohydrate sources. The overall result was that, whilst the Step 1 diet (run-in) resulted in reductions in systolic and diastolic blood pressure, as well as mean arterial pressure (MAP), further significant reductions were induced by all the intervention diets. For MAP, the men showed the greatest response to the combination diet (insoluble and soluble fibre) with a reduction of 10.8mmHg. In contrast the women were most responsive to the barley diet (soluble fibre) for reductions in MAP (Behall et al., 2006).

These two studies appear to show that both soluble and insoluble fibre have positive effects on blood pressure, thus potentially lowering the risk of CVD. However the use of a latin square design may not be the most robust as there could be a multiplicative effect as the participants progress through the different diets. For the Behall study, there were significant differences between the participants at baseline for height, body mass index and body fat %. Given the significant reduction in body weight apparently found in addition to the implications of adipose tissue in the aetiology of CVD, it would have been preferable if these factors could have been controlled for to a greater extent to ensure the effects that are reported are true and not influenced by confounding factors that could have been modified. The evidence from these studies suggests that soluble and insoluble fibres could have an effect on the vasculature, however further research will have to be examined and also conducted in order to create a more detailed picture of how fibres interact with CVD risk factors. No mechanism is put forward by these two studies to explain the results and when compared to the results of the Cross-Over Study, the picture becomes even less clear as to how a beneficial reduction in blood pressure may be initiated simply by eating whole grains. A pathway that could potentially be involved is if there was a subtle reduction in abdominal obesity after consuming the intervention which could then go on to up-regulate adiponectin levels and also down-regulate adipokine levels (Francischetti and Genelhu, 2007). Although the link between obesity and hypertension is not fully explained, if adiponectin and inflammatory markers are beneficially affected by the reductions in abdominal adipose tissue, then this could cascade and eventually reduce (or limit) endothelial dysfunction enough to improve blood pressure (Francischetti and Genelhu, 2007). However, this is a large and complex pathway for one food group to influence, therefore further work should be initiated to fully elucidate the mechanism linking obesity and hypertension and also how diet (in particular whole grains) may affect it.
(ii) **Inflammatory markers**

The inflammatory markers also did not respond to the increase in wheat fibre and inulin consumption. From the serum analysis, the inflammatory markers did not change significantly from baseline for any of the intervention periods, nor were there any significant differences between the responses to the interventions. In terms of assessing CVD risk from the inflammatory marker levels, evidence is available to show that for IL-6 the cut-off to indicate increased risk of CVD is >1.5-3.19pg/ml (Ikonomidis et al., 2005, Ridker et al., 2000, Harris et al., 1999). The median results from the baseline and all interventions for the *Cross-Over Study* were all within this 'high' range. Ridker et al. (2000) determined in their case-control study that for healthy men with the highest quartile of IL-6 plasma concentrations (>2.28pg/ml), they had a risk ratio of 2.7 for developing a myocardial infarction in the future (data obtained from a 6yr follow-up as part of a prospective study). The lowest median level of IL-6 found in this study was 2.33pg/ml (for the inulin intervention), and the implications of this are very interesting. It appears that the participants (according to the current literature) are at a degree of risk from future cardiovascular events, yet this is not supported by other markers such as pulse wave velocity or blood pressure. Nor are the lipid results or glycaemic/insulinaemic indicative of heightened CVD risk. No other data was retrieved from the current literature to indicate the healthy reference range for IL-1β, IL-8 or MCP-1, therefore it is difficult to ascertain whether this single indicator of apparent CVD risk (IL-6) is enough to cause concern, yet with no demonstrable, significant reduction in plasma concentrations from either of the intervention fibres, a mechanism linking IL-6 metabolism and fibre intake cannot be elucidated at present.

For the *ex vivo* cell work, a significant result for the expression of TNF-α was detected. The monocytes (extracted from the participants’ blood samples) were activated at the beginning of the experiment (with lipopolysaccharide, see Section 2.8.1) and then 48 hours later, samples were collected at those time points as well as a sample at 72 hours. The experiment was completed for each participant at baseline, and also after each intervention stage. At the 48 hours time-point all interventions were associated with an increase in TNF-α expression, with the baseline and control samples having the highest concentrations. The wheat fibre and inulin had distinctly lower levels of TNF-α compared to the baseline and control, however this result was not significant. Yet when comparing the levels of TNF-α expression at 48 to 72 hours, the wheat fibre intervention had increased levels, the baseline samples plateaued as had the inulin intervention. In contrast, the control intervention had reduced significantly from the 48 to the 72 hour time point.

This result is surprising as for the control intervention the participants consumed three soft, white bread rolls per day (isocaloric to the inulin and wheat fibre rolls) which is essentially what would be expected to be a close match to that of the baseline dietary intake, yet the monocytes from the baseline and control study sessions appear to have reacted differently to the activation. It is also puzzling how
the inulin and wheat fibre have not mimicked the control sample and had a reduction in TNF-α expression by the 72 hour time point.

There is little evidence within the current literature examining the effects of either inulin or wheat fibre on the inflammatory response. However, when extending the literature search to dietary fibres in general, Neyrinck et al. (2009) explain that Chitosan (obtained from the exoskeleton of white mushrooms) has structural properties that mimic dietary fibre, i.e. resistant to digestion. Chitosan is primarily made up of chitin (found in the shells of insects and crustaceans) combined with beta-glucan—a soluble fibre (Neyrinck et al. 2009). The study focussed on mice (eight per group) where one group were fed a high fat (HF) diet and the other group were fed a HF diet with a Chitosan supplement (5% of intake) for five weeks. Post-intervention it was found that adiposity was significantly less in the mice fed a HF diet and Chitosan, with additional significant reductions in IL-6 and leptin. MCP-1 was reduced but it was not significant. Furthermore, there was no linear relationship between MCP-1 and the adiposity index \( r=0.19 \), but leptin was significantly lowered post-intervention \( p<0.05 \) and correlated strongly with the adiposity index, \( r=0.8 \) (Neyrinck et al., 2009).

Although this is an animal model, it still gives an indication as to the potential effects a dietary fibre could have on the inflammatory response if the most effective dose were to be given. When reflected in human studies, there is some evidence that cereal fibre (insoluble fibre) may have a beneficial effect on the inflammatory response in a cohort of healthy males as shown by Giacco et al. (2010). In a study comparing cereal fibre intake to a control over 3 week intervention periods, a lower level of C-RP (1.8±2.3mg/dl) was detected for the cereal fibre group as opposed to 2.9±4.1mg/dl for the control group. However this difference between interventions was not significant and there is no baseline result to compare against. Yet these results do reflect the MCP-1 data from the Cross-Over Study in so far as there being a reduction for both wheat fibre and inulin compared to baseline with no significance found, yet a possible pattern beginning to emerge. Esposito et al. (2004) have also found a significant reduction in C-RP as well as IL-6, IL-7 and IL-18 in a cohort with metabolic syndrome after consuming a Mediterranean-style diet rich in, among other food, whole grains. Therefore data is available to show that whole grains or indeed insoluble fibre have the potential to ameliorate the inflammatory response. However further work may be warranted to explore whether there is an optimum dose of inulin and wheat fibre in humans from which maximal benefits to health can be conferred. It is known that 15g/day of inulin is the optimal dose before reports of gastrointestinal disturbance are made (Williams and Jackson, 2002) and 31.2g of insoluble fibre per day is required in order to improve insulin sensitivity over just 72 hours (Weickert et al. 2006). However the long-term effects of both fibres, and the balance between tolerance and acceptability versus clear clinical benefit, have yet to be identified.
(iii) Lipid profile and glycaemic regulation

A lot of data within the current literature refers to intervention studies looking at glycaemic regulation and lipid profile responses to fibre consumption. Within the Cross-Over Study, although there were no significant changes for either of the interventions, there were still a number of marked changes in key outcomes.

