The impact of whole grains and their constituents on markers of vascular health and metabolism
— an ex vivo and in vitro investigation

A thesis submitted in accordance with the requirements of the University of Surrey for the degree of Doctor of Philosophy

by

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Statement of originality

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service TurnitinUK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.
Abstract

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in the UK and other Westernised countries. High dietary intakes of whole grains have been associated with a reduced risk of CVD and other associated morbidities including obesity and type II diabetes. The evidence, however, is predominantly observational, with very few randomised controlled dietary intervention studies, and thus can not be used to suggest a causative role for whole grains.

The aim of this research, therefore, was to investigate the effects of whole grains and their components and metabolites on markers of endothelial dysfunction and cardiovascular risk both in vivo and in vitro using a variety of approaches. It was hypothesised that an increase in whole grains and their constituents would reduce CVD risk as reflected in the markers investigated.

The Pilot Whole grain Intervention Study in England (Pilot WISE) trial was a randomised controlled parallel dietary intervention which investigated the effects of 48g whole grain per day on CVD risk markers and circulating metabolite profiles of men and women (aged 48.4 ± 11.7 years, BMI 28.8 ± 3.1kg/m²) over eight weeks. The Fibre and Inulin Trial in Male Adults (FITMA) trial, a randomised controlled cross over designed dietary intervention trial researched the impacts on such CVD risk markers and metabolite profiles of 15g/day wheat fibre or inulin in high risk men (aged 39.8 ± 3.0 years, BMI 30.2 ± 1.0kg/m², n= 10) over 28 days. The effects of a range of physiologically relevant concentrations of the fermentation products of whole grains, the short chain fatty acids (SCFA), on cellular models of the vascular endothelium were also investigated to explore the potential mechanisms behind the proposed risk reduction.
Dietary interventions of whole intact and whole milled grains, wheat fibre or inulin did not significantly impact on circulating levels of inflammatory or haemostatic markers, lipid profiles or markers of insulin sensitivity. Circulating levels of three species of lysophosphatidylcholines, however, were significantly altered by the wheat fibre and inulin in the metabolic profile analyses. Treatment of endothelial cells with SCFA did not significantly alter levels of secreted inflammatory or haemostatic mediators or of cellular adhesion molecules.

In conclusion, no beneficial effects were observed as a consequence of the diets high in whole grains or their components and, as such, these data can not be used to support the dietary health messages associated with high whole grain intakes. Future work could benefit from involving higher numbers of participants or those with a higher CVD risk profile at baseline.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APP</td>
<td>Acute phase proteins</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BALL-1</td>
<td>Blood acute lymphoblastoid leukemia-1</td>
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<td>BHF</td>
<td>British Heart Foundation</td>
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<tr>
<td>BIC</td>
<td>Carbonate/bicarbonate</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>CAM</td>
<td>Cellular adhesion molecule</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>CIU</td>
<td>Clinical investigation unit</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>DEBQ</td>
<td>Dutch eating behaviour questionnaire</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>Endothelial growth factor</td>
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<td>EGM-2</td>
<td>Endothelial growth medium</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbance assay</td>
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<tr>
<td>EpGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell scanning</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FFQ</td>
<td>Food frequency questionnaire</td>
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<td>FHMS</td>
<td>Faculty of Health and Medical Sciences</td>
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<td>FITMA</td>
<td>Fibre and Inulin Trial in Male Adults</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>HAAEC</td>
<td>Human abdominal aortic endothelial cells</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HCAEC</td>
<td>Human coronary artery endothelial cells</td>
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<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HMEC-1</td>
<td>Human microvascular endothelial cell-1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>LC/MA</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Monocyte colony stimulating factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>oxLDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Platelet activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>Pen/strep</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PLSDA</td>
<td>Partial least squared discriminate analysis</td>
</tr>
<tr>
<td>PP</td>
<td>Post prandial</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>RD</td>
<td>Registered Dietitian</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio-immuno assay</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acid</td>
</tr>
<tr>
<td>SIC</td>
<td>Single ion chromatogram</td>
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<tr>
<td>sICAM-1</td>
<td>Soluble intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>T</td>
<td>Tween®</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerides</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine dihydrochloride</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>tPA-1</td>
<td>Tissue plasminogen activator-1</td>
</tr>
<tr>
<td>UPLC-MS</td>
<td>Ultra performance liquid chromatography – mass spectrometry</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VIP</td>
<td>Variable importance on projection</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>vLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<td>WISE</td>
<td>Whole grain Intervention Study in England</td>
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Chapter 1