After both the wheat fibre and inulin intervention stages, it appears that there have been sizeable increases (expressed as percentage change from baseline) in the fasting insulin levels which seem to have gone on to influence the increase in calculated insulin resistance. The wheat fibre intervention had a 21% increase in insulin with a concomitant increase in HOMA-IR of 21%. The inulin intervention is linked to an increase in fasting insulin of 12% with a rise in HOMA-IR of 14%. However, the control intervention also had similar increases in fasting insulin of 11% and 15% for HOMA-IR. Given this control leg result, it is likely to indicate that this insulinaemic response to the OGTT is an overall response to the actual test as opposed to being indicative of any longer-term changes to glucose-handling. Therefore the real concern should be directed towards the wheat fibre intervention that shows a result which is completely at odds with an important study by Weickert et al. (2006) which showed (via an euglycaemic hyperinsulinaemic clamp) a significant improvement in whole body insulin insensitivity in response to an insoluble fibre intervention in 17 overweight/obese subjects (with normal glucose metabolism). However, this study loaded subjects with 31.2g insoluble fibre/day for 3 days versus the control, which is likely to be beyond ‘normal’ eating habits and is double that of the intake for the Cross-Over Study participants. Despite this, the results still show that there is possibly an active factor in the cereal fibre which is apparently improving insulin resistance (Weickert et al., 2006) that the results from the Cross-Over Study have been unable to support.

There is still a mix of evidence referring to the consumption of fibre and Jenkins et al. (2002) showed that when their 23 subjects (all with T2DM) completed a cross-over study (3 month per intervention stage), the high-fibre (wheat bran) diet had no significant effects on any markers of CVD (Jenkins et al., 2002). This included markers of the lipid profile, inflammation and glycaemic control. The authors do note that the intervention may have been too short to show measurable change and that the pursuit of insoluble fibre to distinguish its metabolic effects is still warranted (Jenkins et al., 2002).

Another earlier study by Weickert et al. (2005) looked at the possible mechanism linking insoluble fibre intake and glucose regulation by monitoring the effects of the intervention on insulin and glucose responses, glucose-dependent insulino tropic polypeptide (GIP) and glucagon-like peptide 1 (GLP1) and colonic fermentation within the acute phase (24 hours). The study utilised a cross-over design and included 14 women with normal glucose tolerance. Study sessions ran over two days: On day 1 of the study participants consumed either the control bread or a bread with additional wheat or oat fibre.
(10.5g and 10.6g respectively) at three separate occasions within 24 hours (i.e. at each mealtime). The following day a further portion of control bread was consumed at breakfast. Blood sampling was commenced for 300 minutes post-ingestion at the beginning of day 1 and 2 to compare the short-term effects. The acute response on Day 1 of the study found that the insulin response to both the wheat (p=0.026) and oat (p<0.001) fibre were significantly earlier than to the control intervention but there was no overall change to the area under curve for the insulin response. For glucose, a fibre x time interaction was found after both the wheat and oat fibre (p=0.021) for the early glucose response, however, the area under curve was not affected. There was also an earlier GIP response for both the wheat fibre (p=0.054) and oat fibre (p=0.02).

After the 24hrs of increased insoluble fibre intake, there was a significant reduction in glucose post-prandial response after the consumption of the control meal; p=0.007 for wheat fibre, p=0.01 for oat fibre. There were no significant shifts in the insulinaemic response post-prandially. Considering the results, the authors conclude that the consumption of insoluble fibre enables improved carbohydrate handling (Weickert et al., 2005) however there is further work required due to the small cohort size and acute study design.

The results of the Cross-Over Study for the glycaemic response are largely non-significant, with minimal differences between interventions at fasting levels for glucose. However, when analysing the response to the oral glucose tolerance test, there was a significant difference between the response for the wheat fibre and control interventions from baseline. The wheat fibre reduced by 2.2% from baseline and the control results showed a 13.8% increase. The relatively small reduction in glucose AUC from the wheat fibre is magnified when compared against the control, with a 16% difference between the two interventions. This result appears to indicate a benefit to consuming wheat fibre, and also seems to support the evidence base currently available that reports that the effects of wheat fibre are largely centred around glycaemic control.

Therefore three intervention studies and the Cross-Over Study have concurred as to the beneficial effects of wheat fibre on glycaemic control and/or insulin sensitivity. However, each have their weaknesses such as short intervention periods and small cohort numbers. A parallel study design may also be more suited for a longer-term intervention period to truly establish the effects of these fibres. The effectiveness of the cross-over study design may also be reduced in these circumstances as the adaptation period for the body to the consumption of the wheat fibre and inulin may be longer than thought.

The data already available on inulin tends to focus on an effect on the lipid profile. In the Cross-Over Study, there were no significant effects from either interventions however as for the insulin results, there were important effects noted. The wheat fibre reduced total cholesterol by 5% with a 4%
reduction in HDL-cholesterol which clearly could have implications for CVD risk if this pattern were to be continued in the longer-term. In addition, there was a 37% increase in triglycerides. For the inulin intervention, although there was a similar increase in triglycerides of 29% compared to the wheat fibre intervention, the inulin performed much better for HDL-cholesterol. The HDL-cholesterol level was increased by 2% whilst the total cholesterol level was increased by a marginal 0.5%. The control group's results were very similar to those of the wheat fibre intervention with a 3% reduction in total cholesterol and a 3% reduction in HDL-cholesterol.

The triglyceride levels recorded after each intervention phase are of concern as prior to commencing the study, the triglyceride levels of the participants were classified as healthy (i.e. <1.7mmol/L). Post-intervention the triglyceride levels increase to 2.23mmol/L, 2.08mmol/L and 1.79mmol/L for the wheat fibre, inulin and control intervention phases respectively. This is unexpected as a recent meta-analysis on the effects of dietary inulin-type fructans on serum triglycerides showed that with increasing intakes of inulin, there is an associated reduction in serum triglycerides of 7.5%. Although the authors note that there were a limited number of studies (15) in which to derive this calculation, most of the studies used to compile the meta-analysis show a reduction in triglycerides to a certain degree. Therefore it would be expected that the serum triglyceride levels in the Cross-Over Study would also mimic the results of the meta-analysis in a similar way. However this is not the case and so this result from the Cross-Over Study should be heeded for future research into the effects of these fibres on health. It may be an anomalous result and won't be repeated in any other future studies, however it could also be indicating that there are elements to these fibres that are not fully understood. Many factors could have influenced this deleterious result, for example, the background diet consumed alongside the inulin or wheat fibre, as other dietary components may have had a negative effect on TAGs, and the possibility of an general 'study effect' whereby all interventions resulted in an elevation of triglycerides. What is known is that when comparing the results for the lipid profile from the Cross-Over Study with other studies within the literature, it is clear that there is not yet a consensus as to the true effects of inulin (nor for wheat fibre).

Letexier et al. (2003) conducted a study in eight healthy participants in a cross-over design comparing the intake of 10g of inulin versus a placebo against a background high-carbohydrate, low-fat diet for 3 weeks (per leg). The results showed that fasting triglyceride levels were significantly reduced after the inulin stage (p<0.05), in addition after an isotope study (using deuterium-enriched water), a significant reduction in hepatic lipogenesis (i.e. contributing to the plasma triglyceride levels) was found, p<0.05 (Letexier et al., 2003). This important step in elucidating how inulin affects lipid levels is not supported by Forcheron et al. (2007), who investigated the effects of an inulin-type fructan. Over a period of 6 months, 17 healthy participants were asked to consume daily either a 10g mix of inulin and oligofructose powder or a placebo equivalent. Despite extensive investigation via isotope study, lipid
profiles, and anthropometrics, no significant changes were detected for any designated outcome marker (Forcheron and Beylot, 2007). However, this study largely differs from those previously described as they used a mix of inulin and oligofructose of unknown proportions rather than pure inulin. The study population is also considerably smaller than the other studies \( (n=8 \text{ placebo, } n=9 \text{ intervention}) \), for a parallel design such as this study and for the length of time for the intervention phase, the numbers may not have been sufficient to ensure enough power in the results.

As no overall significant benefit to health was found from this study, coupled with the evidence of no consensus within the literature, it is difficult to come to a conclusion as to the mechanism of the effect of these fibres. The results, especially for the glycaemic response and lipid profile, are very mixed and contradictory. For example, inulin raised total and HDL cholesterol whereas wheat fibre lowered both. Clearly for optimum benefits, the total cholesterol levels would need to be reduced with an increase in HDL-cholesterol. Likewise for the glycaemic response, minimal increases in glucose were noted however the insulin levels post intervention were much higher which is opposite to what was expected. The participants also did not improve their insulin sensitivity, arterial stiffness or inflammatory markers which were the end-points expected also to be beneficially affected.

Without robust proof either way of the effects of inulin and wheat fibre on the metabolism, there is an obvious gap in the literature to conduct further intervention studies in order to confirm if and how these fibres have effects on the markers of CVD risk. More specifically, focussing on the lipid profile as found in this study, there were changes to HDL and total cholesterol levels, as well as triglycerides that were not entirely expected. Therefore to save the unnecessary expense of merely repeating the study in order to increase participant numbers or study design (parallel vs cross-over), it may be beneficial to examine the effects of inulin and wheat fibre on not only the main fractions of the lipid profile but to also investigate the wider lipid profile to include LDL and HDL sub-classes. By understanding how lipid metabolism is influenced by the consumption of different fibres, it may help provide a wider understanding of the true health benefits of increasing insoluble fibre and inulin within the diet and the eventual effects on CVD risk.

(iv) Dietary analysis

An analysis of the dietary intake of the participants at specific junctures within the study is critical in assessing both the compliance of the participants to consuming the intervention rolls whilst also gauging the impact that the change in diet may have had when compared to their habitual intake.

Overall the compliance from the participants to consuming the intervention rolls appears good. There was a return rate of 67%, with one participant not returning any diet diaries for the intervention periods. However from the remaining nine participants who did return diaries, five fully accounted for consuming the correct quantity of rolls in the diet diaries throughout the study and two participants did
not indicate any consumption of rolls in any diary. The final two participants indicated they consumed a variable amount – one participant consumed all the correct rolls for one intervention phase but none for the other two interventions and the other participant consumed between four and eight rolls (out of a possible nine) for their intervention phases.

When comparing qualitatively between the diaries, the impact of the rolls on the diets of the participants seemed variable. There was evidence of participants incorporating the rolls into their diet by substituting their habitual foods for the rolls, whereas other participants appeared to be consuming the rolls in addition to their regular intake. In all cases, the participants were using high fat/high sugar products to consume with the rolls such as peanut butter, jam and butter. This could have potentially had an impact on their macronutrient intake, in addition to their overall energy intake, however statistical analysis indicated there were no significant differences in dietary intake when comparing the intervention phase dietary intake to the intake at baseline. Therefore the rolls were successful in not causing a detrimental alteration to the participants' diets. The main impact of the rolls was the significant increase in dietary fibre during the wheat fibre intervention compared to the control phase and the higher level of dietary fibre consumed during the inulin phase compared to baseline. These results give additional assurance that the participants must have had a consistent intake of rolls and so assists in strengthening the proof of participant compliance when there is the clear increase in fibre intake during the fibre-based intervention phases compared to the control/baseline phases.

When assessing the diaries and noting the habitual intakes of the participants, almost all diaries indicated poor food choices. High fat meals and snacks were common place with limited fruit and vegetable intake. Routine mealtimes were lacking and large amounts of carbonated drinks as well as caffeine were consumed. Yet when assessing the percentage energy that is contributed to the diet from fat, the highest level was found at baseline, with 37% of energy intake being attributed to fat. All other intervention phases ranged from 30-32%, which is an acceptable level. A recent concern for health is salt intake due to excess amounts consumed within the diet being linked to the incidence of hypertension (FSA. However, at no point was the average salt intake far above the recommended level of 6g/day. Over the intervention phases, the participants intake of salt ranged from 5.1g-6.1g per day. When comparing the qualitative assessment and the actual data from the dietary analysis the relatively poor diet choices of the participants do not entirely concur with the statistical outcomes. Despite eating high fat foods which are likely to contain high levels of salt and possibly sugar, the data from the diaries indicates a healthier outcome. No macronutrients were over recommended government levels and the overall energy intakes appeared low. Therefore, in order to gain some view on the validity of the diaries and whether there was a degree of 'under-reporting', the Goldberg equation (Black, 2000) was implemented and some discrepancies were found between the average expected energy intake and the average actual energy intake that was reported.
It was determined that for all four stages of diet diary analysis the participants were reporting far lower energy intakes than would have been expected for the average weight and age of the participants (mean weight 101.6kg, mean age 39 years). For the baseline, wheat fibre, inulin and control phase diet diaries, there was a discrepancy between the expected and actual energy intakes of 35%, 25%, 30% and 35% respectively. These calculations are based on the mean BMR for the group (8530kJ/day) with the widely recognised ‘average’ physical activity level (PAL) of 1.55 (Black, 2000). Using these values, the expected energy intake would have been 13,221kJ/day, yet the diet diaries reported intakes much lower than this. This may be explained by the fact that both the methodology of diet diaries and the assessment of under-reporting using the Goldberg equation are subject to a number of assumptions and sources of error. The researcher relies on the participants completing the diet diaries as clearly as possible, giving all the detail available so that their dietary intake can be accurately assessed. However, the participant may inadvertently introduce error by altering their diet to a simpler variety to enable them to complete the diary more efficiently, even though the diet represented in the diary is no longer a version of the participant’s habitual diet as is required. The researcher may under or over estimate quantities when analysing the diaries. The Goldberg equation, though useful, is based on assumptions of required energy intakes and estimated activity levels. Therefore the results of this diet diary analysis may be of limited value to the study from a quantitative point of view. However, the actual descriptions of food and comparisons of food choices over the course of the intervention gives an interesting and valuable insight into how the implementation of an intervention can affect participants’ food choices and how their behaviour and eating patterns may adapt under study conditions.

6.4.2 Limitations
This study was sufficiently powered in terms of insulin sensitivity however it is still a small scale study which could have possibly benefited from an increase in the number of participants. Instead of powering the study based on insulin sensitivity, pulse wave velocity could be used instead. Liang et al. (1998) have calculated that when conducting a study powered specifically for PWV, a cohort of eight would be sufficient in a 2-sided cross-over design to detect a 10% change. This therefore gives reassurance that the Cross-Over Study is adequately powered, however given the nature of the results produced it may still be advisable to increase the cohort.

Another possible issue was the cross-over design itself. Even though there were wash-out periods contained within the study design of the same length of time as the intervention periods, it may have been beneficial in this instance to increase the wash-out stages to perhaps 8 weeks or more (if keeping the intervention stage at 4 weeks). The wash-out time period coupled with one baseline could mean that if a participant responded well to a treatment phase and thus had a lowered PWV, this effect could continue for the rest of the study, independent of further treatment. The only way to control for this
would be to complete a baseline assessment prior to each intervention phase however this was not possible as explained below. In order to take into account the possibility of a ‘carried over’ effect, intervention-phase results were analysed in visit order (as opposed to being grouped per intervention) to assess for any possible effect. No time effect was found for any marker when comparing baseline to the second, third and fourth visit.

It could have also been beneficial to include a baseline assessment before each intervention stage, not just at the beginning of the entire study. The reason that the Cross-Over Study had only one baseline was because of the relatively intensive study design, multiple blood samples were required. Ethically it was not justified to then include a baseline prior to starting each intervention stage as it would have been too big a burden for the participants in terms of blood donations. The number of visits involved may have also become prohibitive as the length of time the participants would have needed to use to attend may have caused problems with their working or personal schedules. Therefore it was deemed sufficient for one baseline to be taken at the beginning of the study with the matching wash-out stage which left the assumption that once the participants had completed the wash-out phases, they had essentially returned to their baseline measurements.