Introduction
1.1 Cardiovascular disease

Cardiovascular disease (CVD) encompasses multiple arterial diseases, both congenital and acquired, of the heart and blood vessels, including coronary heart disease (CHD), rheumatic heart disease, atherosclerosis and vascular inflammation. CVD is the largest single cause of mortality and morbidity in the UK and several other Westernised countries (Truswell A 2002) accounting for over half the deaths in middle age and one third of all deaths in old age in most developed countries. CHD alone kills approximately 94,000 people in the UK per annum (BHF 2008). The pathology of CVD involves the inflammatory condition atherosclerosis, the build up of fatty plaques within the artery wall and the dysfunction of the cardiovascular endothelium with disruption of the control of vasodilation, thrombosis, haemostasis and tissue perfusion (Rush J 2005). CVD is often subclinical, remaining undiagnosed for decades without producing symptoms severe enough to prompt a diagnosis. Often the first symptom may be the narrowing of the coronary arteries, angina pectoris, characterised by pain in the chest on physical exertion due to the restriction of the arterial lumen by the atherosclerosis and subsequent reduction in blood flow to the myocardium.

During the inflammatory response associated with atherosclerosis, the expression of a range of adhesion molecules is transiently altered thus increasing the extravasation of white blood cells, platelets and other small molecules into the neointima, increasing the size and fragility of the associated plaques. Levels of some cytokines, chemokines and haemostatic factors in the blood such as the acute phase reactant, C-reactive protein, can also be increased during inflammation due to the increased demands associated with
repair, and can predict major coronary events (Danesh J 1998; Ross R 1999; Danesh J 2000).

The initial trigger of these events is poorly understood, however, CVD is associated with elevated dietary saturated fats and cholesterol. It is, therefore, further associated with circulating levels of LDL-cholesterol, lipoprotein(a) and remnant lipoproteins, in association with decreased levels of HDL-cholesterol. These abnormal levels of fats in the blood are known as dyslipidaemia and contribute to a build up of cholesterol within the smooth muscle cells of the artery wall. Common co-morbidities of CVD include hypertension, diabetes and obesity showing associated elevated levels of risk markers such as homocysteine and total and LDL-cholesterol.

CVD is a multifactorial disease with both modifiable and non-modifiable risk markers. Non-modifiable risk markers include age, sex, socio-economic status, genetic predisposition and ethnic background. Modifiable risk marker include smoking status, exercise status, blood pressure, type II diabetes and diet, many of which are interrelated, and which provide possible areas for interventions to reduce risk. Certain dietary patterns, for example, are associated with reduced risk of cardiovascular disease. One of these dietary patterns associated with lower risk of CVD is a diet high in whole grains (Jacobs D 1998; Liu S 1999; Liu S 2000; Liu S 2003; Steffen L 2003).

1.2 The endothelium — structure, function and indicators of vascular health

As CVD is an inflammatory disorder characterised by dysfunction of the vascular endothelium, understanding the normal physiology of the vasculature is important. The
vascular endothelium is a monolayer of approximately $10^{13}$ cells forming the lining of the blood vessels, situated between the intima and the basal lamina (a thin layer of collagen containing extracellular matrix) which gives structure to the lining and separates it from the underlying tissue (figure 1.1). The endothelium has an estimated mass of 1.5kg and a surface area equivalent to a football pitch in adults (Anggard, 1994).

![Figure 1.1: Representation of a blood vessel in cross section.](image)

Endothelial cells themselves differ in their properties at different anatomical sites within the vascular tree (Thorin and Sreeve 1988, Vercellotti 1988) which may be partially explained by site specific variations in functional shear forces. These differences include variations in cytokine expression and cell/endothelial permeability. With the
exception of the specific region of the blood-brain barrier, the endothelium is a relatively permeable membrane, forming a mechanical barrier between the blood and the proximal tissues and actively managing the passage of cells, nutrients and other components between them.