The level of fibre intake per day could also be re-assessed as the participants were consuming 15g per day. This was the level found to be tolerable in previous studies (Williams and Jackson, 2002) and is fairly reflective of the amount of fibre consumed within the recommended amount of whole grains (48g per day). Therefore the levels are physiological and a realistic representation of possible intakes. Yet these amounts did not cause an effect therefore it could be beneficial in this instance to increase the amount of fibre to force a metabolic change so to enable a mechanistic pathway to be found.

6.4.3 Conclusion
Overall, no conclusive effect from this intervention study assessing the effects of an increase in the consumption of inulin or wheat fibre on CVD risk factors was found. Comparing the results of the study to the current literature there are contradictory results found for the lipid profile which indicates a possible deleterious effect of consuming these fibres on CVD risk. However, an interesting reduction in the glucose area under the curve for wheat fibre may be indicating that there is a beneficial effect on glycaemic control. Therefore at present, it is not possible to say whether overall these fibres impart positive or negative effects to CVD health due to the relatively few metabolic changes post-intervention. However, given the gaps in the current literature regarding the use and effects of these fibres, more research based on dietary intervention trials is justified especially to focus in detail on how the fibres affect individual CVD risk markers.
Chapter 7

7 General Discussion

Through a series of dietary intervention studies, the SLOWCARB programme of research hoped to elucidate a mechanism to explain how the consumption of whole grains (and their constituent fibres) within the diet may confer benefits to cardiovascular health. It was also a unique opportunity to explore and develop a novel approach to introducing a dietary intervention to the habitual diets of the participants.

Overall the SLOWCARB project has not identified a consistent reduction in CVD risk over the medium-term following daily dietary supplementation with food sources of either whole grain or two of its constituents. This applies to population groups at both high and low risk of CVD and therefore disproves the overall hypothesis that whole grain intake would positively influence risk factors for CVD that include inflammatory status and arterial stiffness. Although no significant benefit was found after the consumption of the whole grains, the data obtained supports other intervention studies that also found no effect from whole grain consumption (Brownlee et al. 2010, Andersson et al. 2007).

Although there is convincing data within the current literature establishing the importance of arterial stiffness as an independent risk factor for CVD (Safar et al., 2002, Laurent et al., 2001), the positive ‘whole-body’ benefits of whole grain consumption (Liu et al., 1999) and the implication of an inflammatory response in the development of atherosclerosis (Libby, 2002), there are currently no studies attempting to investigate these factors within one comprehensive design. Therefore this programme of work represented a unique opportunity to examine the benefits of consuming a diet rich in whole grains on global risk, as categorised by markers of arterial stiffness, inflammation, lipid profile and glucose control.

Furthermore, the novelty of this work extends to the unique dietary intervention delivery system employed within the SLOWCARB programme with the use of specifically produced bread rolls. Historically within whole grain intervention studies, a like-for-like food replacement plan using commercial products is frequently implemented however participants must have a good understanding of portion sizes, and of which foods contain whole or refined grain, in order to calculate the correct servings necessary. In contrast this programme of work utilised bread rolls loaded with specific
amounts of the ‘active ingredient’ i.e. 24g of whole grain per roll for the *High Risk Study/Low Risk Study* and 5g of inulin or wheat fibre per roll for the *Cross-Over Study*. These rolls provided flexibility in how the active ingredients were presented to the participants within the intervention studies. For example, it was possible to deliver grains where the structure had been altered by crushing them yet they still retained the main components of the grain in the correct proportions and in an amount to match that of the intact grain. In the case of the *High Risk Study* and *Low Risk Study* both a whole grain intervention (i.e. grains intact) and a milled grain intervention (an identical roll with the same amount of grains but milled in order to separate the germ, endosperm and bran components) were used against an isocaloric control roll. This approach enabled investigation of the impact of grain structure on the overall health effects imparted by the whole grains, an understudied area of the wholegrain field.

One early acute response study by Jenkins et al. (1988) did find that when breads that were enriched heavily with barley or cracked wheat (up to 75% of the content) were consumed, there was a lower glycaemic response compared to the breads with a lower density of grains. Although not specifically looking at the structure or degree of milling of the grains, the study by Jenkins et al. (1988) is interesting as it shows that the concentration of grains within the food item is important. What was also found was that even though the bread used in the Jenkins study was heavily packed with whole grains, it was still deemed palatable. This was also found for the rolls used within the SLOWCARB studies, as a sensory evaluation (Section 2.3) was conducted to compare the qualities of the experimental rolls used within the studies to the market equivalent. Even though the bread rolls used as part of the *High and Low Risk studies* are clearly only for investigational purposes in this instance, it was found that after evaluating all the outcomes, the whole grain rolls (as well as the milled grain and refined grain rolls) were palatable and so there should be no major barriers to consumption for the participants. Looking to the future, this could have implications for future research as the use of these rolls should mean that there is no significant negative effect on participant compliance to the intervention. If the increased consumption of whole grains is found to have a definite positive effect on CVD health from further research, then there could be real applications possible within the wider population, with commercial opportunities to develop similar products for general the general population.

The use of the rolls also conferred advantages in terms of subject compliance with the intervention. Using the system of bread rolls, the portion and amount of whole grains (or constituent fibres) consumed by the participant can be easily controlled, monitored and quantified by the researcher. This was also found to avoid the potential issues of portion size distortion that can occur with the alternative and commonly used food exchange system (i.e. swapping refined grain products for equivalent whole grain product), reducing participant burden by cutting out the need for weighing,
counting or estimating portion sizes. Whilst the specific wholegrain interventions resulted in few significant changes in health markers, there is no evidence that this was a function of unsuccessful intervention strategies and so the intervention model developed here, through a successful collaboration between academia and industry, can be recommended for future dietary supplementation or enrichment studies.

As pulse wave velocity (PWV) was chosen as one of the primary measures for the SLOWCARB programme, it was important to understand the reproducibility of PWV within the target population groups of the proposed dietary intervention studies. Within the available literature there was no study that recorded the reproducibility of PWV over a period of time such as 4-6 weeks (existing evidence focused on a two-week research period, for example Liang et al. (1998) and Woodman et al. (2005) or which detailed how PWV may fluctuate within a short space of time (i.e. 1-3 hours), such as that found within a clinical trial study session. Therefore, despite the support for the use of PWV, a gap in the literature was identified with respect to the validity and reproducibility of its specific application in population groups at different levels of CVD risk, over the time course represented by a medium term intervention study.

Thus an exploratory validation study was completed of which the main aim was to examine the reproducibility of PWV in a variety of controlled conditions so to reproduce typical study conditions and normal metabolic processes (fed vs. fasted, sedentary vs. active), as well as measuring the time interaction. This should allow for the natural variation in arterial stiffness and its response to metabolic stimuli within the study population to be assessed, allowing the findings from the intervention studies to be interpreted appropriately.

The validation study was unique in its approach and overall found PWV to be highly reproducible when compared to other data sources within the literature. It was found that over the longer time-course of 6 weeks, that there was no significant variation for either central or peripheral arterial stiffness. The variation within a PWV recording session was slightly higher at 9-10% (compared to ≈5% for the inter-session variation) although not significantly so. Both sets of results confirmed the hypothesis that the co-efficient of variation for both intra and inter-session PWV would not exceed 10%. Given the low level of variability in PWV over time and the good reproducibility, this should mean that it is a reliable marker for using as a basis for power calculations in future studies.

Further work here may be required, however, to examine whether the larger intra-session variability is influenced more by the operator than the actual level of arterial stiffness of the participant, which is more an issue of repeatability. In the meantime, sufficient data has been collated to reassure that when applying this knowledge of PWV reproducibility to the PWV data obtained from intervention studies, it can be confidently assumed that any significant changes found post-intervention should not be
influenced by anomalous variations in PWV over time. This is also pertinent when considering the blood pressure fluctuations post-exercise and post-prandial as part of the PWV validation study. PWV was far less variable, with no significant changes over time or in response to acute metabolic changes which is indicative that PWV is more likely to respond to chronic changes. In contrast, the blood pressure (taken pre and post-exercise/meal) was instantly responsive and is clearly in a constant state of flux, which is also demonstrated by the diurnal variation found within the 24-hour ambulatory blood pressure data from the Low Risk Study and the Cross-Over Study.