The endothelium plays an active role in maintaining haemostasis and regulates vascular tone via a range of mediators, most of which are dilatory, including nitric oxide (NO). Many other factors, including nitroglycerin, prostacyclin and acetylcholine, are also involved in the regulation of vascular tone. Bleeding at any site in the vascular tree must be rapidly and effectively halted by the platelet and coagulation pathways. This response must also be very closely monitored to prevent hyper-coagulation. Thus, the balance of pro- and anti-coagulant mechanisms must be tightly maintained in the haemostatic system.

On damage to the vessel wall, vasoconstriction occurs to reduce blood loss. Circulating platelets (produced in bone marrow by the fragmentation of the cytoplasm of megakaryocytes) adhere to any exposed subendothelial matrix proteins via the interaction of specific adhesive glycoproteins (GPIb, GPIIb and GPIIIa) with matrix-coating von Willebrand Factor (vWF). Platelets are activated which induces a change of morphology and initiates granule secretion. Collagen also binds to this aggregated platelet-vWF complex and this ligation activates GPIIb and GPIIIa, resulting in further activation of platelets and the production of an unstable primary plug. Thromboxane A2 then leads to further vasoconstriction and platelet aggregation (figure 1.2). Vessel damage also releases tissue factor, initiating the coagulation cascade, an amplification system in which
initiation substances activate a cascade of circulating precursor proteins, via proteolysis, to their active protein forms, resulting in the generation of thrombin. Thrombin then converts soluble plasma fibrinogen to fibrin, which enmeshes with the aggregated platelets to convert the primary plug into a firm, definitive and stable haemostatic plug (Furie B 2005).

Figure 1.2: Endothelial cells form a barrier between the endothelial connected tissues and the contents of the lumen such as platelets and plasma clotting factors. Endothelial cells produce substances that can initiate coagulation, cause vasodilation, inhibit platelet aggregation or haemostasis, or activate fibrinolysis. Reproduced from (Hoffbrand A 2007).

The endothelium limits local thrombosis by production of anticoagulant mediators including heparins, thrombomodulin and tissue plasminogen activator-1 (tPA-1) and...
prevents platelet aggregation via the secretion of NO. At sites of injury the endothelium ceases secretion of these coagulation and aggregation inhibitors and instead secretes vWF and tissue thromboplastin. This aids coagulation and healing. NO is released from macrophages and platelets as well as endothelial cells. Its activities include platelet activation and promotion of vasodilation. Prostacyclin, also produced by the endothelial cells, enhances this inhibition of platelet function and further promotes vasodilation. The adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM-1) further inhibits platelet activation. Hageman factor, factor XII, circulates as an inactive plasma protein. It is the zymogen form of factor XIIa, a serine protease class enzyme. Activation to factor XIIa is triggered by collagen, platelets, or exposed basement membranes and occurs via conformational change. Activated XIIa is able to activate the kinin system supporting inflammatory processes including vasodilation, the fibrinolysis system (acting to oppose the coagulation system) and the coagulation system or clotting cascade.

Tissue plasminogen activator-1 (tPA-1) activates clot dissolution in the presence of fibrin by catalyzing the conversion of plasminogen to plasmin and cleaving cross-linked fibrin to fibrin degradation products (including D-dimer). tPA-1 is used in clinical medicine to treat embolic or thrombolytic stroke and is a serine proteinase which maintains a negative charge on the blood vessel surface (Cipollone F 2007). It is also critical in the immune response to injury and infection within the vasculature (Kumar V 2003; Majno G 2004). Normal ranges of circulating tPA-1 have been found to be 7.77 ± 1.63ng/ml with CHD risk determined at 9.67 ± 1.55ng/ml (Gram J 2000). Another study associated angina pectoris and angiographically verified coronary artery disease (CAD) with levels of 9.3 ± 3.9ng/ml. This level was raised to 10.9 ± 3.5 in those with angina and...
CAD who died within a seven year follow up (Jansson J 1993). High plasma levels of tPA-1 have been shown to precede a first acute myocardial infarction in both men and women (Thogersen A 1998).

Increased serum levels of tPA-1 antigen have been positively associated with incidence of CHD (Folsom A 2001) in the Atherosclerosis Risk in Communities study. A further study showing significantly higher serum concentrations of tPA-1 and CRP in individuals with CHD than in controls without CHD (Gram J 2000) also concluded that tPA-1 is an independent risk marker the evolution of CHD, in that it can be used to predict increased risk of CHD even when other markers are controlled for. Furthermore, tPA-1 and the tPA-1/plasminogen activator inhibitor-1 complex (tPA-1/PAI-1) have been associated with increased risk of first stroke (Johansson L 2000), an association which remained after adjustment for traditional risk markers, showing the tPA-1/PAI-1 complex as an independent risk marker for first stroke. This complex was also determined as a risk marker for recurrent myocardial infarction in both sexes (Wiman B 2000).

Plasminogen activator inhibitor-1 (PAI-1) is the principal inhibitor of the serine tPA-1 and urokinase, the activators of plasminogen and hence fibrinolysis. It is a serine protease inhibitor mainly produced by the endothelium but also secreted by other tissue types, such as adipose tissue. PAI-1 is present in increased levels in various disease states including certain cancers and obesity. It has been linked to the increased occurrence of thrombosis in patients with these conditions. In inflammatory conditions in which fibrin is deposited in tissues, PAI-1 appears to play a significant role in the progression to
fibrosis (pathological formation of connective tissue). Angiotensin II increases synthesis of PAI-1 and so accelerates the development of atherosclerosis.

vWF, is a large (250kD) multimeric blood glycoprotein involved in haemostasis which can be used as a marker of endothelial dysfunction. It is produced constitutively by the endothelium, in the Weibel-Palade bodies, and also by the sub-endothelial connective tissue (Sadler J 1998). vWF plays a major role in blood coagulation with deficiency or dysfunction (von Willebrand disease), therefore, leading to a bleeding tendency. vWF is bound to factor VIII while inactive in the circulation. Factors such as thrombin release vWF from factor VIII (which then rapidly degrades) leaving functional vWF.

Functional vWF is known to bind to many active particles involved in blood coagulation including several platelet receptors providing a possible role for it in triggering thrombus formation in the advancement of CVD. vWF binds to collagen on exposure, as in the case of injury to endothelial cells and appears to uncoil, decelerating and attracting passing platelets and binding to their receptors including GPIb (Sadler J 1998) thus propagating any developing atherosclerosis. Prospective evidence suggests a relationship between increased vWF levels and increased risk of vascular disease in individuals with type II diabetes (Jager A 2001). Higher than population average levels of vWF are more common among people who have had a single incidence of ischaemic stroke (Bongers T 2006) with vWF antigen levels significantly higher in cases (1.47 ± 0.66IU/ml) compared with controls at (1.23 ± 0.50IU/ml). Other studies have determined healthy levels of vWF as 1.19 ± 0.42IU/ml with increased levels of 1.28 ± 0.44IU/ml in those with diagnosed ischaemic heart disease (Rumley A 1998). vWF is a considered an
established risk marker for CHD (Meade T 1994; Thompson S 1995) and an important
risk marker for recurrent myocardial infarction (Wiman B 2000) with levels in healthy
males and females at $1.34 \pm 0.54$ IU/ml and $1.36 \pm 0.48$ IU/ml respectively, raised to $1.69
\pm 0.77$ IU/ml and $1.66 \pm 0.43$ IU/ml respectively in counterparts who had suffered a
myocardial infarction.

Nitric oxide (NO) was formerly referred to as ‘endothelium derived relaxing
factor’ and is known to be expressed by endothelial cells for the regulation of vascular
tone and blood homeostasis (Bachetti T 2004). Endothelial nitric oxide synthase (NOS) is
referred to as eNOS, ecNOS or NOS III and was discovered first in endothelial cells
(Forstermann U 1991; Pollock J 1991) and followed by other cells such as epithelial cells
(Forstermann U 1995; Sakai M 1996). Although eNOS is constitutively expressed, its
expression can be influenced by a number of factors including cytokines e.g. tumour
necrosis factor-α (TNF-α), oxidised lipoproteins and oestrogens (Forstermann U 1995;
Harrison D 1996).