Each of the intervention studies produced some interesting, and sometimes contradictory, results. For the High Risk Study, the whole grain group experienced an increase in diastolic blood pressure (BP) in addition to rises in mean arterial pressure (MAP) and the inflammatory cytokine IL-1β and a reduction in pulse pressure (PP). As previously discussed in Chapter 4.4, the attempt to elucidate a possible mechanism to explain the benefits of whole grains on health was not possible in this case as the BP, MAP and PP results did not fit with the established physiology of blood pressure. The IL-1β increase also appeared anomalous since it was not supported by the concomitant increases in other cytokines such as TNF-α and IL-6 that would have been expected since they all combine together to form an organised inflammatory response based around the endothelium (Hansson 2001). For the Low Risk Study, there was a significant reduction in central PWV within the whole grain group but there were no other indications of an effect from whole grains for any other outcome markers.

When considering these results from both the High and Low Risk studies together, it is clear that there is no definitive relationship between whole grain intake and risk of CVD. This finding is in direct disagreement with a large proportion of the epidemiological data available which suggests an inverse link between whole grain consumption and CVD risk (Liu et al., 2000). The possible mechanistic pathway linking whole grains and CVD risk has already been discussed within the respective High and Low Risk Study chapters. Yet irrespective of proposed pathways, considering the current literature and the results from these studies, it is clear that both population groups (low and high risk of CVD) require further research targeted to ascertain whether there is any conceivable benefit to health in consuming whole grains. As found in the High Risk Study, deleterious effects were found mainly relating to whole-body measurements of vascular health (diastolic blood pressure, mean arterial pressure etc.). In contrast the Low Risk Study demonstrated beneficial effects of whole grains on pulse wave velocity, requiring a more in-depth investigation into the basis of these results and specifically whether they were directly linked to the consumption of whole grains is very important at this point. Surveying the current literature, the most powerful and robust study conducted to date focusing on whole grains and CVD risk is the WHOLEheart study (Brownlee et al. 2010). Matching the cohort size and length of future intervention studies to that of the WHOLEheart study, whilst also focusing intensively on whole-body measures of vascular health, would create an excellent opportunity to
produce truly comparable data across the population groups which is something that is currently missing from the literature to date. Until larger cohorts are assessed via highly detailed data and analysis, the intervention dose of whole grains should remain at the level of the current USDA recommendation. This is also justified because from the epidemiological data lower amounts than the 48g used in the High Risk Study were consumed by their cohorts, yet positive outcomes for health were achieved. Without clarifying whether this level is sufficient to improve health beyond any doubt, there is little point progressing to higher or indeed lower amounts of whole grain intake if the current guidelines cannot be proven categorically.

The Cross-Over Study is equally unique through its aim to investigate whether certain fibres found within whole grains are influencing CVD risk markers within a synergistic relationship or on an individual basis. Currently the literature appears to show that there is some evidence beginning to show that consuming inulin is beneficial to the lipid profile (Letexier et al., 2003), as is wheat fibre (Giacco et al., 2010). However, the literature available reporting intervention trials focusing on inulin (non-digestible carbohydrate) and wheat fibre (insoluble fibre) do not attempt to assess the overall CVD risk by measuring a range of markers covering the same aspects of the metabolism as the High and Low Risk studies, such as arterial stiffness, inflammatory response, blood pressure and glycaemic control.

The overall results of the Cross-Over Study showed that there was a small significant effect of the consumption of both inulin and wheat fibre. As fully discussed in Chapter 6.4, the effect was identified in relation to glucose handling following the OGTT. Although there were no significant differences in total glucose or insulin release post glucose challenge (as assessed via AUC), when looking at the difference in percentage change from baseline between the interventions there was a significant reduction in AUC following the wheat fibre interventions alongside a relatively large increase in AUC following the control intervention. A marginal increase in AUC post-inulin intervention was also observed. Without both interventions showing simultaneous reductions or indeed increases in AUC, it is not possible to determine an underlying mechanism. Further research is justified to determine whether the different glycaemic responses elicited by the wholegrain components is a true reflection of the impact these fibres can have on health. In terms of a mechanism behind this particular result, since the OGTT glucose AUC was lower compared to baseline, this could be indicating an improvement in glucose handling due to prolonged daily consumption of wheat fibre. If the participants continue to have this reduced glycaemic response to the wheat fibre on a consistent basis in the longer-term, then real changes to their health may occur since insulin resistance is linked to the development of a raised lipid profile and type 2 diabetes (Lionetti et al., 2009). Insulin resistance is also the end result of an inflammatory process involving adipose tissue (Lionetti et al., 2009), thereby inferring that if insulin resistance can be reduced, then the inflammatory response can
also be dampened and so reducing the risk of CVD overall.

By not finding any real difference between the inclusion of inulin or wheat fibre in the diet versus a control, it can be concluded that these fibres individually are yet to be fully defined as to how they exactly exert an effect on health. And as already discussed above, data has been shown that when these fibres are consumed together within the whole grain, this scenario also does not seem to confer substantial benefits to lowering CVD risk within the identified population groups.

There are potential limiting factors to consider for dietary intervention studies such as the *High Risk Study* and the *Cross-Over Study* which may have moderated the effects observed. There may be an optimal background dietary intake (i.e. vitamin and mineral intake, antioxidant status etc.) within which whole grains have to be consumed in order to maximize their effects. As shown in the dietary analysis from the *Cross-Over Study* (Section 6.6), the fat content of the participants' diets were relatively high, alongside low levels of fibre, therefore their dietary intake may have been inhibitory and thus a potential confounding factor. For future dietary interventions, it may be beneficial to implement a standardised habitual diet for the participants to consume in addition to the intervention so creating a controlled environment and optimising the opportunity to evidence any metabolic effects from the fibres, or indeed whole grains.

The ongoing collection of evidence from interventions trials investigating the use of whole grains and their constituent fibres appears to be weakening, rather than strengthening the previously perceived relationship between regular dietary consumption of whole grains and health benefits. This benefit of consuming whole grains was initially given credence by the numerous and very large prospective observational studies running up to the 1990's. As whole grains began to be correlated with reductions in CVD risk (Liu et al., 2000), naturally intervention trials were then set-up to test the proposed relationships. However, the whole grain field is largely now in disagreement as to the scale of the impact whole grains can have on health and even if they have any benefit at all. There also remains a large gap in the literature in terms of large-scale intervention trials that need to be conducted over many months, if not years to give a real chance for the whole grains to confer any possible benefits.

The difference in outcome between the observational and intervention studies within the current literature may be due to a series of factors relating to cohort size, the population groups targeted, length of observation/intervention and the study outcomes chosen to quantify the effects of the whole grains. Naturally an observational study is far cheaper to conduct than an intervention study, therefore larger numbers of participants are able to be recruited and retained for much longer periods of time compared to a typical intervention study. Observational studies are then more likely to pick up patterns within the cohort's results to detect any possible influence from the reported consumption of whole grains. The intervention study is intensive in terms of research activity for both the researcher and
participant; thus this can have a limiting effect on resources and the numbers of participants to be recruited. Maintaining cohort compliance with an intervention product can prove troublesome if the duration of the study is too long. Both study designs provide interesting data, however observational studies are only ever ‘hypothesis-generating’ and will never be able to truly ascertain any form of causal relationship between whole grains and CVD health. Comparing this to the intervention studies (hypothesis-testing), these studies are designed to piece together a mechanism (if one is present) linking whole grain to CVD risk. Therefore any outcome, no matter what it shows and provided the methodology is robust, should be argued as having the greater value compared to the observational data available. This is clear within the field of whole grain and CVD research. Despite positive benefits to health (of increased whole grain consumption) described in the epidemiological data, this is not translated into the intervention trials which are the real test as to any cause-effect relationship that may be present.