Endothelial NO, released into the vessel lumen, is rapidly degraded to nitrate and
nitrite, which further interchange within the circulation. Thus serum concentrations of
nitrate and nitrite are often measured as surrogate markers of NO concentration. NO is
synthesized in biological systems by the constitutive, calcium- and calmodulin-dependent
enzyme NOS (Moncada and Higgs 1993), a highly complex enzyme which acts on
molecular oxygen, arginine and NADPH to produce picomols of the short lived radical
NO (Nathan C 1994). Also produced in this process are citrulline and NADP+. This
reaction requires the cofactors flavin mononucleotide, flavin adenine dinucleotide,
calmodulin and tetrahydrobiopterin, and the divalent cations calcium and haem iron. Further to eNOS, two distinct isoforms of NOS have been identified: neuronal (nNOS), inducible (iNOS) (Andrew P 1999). Both nNOS and eNOS are constitutively expressed but expression of both is inducible to a certain extent, while, iNOS may be induced only under pathological conditions (Michel T 1997).

NO produced in endothelial cells by eNOS is crucial for maintaining normal endothelial health and function (Monacada S 1991; Schmidt H 1994), is involved in blood pressure regulation (Huang P 1995) and is known to exert protective effects on the cardiovascular system (Gibbons G 1996). The iNOS present in phagocytes is known to be activated by IFN-γ alone, or by TNF-α and an accompanying signal (Kaibori M 1999). NO is secreted as a free radical and is toxic to bacteria via mechanisms including DNA damage (Wink D 1991).

Known functions of NO include the inhibition of platelet aggregation and adhesion, modulation of smooth muscle cell proliferation, blood pressure regulation (Huang P 1995), attenuation of endothelin generation and reduction of leukocyte-endothelium adhesion. It has protective effects on the endothelium (Gibbons G 1996) with decreased production levels or impairment of production pathways associated with pathophysiological conditions e.g. type II diabetes, atherosclerosis and hypertension (Dessy C 2004). The progression of CVD precedes the development of type II diabetes, with common features including NO-dependent endothelial dysfunction and impaired vascular responses to NO donors (Williams S 1996). As NO is associated with good cardiovascular health, investigating methods of increasing stimulation are of interest.
1.3 The endothelium - response to injury or infection

1.3.1 Inflammation

The normal response to injury or infection is the initiation of inflammation, a complex series of events involving soluble mediators and cells working synergistically to increase vascular permeability, blood flow to the affected area and white blood cell migration. The five signs of localized acute inflammation were first recognised two millennia ago and are calor (heat), dolor (pain), rubor (redness), tumor (swelling) and a reduction in function. Acute inflammation is generally associated with a systemic reaction, the acute phase response, characterised by rapid alterations in the levels of plasma proteins. The complement cascade protein C3 cleaves to C3a and C3b and stimulates the release of histamine by mast cells, thus producing vasodilation and increasing blood flow to the area. Neutrophils are generally the first leukocytes to migrate into tissues within the first six hours, with an associated increase in production within the bone marrow of up to ten-fold. Extravasated neutrophils phagocytose invading pathogens and release further pro-inflammatory mediators. This haemostasis phase usually leads to healing and repair, however, prolonged immune activation can precipitate and encourage chronic inflammatory disorders.

Acute inflammation can be triggered by resident macrophages and monocytes in tissues, mediators of inflammation and endothelial damage, via leukotrienes, prostaglandins, bradykinin and histamine. Bradykinin is a vasoactive protein responsible for the induction of vasodilation, increased permeability, contraction of smooth muscles and the induction of pain. Interleukin-8 is responsible for the activation and chemoattraction of neutrophils to the site of inflammation. It also has a weak activating
and chemoattractant effect on eosinophils and monocytes. Interleukin-6 is associated with adipose tissue production in obesity (Bastard J 2000) and has been proposed as a mediator in obesity related insulin resistance. TNF-α and IL-1 affect a variety of cells to induce a range of inflammatory reactions including the further production of cytokines. Cytokines are also responsible for increased permeability of the endothelium. Localised effects include leukocyte adherence, fever, activation of fibroblasts and chemotaxis. Systemic effects can include an increased heart rate and the loss of appetite. Inflammation is also associated with high circulating levels of acute phase proteins (APP) which, although beneficial in acute inflammation, can be pathological in chronic inflammation. These APPs include C-reactive protein (CRP), vasopressin, serum amyloid A and serum amyloid P and cover a range of symptomatic effects including increased blood pressure, loss of appetite, fever and malaise.

Growth factors including IL-6, granulocyte-macrophage stimulating factor (GM-CSF) and monocyte colony stimulating factor (M-CSF) are involved in the proliferation and maturation of granulocytes produced from bone marrow progenitor cells. Such production is upregulated in inflammation in response to increased expression of growth factors from the stromal cells and T lymphocytes, on stimulation by IL-1, endotoxins or TNF. These cells then migrate via the blood stream to the site of the inflammation.

CRP, the 25,106kDa member of the pentraxin family produced in the liver, is an acute phase reactant and as such its circulating levels are increased in inflammation by up to 1000 fold. CRP production is regulated by the cytokine IL-6 (Pepys M 2003). Circulating concentrations of CRP have been determined as $1.68 \pm 1.42$mg/l with
increased levels of 3.35 ± 1.56mg/l in subjects with established CVD (Glurich I 2002) and epidemiological data have shown an independent association between increased levels of CRP and risk of coronary pathology (Ridker P 1997; Tracy R 1997; Ridker P 1998). Human atherosclerotic lesions, coronary artery smooth muscle cells, aortic endothelial cells and adipocytes have all been shown to express CRP (Yasojima K 2001; Carabro P 2003; Carabro P 2005). CRP has been shown to induce expression of ICAM-1 and VCAM-1 by endothelial cells suggesting a direct role in promoting the inflammation associated with atherosclerosis (Pasceri V 2000). Studies have identified CRP as a possible risk marker for CVD in women (Ridker P 2000) and CHD in previously healthy but high risk men (Koenig W 1999) and a meta-analysis in 1998 determined raised CRP levels as a risk marker for CHD (Danesh J 1998). One study suggested that CRP may actively promote the formation of atherosclerotic lesions by participation in the activation of the complement system and the formation of foam cells (Torzewski J 1998).

Individuals with genetically increased levels of CRP have been shown not present with an increased risk of CVD (Zacho J 2008). Meta-analysis of long term prospective studies report the risk of CHD is approximately 90% (50% – 130%) increased in individuals with moderately raised circulating CRP when comparing those in the highest third of concentration of these factors with those in the bottom third (Danesh J 1998; Danesh J 2000).

1.3.2 Cell migration

The movement of circulating leukocytes into the subendothelial tissue has been well characterised. Tissue infiltration by leukocytes occurs in the three distinct phases of rolling, attachment and extravasation which are largely regulated by the differential.
expression of a range of adhesion molecules. Leukocyte rolling within the lumen is mediated by low affinity bonds between leukocyte surface molecules and activated endothelial cells. This is predominantly mediated by E- and P-selectins (Tedder T 1995; McEver 2002), chemokines and addressins (Vestweber D 2003) but can, alternatively, be mediated by the endothelial intracellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) (Springer T 1994) and its leukocyte surface ligand very late antigen-4 (VLA-4) or leukocyte α4β1-integrin (Alon R 1995).

ICAM-1, CD54, a 90kd inducible single chain, surface glycoprotein is a member of the immunoglobulin (Ig) super gene family consisting of five extracellular Ig-like domains, a single transmembrane region and a short (29 amino acid) cytosolic tail. It is expressed constitutively on resting endothelial cells and expression is upregulated on activation by injury or infection to promote healing, increasing the attraction and binding of leukocytes (Yang L 2005) via leukocyte β2-integrins lymphocyte function-associated antigen-1 (ICAM-1/LFA-1) or macrophage antigen-1 (Mac-1) via its first or third Ig-like domains respectively. Improper function of ICAM-1 results in an impaired immune response in inflammation in ICAM-1 deficient mice (Thompson P 2002). Following adhesion, ICAM-1 translocates from the apical surface to the basal membrane in areas close to the ends of actin stress fibres (Millan J 2006). Normal circulating levels of sICAM-1 in healthy adults are 115 to 306ng/ml (Glurich I 2002). ICAM-1 triggers various signalling pathways for cell motility and act in the induction of microvilli-like projections which extend out from the luminal surface of the endothelial cell towards the leukocyte as a docking structure (Carman C 2003).
The sialoglycoprotein VCAM-1, CD106, a 110 kD member of the
immunoglobulin super-gene family, has a short (19 amino acid) cytosolic tail. This type 1
membrane protein is not expressed in resting cells but is expressed in inflammation via
cytokine activation of the gene of the same name, increasing both gene transcription (on
activation by TNF-α or IL-1) and stabilisation of the mRNA (on activation by IL-4). Its
extracellular portion binds primarily to the leukocyte integrin VLA-4. VCAM-1-VLA-4
ligation results in leukocyte-endothelial cell adhesion, promoting the adhesion of
lymphocytes, monocytes, eosinophils and basophils to the endothelium. Expression of
VCAM-1 has also been observed in smooth muscle cells. VCAM-1-ligand binding also
results in signalling via the cytosolic tail, producing reactive oxygen species (ROS) via
NADPH oxidase activation (van Buul J 2004). ROS are highly reactive molecules
containing oxygen, including oxygen ions and peroxides, produced as a result of normal
metabolism. Low levels of ROS are thought to be protective by preserving endothelial
barrier function and preventing migration and angiogenesis. Higher levels of ROS can
activate matrix metalloproteinases (MMPs) which degrade surrounding extracellular
matrices, resulting in loss of barrier integrity and increased leukocyte passage (Hordijk P
2006). Transendothelial migration steps involving VCAM-1 can be enhanced via prior
expression of chemokines by the endothelium (van Buul J 2004) by the activation of
Rap1, a signalling protein which results in increased affinity of the leukocyte integrin
VLA-4 to endothelial VCAM-1.