**Limitations:**

Overall, the SLOWCARB research programme was well developed with clear goals due to the recognition of a lack of available data examining the effects of whole grains on the risk of CVD via a global health assessment. As discussed in Chapter 4 referring to the High Risk Study, target sample sizes were difficult to meet and after extensive efforts, it was decided to cease recruitment in order to maintain the progress of the overall research project. In addition to the smaller cohort size, a further issue was the mixed cohort of men and women participants involved in the study. Even though the randomisation was stratified to ensure equal spread of men and women across the intervention groups, in hindsight, it may not have been conducive to a robust study design to recruit both men and women. The main problem is the imbalance of numbers, with 15 men and 6 women taking part, with each intervention group containing 5 men and 2 women. Both genders were recruited due to their apparently high CVD risk according to their gender (mainly based on age and waist circumference; men with waist circumference >94cm, women >80cm). However, the level of CVD risk that men and women experience at different ages could vary and so the intervention groups may have become too heterogenous in terms of actual CVD risk, with too much natural variation between the individuals to show a true effect from whole grains. However, considering the relatively small, mixed gender cohort (women were post-menopausal thus eliminating hormonal influence) for the High Risk Study, the large cohort intervention trial the ‘WHOLEheart study’ by Brownlee et al. (2010) also had a mix of men and women with an even larger age range of 18-65yrs yet they too found no significant changes. With a male to female ratio of nearly 1:1 and a minimal inclusion criteria of requiring a BMI >25kg/m², the cohort was very heterogenous in nature. However, it would be rational to think that by recruiting 266 participants (total number who completed the WHOLE heart study), that the background ‘noise’ created by the inclusion of a diverse population of people at varying levels of CVD risk would actually
be cancelled out to some degree by the size of the cohort. This is especially interesting as the study was highly powered, with the cohort size required based on expected reductions in LDL cholesterol observed from a previous viability study (Brownlee et al., 2010). However, in this case, no effect from whole grains on CVD risk was found and so for future study designs it is important to consider the implications of recruiting both genders within the same study.

The monitoring of participant compliance for the duration of the intervention studies may have benefitted from additional measures to ensure that the participants were consuming the intervention rolls in a consistent manner throughout the intervention. Apart from the measures put in place as part of the SLOWCARB intervention studies (i.e. diet diaries, regular communication with participants), it may also have been of benefit to include the analysis of biomarkers indicative of whole grain intake. One possibility is to monitor the plasma levels of alkylresorcinols which are phenolic lipids found only in wheat and rye (Landberg et al., 2008). The results of a study by Landberg et al (2008) showed that via the consumption of increased whole grain, there was a proportionate increase in plasma levels of alkylresorcinols (Spearman’s $r=0.58, p<0.001$). When consuming refined grains, the alkylresorcinol levels reduced. Therefore regular assessment of alkylresorcinol levels could be a key unobtrusive tool in which to monitor compliance. The current limitation with the use of this biomarker is the need for further, large-scale investigation to confirm its position as a genuine biomarker of whole grain consumption. There is also no indication as to how long it takes for the plasma levels to be indicative of whole grain consumption, nor if levels are affected by any other factors. For example, the measurement of enterolactone (another possible biomarker for whole grain intake) is affected by the participant taking antibiotics (plasma levels reduce) and is also reliant on adequate gut microflora to ensure conversion from the plant lignans (found in the whole grains) to the mammalian lignans circulating in the plasma (Jacobs et al., 2002).

Despite the need for further clarification as to the most appropriate biomarkers of whole grain intake the field is growing rapidly, and so developing this area for use in confirming participant compliance should result in a very useful tool for future dietary intervention trials involving the spectrum of whole grains and their constituents.

**Future work:**

For future research, there may be scope to extend this research further. Despite detecting no beneficial effects of whole grains overall, the issues with subject recruitment and sample size means that it remains justified to repeat the study design of the *High Risk Study* in a much larger cohort depending on a review of how to power the study, possibly using data from the pulse wave validation study (Chapter 3). Due to the smaller than anticipated study cohort, any beneficial effects may not have been detected due to a lack of power, therefore justifying the possibility of repeating the approach but
having a much wider-ranging and better funded strategy for recruiting participants. With greater resources in advertising and forming key partnerships with local healthcare providers, such as general practitioners, recruiting a larger cohort should be possible. An alternative would be to create a multi-site study comparing separate local populations that are differentiated by the socioeconomic climate (i.e. unemployment or employment) or public health issues such as levels of obesity or CVD mortality etc.

In terms of emerging areas of research that may tie in with this project, the detection of matrix metalloproteinases (MMP) and endothelial progenitor cells (EPCs) could provide interesting avenues and additional information as to the possible effects of whole grains that may not have been identified within the remit of this thesis. It has already been shown that there is a strong inverse relationship between pulse wave velocity and serum levels of MMP-2 and MMP-9 (Vlachopoulos et al., 2007). Since MMPs are responsible for driving the degradation of the extracellular matrix within the arteries (Yasmin et al., 2006) and contributing to atherosclerotic plaque stability (Pasterkamp et al., 2000), it appears that this action may in turn affect the elasticity of the artery thus increasing arterial stiffness.

An important review by Timmermans et al. (2009) describes the presence of endothelial progenitor cells (EPCs) which apparently circulate in the blood (Asahara et al., 1997) and are implicated in neovascularisation of the artery wall (Timmermans et al., 2009). However, there is still much controversy and uncertainty as to the true applicability of the EPCs in terms of understanding their link with CVD as most of the work referring to them seems to focus on in vitro animal cell culture models. However, within a standard dietary intervention, isolating EPCs from the blood samples obtained from participants in an ex vivo experiment may provide an exciting challenge. This would be an opportunity to develop this area further and gain understanding as to the prevalence and activity of EPCs within the human body as opposed to the animal models currently used. The observation of the activities of MMPs and EPCs is important in terms of looking at additional possible mechanisms linking whole grains to health. However this research would be far more effective if it could be established first that there is an overall effect of whole grains on whole-body measurements.

By expanding the molecular aspect of this research, the context in which the inflammatory response and the development of atherosclerosis and arterial stiffness occur may become clearer. The continued development of the ‘omics’ (proteomics, genomics and metabolomics) within nutrition is an absolutely critical avenue of research as they provide highly detailed data that is not possible to obtain within the standard dietary intervention studies. Within the High Risk Study metabolomics has been initiated (by another researcher) to identify a biomarker for whole grain consumption in addition to profiling those at high risk of CVD. However it also has many other applications and the ideal main area of development within whole grain and CVD research would be to definitively establish the typical profiles of not only those at risk of CVD but also those that are not and those that are...
symptomatic of CVD (i.e. post-myocardial infarction). By identifying the elements that separate the CVD risk profiles but also those that are present throughout, the data provided could help steer intervention studies towards a direction where they become more efficient and effective in their design and overall execution.

Conclusion:

Despite the epidemiological evidence suggesting a role for whole grains in reducing CVD risk, this collection of studies adds to the evidence base which has failed to provide evidence that supplementation with achievable levels of dietary whole grain can illicit measurable improvements in CVD risk. This is despite the use of robust intervention study designs (run over the medium term) coupled with the measurement of a wide range of markers used to identify variations in arterial stiffness, inflammation, blood pressure, lipid metabolism, glycaemic regulation and body composition.

Future research must focus on the final confirmation as to the effectiveness of whole grains in reducing CVD risk through further dietary intervention studies whilst incorporating the unique data provided by nutrigenomics (i.e. nutrient-gene interactions related to whole grains) and metabolomics in order to inform appropriate and effective public health policy to reduce the burden of CVD within the UK.
References


References


References


References


Published Work

Abstracts


Appendix A

Appendix A

Appendix A.1 – Generic consent form used for all studies and altered as appropriate

Consent Form

I the undersigned voluntarily agree to take part in the study: The acute effects of the soluble and insoluble fibres found within whole grain on the markers of cardiovascular disease risk in adult men.

- I have read and understood the Information Sheet provided. I have been given a full explanation by the investigators of the nature, purpose, location and likely duration of the study, and of what I will be expected to do. I have been advised about any discomfort and possible ill-effects on my health and well-being which may result. I am aware of the procedure involved regarding Pulse Wave Velocity tests. I have been given the opportunity to ask questions on all aspects of the study and have understood the advice and information given as a result.
- I agree to comply with any instruction given to me during the study and to co-operate fully with the investigators. I shall inform them immediately if I suffer any deterioration of any kind in my health or well-being, or experience any unexpected or unusual symptoms.
- I agree to the investigators contacting my general practitioner about my participation in the study, and I authorise my GP to disclose details of my relevant medical or drug history, in confidence.
- I understand that all personal data relating to volunteers is held and processed in the strictest confidence, and in accordance with the Data Protection Act (1998). I agree that I will not seek to restrict the use of the results of the study on the understanding that my anonymity is preserved.
- I understand that I am free to withdraw from the study at any time without needing to justify my decision and without prejudice.
- I acknowledge that in consideration for completing the study I shall receive the sum of £120. I recognise that the sum would be less, and at the discretion of the Principal Investigator, if I withdraw before completion of the study.
- I understand that in the event of my suffering a significant and enduring injury (including illness or disease) as a direct result of my participation in the study, compensation will be paid to me by the University subject to certain provisos and limitations. The amount of compensation will be appropriate to the nature, severity and persistence of the injury and will, in general terms, be consistent with the amount of damages commonly awarded for similar injury by an English court in cases where the liability has been admitted.
- I confirm that I have read and understood the above and freely consent to participating in this study. I have been given adequate time to consider my participation and agree to comply with the instructions and restrictions of the study.

Name of volunteer (BLOCK CAPITALS) ..........................................................
Signed ..............................................................................................................
Date ..................................................................................................................

Name of researcher/person taking consent (BLOCK CAPITALS) ............................
Signed ..............................................................................................................
Date ..................................................................................................................
Appendix A.2 - Dutch Eating Behaviour Questionnaire (DEBO)

Participant code______________________ Date__/__/__

Please answer the following questions as carefully and honestly as possible.

Read each question and simply fill in the column which best applies to you.

<table>
<thead>
<tr>
<th>Question</th>
<th>Never</th>
<th>Seldom</th>
<th>Sometimes</th>
<th>Often</th>
<th>Very often</th>
<th>Not relevant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. If you have put on weight, do you eat less than you usually do? *</td>
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<tr>
<td>2. Do you have a desire to eat when you are irritated? *</td>
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<td>3. If food tastes good to you, do you eat more than you usually do? *</td>
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<tr>
<td>4. Do you try to eat less at meal times than you would like to eat? *</td>
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<td>5. Do you have a desire to eat when you have nothing to do? *</td>
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<tr>
<td>6. Do you have a desire to eat when you are depressed or discouraged? *</td>
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<td>7. If food smells and looks good, do you eat more than you usually eat? *</td>
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<td>8. How often do you refuse food or drink offered because you are concerned about your weight? *</td>
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<td>9. Do you have a desire to eat when you are feeling lonely? *</td>
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<td>10. If you see or smell something delicious, do you have a desire to eat it? *</td>
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<td>11. Do you watch exactly what you eat? *</td>
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<tr>
<td>12. Do you have a desire to eat when somebody lets you down? *</td>
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<td>13. If you have something delicious to eat, do you eat it straight away? *</td>
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<td>14. Do you deliberately eat foods that are slimming? *</td>
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<tr>
<td>15. Do you have a desire to eat when you are cross? *</td>
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<td>16. Do you have a desire to eat when you are approaching something unpleasant to happen? *</td>
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<tr>
<td>17. If you walk past the baker do you have a desire to buy something delicious? *</td>
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<tr>
<td>18. When you have eaten too much, do you eat less than usual the following days? *</td>
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<td>19. Do you get a desire to eat when you are anxious, worried or tense? *</td>
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<tr>
<td>20. If you walk past a snack bar or café, do you have a desire to buy something delicious? *</td>
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<tr>
<td>21. Do you deliberately eat less in order not to become heavier? *</td>
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<tr>
<td>22. Do you have a desire to eat when things are going against you, or things have gone wrong? *</td>
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<td>23. If you see others eating, do you have also the desire to eat? *</td>
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<tr>
<td>24. How often do you try not to eat between meals because you are watching your weight? *</td>
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<tr>
<td>25. Do you have a desire to eat when you are frightened? *</td>
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<tr>
<td>26. Can you resist eating delicious food? *</td>
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<tr>
<td>27. How often in the evening do you try not to eat because you are watching your weight? *</td>
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<tr>
<td>28. Do you have a desire to eat when you are disappointed? *</td>
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<tr>
<td>29. Do you eat more than usual when you see other eating? *</td>
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<tr>
<td>30. Do you take your weight into account when you eat? *</td>
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<tr>
<td>31. Do you have a desire to eat when you are emotionally upset? *</td>
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<tr>
<td>32. When preparing a meal are you inclined to eat something? *</td>
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<tr>
<td>33. Do you have a desire to eat when you are bored or restless? *</td>
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</tbody>
</table>

Structure:

The DEBQ questionnaire consists of 33 items, all with the same response format – never, seldom, sometimes, often and very often, together with a non relevant category in items that are presented in a conditional format involving particular experiences. The 33 items are distributed as follow by the three behavioural patterns groups aimed to be investigated:
* Emotional eating - 13 items
* External eating - 10 items
* Restrained eating - 10 items

**Scoring:**
Each item is scored as follows, with the exception of item 26 which scoring has to be reversed.

- Never - 1
- Seldom - 2
- Sometimes - 3
- Often - 4
- Very often - 5

Scores are then added up for each group of items (emotional, external and restrained) and the final score for that group calculated, individually for each subject, as follows:

e.g. Restrained score = total score from “restrained” items

\[
\text{number of restrained group items}
\]

e.g. Restrained score = \( \frac{28}{13} = 2.2 \)

If a subject chose the “non relevant” option, this is given a score of 0 and the number of items used as the division factor in the above described equation is reduced by 1 for that particular group.
Appendix A, 3 - Sensory Evaluation - Hedonic testing

Date: ________________  Age: ________________

Please taste test these samples and check how much you like or dislike each one. Please tick the statement that best describes your feeling about the sample and provide a reason for this choice.

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Like extremely</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Like very much</td>
<td></td>
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<tr>
<td>Like moderately</td>
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<tr>
<td>Like slightly</td>
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<tr>
<td>Neither like nor dislike</td>
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<tr>
<td>Dislike slightly</td>
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<tr>
<td>Dislike moderately</td>
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<tr>
<td>Dislike very much</td>
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<tr>
<td>Dislike extremely</td>
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</table>

Reasons:
**Appendix A.4 - Sensory evaluation - Difference Test**

Date: __________   Age: ___________   Sample: ___________

For each of the following words, please indicate which numbers best describe the samples tested by circling them:

1) **Moistness**
   - Very dry
   - Just right
   - Very moist
   
   1  2  3  4  5  6  7  8  9  

2) **Chewy**
   - Very chewy
   - Just right
   - Very soft
   
   1  2  3  4  5  6  7  8  9  

3) **Hard**
   - Very hard
   - Just right
   - Very soft
   
   1  2  3  4  5  6  7  8  9  

4) **Roughness**
   - Very bitty
   - Just right
   - Very smooth
   
   1  2  3  4  5  6  7  8  9  

5) **Salty**
   - Very Salty
   - Just right
   - Very bland
   
   1  2  3  4  5  6  7  8  9  

6) **Brown**
   - Cooked too much
   - Just right
   - Not cooked enough
   
   1  2  3  4  5  6  7  8  9  

7) **Filling**
   - Very filling
   - Just right
   - Not filling enough
   
   1  2  3  4  5  6  7  8  9  

8) **After taste**
   - Strong after taste
   - Just right
   - No after taste
   
   1  2  3  4  5  6  7  8  9  
Appendix A.5 - Sensory evaluation - Visual

Please look at the scale photographs (following page) of 6 different types of bread (A-F). Rank each bread in order of your preference.

The bread which you feel looks the most appetising would be 1st, the least appetising 6th (last).

Please also indicate your reasons for your choices.

1st -
Reason:

2nd -
Reason:

3rd -
Reason:

4th -
Reason:

5th -
Reason:

6th -
Reason:
Appendix A.6 - Sensory evaluation - Photographs

A

B

C

D

E

F
Appendix A.7 - Health and Lifestyle Questionnaire – Generic used for all studies

A Pilot Study: The influence of whole grain intervention on markers of cardiovascular disease risk in adult males.

Name: __________________________ DOB: __________________________

Address: ___________________________________________________________________

____________________________________ Daytime Tel: __________________________

________________________ Evening Tel: _______________________________________

GP Name: __________________________

GP Address: __________________________

Please find below a short health and lifestyle questionnaire that will be used as part of the screening process for this study.

Please tick all that apply:

| I have no prior/present history of coronary heart disease, angina, heart attack or stroke |
| I have no prior/present history of Type 1 and Type 2 Diabetes. |
| I have no prior/present history of a gastrointestinal disorder, such as Crohn's Disease, Coeliac Disease or Irritable Bowel Syndrome. |
| I have no prior/present history of liver or kidney disease. |
| I have no prior/present history of, or I am not currently being treated for clinical depression or other psychological disorders. |
| I have no prior/present history of eating disorders. |
| I have no prior/present history of drug or alcohol abuse within the last 2 years. |
| I am currently not taking any regular medication prescribed by my GP. |

Do you take any dietary supplements such as vitamins, minerals or fish oils? YES/NO

If yes, please state which type and how often.

Are you currently on a weight-reducing diet or other dietary restrictions? YES/NO

If yes, please give details.
Appendix A

Do you regularly include whole grain foods in your normal diet? YES/NO
If yes, how many times a week do you have whole grain foods?
(Please see attached list)

Do you exercise regularly? YES/NO
If yes, what type of exercise and how often?

Have you been involved in a clinical trial in the last 3 months? YES/NO

Do you smoke? YES/NO
If yes, how many per day?

Do you drink alcohol? YES/NO
If yes, how many units per week? (See below)

Thank you for your time in completing this questionnaire. All information will be kept strictly confidential at all times.

What is a unit of Alcohol?

The list below shows the approximate number of units of alcohol in common drinks:

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Measure</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordinary strength lager (4%) e.g. Carling, Fosters</td>
<td>Pint</td>
<td>2.3</td>
</tr>
<tr>
<td>Strong lager (5.2%) e.g. Stella Artois, Kronenburg</td>
<td>Pint</td>
<td>3</td>
</tr>
<tr>
<td>Strong lager e.g. Stella Artois, Carlsberg Export, Grolsch</td>
<td>440ml can</td>
<td>2.2</td>
</tr>
<tr>
<td>Beer/ordinary strength Ale e.g. John Smith’s, Guinness</td>
<td>Pint</td>
<td>2.3</td>
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<tr>
<td>Red/White Wine</td>
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<td>Red/White Wine</td>
<td>Lg. 250ml</td>
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<td>Spirits</td>
<td>Std 25ml</td>
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<tr>
<td>Spirits</td>
<td>Lg. 35ml</td>
<td>1.4</td>
</tr>
<tr>
<td>Alcopop e.g. Smirnoff Ice, Bacardi Breezer, Reef</td>
<td>275ml</td>
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## Appendix A.8 - Food Frequency Questionnaire:

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<th>How many spoons/slices of the food do you have per portion?</th>
<th>Daily S+</th>
<th>Daily 4</th>
<th>Daily 3</th>
<th>Daily 2</th>
<th>4 x week</th>
<th>2 x week</th>
<th>1 x week</th>
<th>Fortnightly</th>
<th>Once a month</th>
<th>Quarterly</th>
<th>Yearly</th>
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<td>Food (1 portion)</td>
<td>How many spoons/slices of the food do you have per portion?</td>
<td>Daily 5+</td>
<td>Daily 4</td>
<td>Daily 3</td>
<td>Daily 2</td>
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</table>
Appendix A.9 - Standard Meal Options (for all dietary intervention studies)

As discussed, please find below the selection of meals we can offer for your standard meal (to be eaten the night before each study morning.) Please choose one main and one dessert/side dish and you will need to take into account that you will need to have the same meal before every study morning (3 times).

**Sainsbury’s**

**Mains**
- Be Good To Yourself – Chicken Tikka Masala and Rice (400g)
- Be Good To Yourself – Lasagne (400g)
- Be Good To Yourself – Vegetable Curry (450g)
- Sainsbury’s Beef Stroganoff and Rice (400g)

**Desserts**
- Sainsbury’s Strawberry Trifle (125g)
- Onken Lemon Mousse (150g)
- Taste the Difference – Raspberry Yoghurt (150g)
- Sainsbury’s Blackcurrant Cheesecake (100g)

**Tesco’s**

**Mains**
- Naturally Good For You – Chicken Tikka Masala and Rice (400g)
- Italian – Lasagne (400g)
- Italian – Spaghetti Bolognese (400g)
- Italian – Spinach and Ricotta Canneloni (400g)
- Naturally Good For You – Beef Stroganoff and Rice (400g)

**Tesco’s Vegetable Curry (225g) and Tesco’s Mushroom Rice (250g) – These two items must be eaten together**

**Desserts**
- Tesco’s Strawberry Dream (140g)
- Finest Lemon Mousse (130g)
- Finest Raspberry Yoghurt (150g)
- Tesco’s Blackcurrant Cheesecake (100g)

**Alternative Side dishes**
- 2 x Slices White Bread with margarine
- Cheese (60g) with 4 x cream crackers
Appendix A.10 - Nutritional composition for the bread rolls used as part of the *High Risk Study* and *Low Risk Study* dietary interventions

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Whole-grain Roll</th>
<th>Milled-grain Roll</th>
<th>Control Roll</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per Roll (80g)</td>
<td>Per 100g (97g)</td>
<td>Per Roll (106.9g)</td>
</tr>
<tr>
<td>Energy kcal</td>
<td>234</td>
<td>275</td>
<td>276</td>
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<tr>
<td>Energy kJ</td>
<td>994</td>
<td>1164</td>
<td>1168</td>
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<tr>
<td>Moisture (g)</td>
<td>17.44</td>
<td>23.77</td>
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<td>Carbohydrate (g)</td>
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<td>55.68</td>
<td>57.83</td>
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<td>Sugar (g)</td>
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<td>1.46</td>
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<td>Starch (g)</td>
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<td>Protein (g)</td>
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<td>Fat (g)</td>
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### Appendix A.11 - Nutritional composition for the bread rolls used as part of the Cross-Over Study

#### Inulin - Nutritional Composition

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<th>Calories (kJ)</th>
<th>Moisture</th>
<th>Carbohydrate (of which)</th>
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<td>1015</td>
<td>32.2</td>
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<td>Energy (kJ)</td>
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#### Control - Nutritional Composition

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<th>Calories (kJ)</th>
<th>Moisture</th>
<th>Carbohydrate (of which)</th>
<th>Protein</th>
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#### Wheat fibre - Nutritional Composition

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<th>Calories (kcal)</th>
<th>Calories (kJ)</th>
<th>Moisture</th>
<th>Carbohydrate (of which)</th>
<th>Protein</th>
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Fat (of which) 1.3

All rolls weighed approximately 75g