As VCAM-1 and ICAM-1 appear in close proximity on cell surfaces, it has been
suggested there may be interaction between their functions (Hordijk P 2006). Both
molecules appear to upregulate certain transcription factors. VCAM-1 upregulates NF-
κB, one of the main transcription factors that affects innate immunity, cell cycle control and apoptosis, and c-fos, which upregulates MMPs and cell adhesion molecules. ICAM-1 upregulates p38 MAP kinase, resulting in cytoskeletal changes (van Buul J 2004) and is also able to upregulate this pathway via the ROS it generates (Cook-Mills J 2005). It thus appears that, at the transcriptional level, ICAM-1 and VCAM-1 are in some sort of cooperation with one another, however, the precise mechanisms remain unclear. ICAM-1 and VCAM-1 are found more commonly in human atherosclerotic lesions than in healthy arterial tissue (Poston R 1992; Davies M 1993) and knockout mice deficient in E-selectin, P-selectin or ICAM-1 develop fewer arterial lesions than normal mice (Johnson R 1997; Nageh M 1997; Dong Z 1998; Collins R 2000) suggesting a possible role for these endothelial ligands in the development and progression of CVD.

The velocity of leukocytes rolling on the endothelium is 100-1000x lower than the mean blood velocity. The binding of selectins to leukocytes stimulates ‘outside in’ signals on the leukocytes increasing the affinity of the integrin receptor family, which in turn bind to adhesion molecules on the endothelial cells, ICAM-1 and VCAM-1 (Alon R 2002; Shamri R 2002). Chemokines displayed on the surface of endothelial cells trigger ‘inside out’ signals from leukocyte chemokine receptors which rapidly increase leukocyte integrin affinity (Alon R 2003). This integrin receptor affinity arrests the rolling leukocytes on the luminal surface of the endothelial cells. The chemokine gradient on the surface of the endothelial cell then stimulates the arrested leukocytes for chemotaxis (Middleton J 2002).
The projection of microvilli like pseudopodia from the endothelium, involved in leukocyte adhesion (Carman C 2003), involves endothelial surface molecules CD99, VE-cadherin, junctional adhesion molecules and the cellular adhesion markers platelet/endothelial cell adhesion molecule-1 (PECAM-1), ICAM-1 and VCAM-1 (Engelhardt B 2004). PECAM-1, CD31, a 130kDa transmembrane glycoprotein found in endothelial cell-cell contacts, is a mediator of various migration steps including trans-endothelial migration and leukocyte migration through the extracellular matrix (Liao F 1995). Its role in migration has been demonstrated both in vitro and in vivo. Blocking PECAM-1 interactions (via antibodies or competitive inhibition) blocks the movement of molecules out of the blood vessels via the endothelium by approximately 90%; leukocytes are tightly adhered but can not extend pseudopodia into the junction (Muller J 2003). PECAM-1 knockout mice, however, respond similarly to wild type mice which can be explained by an expanded function of other adhesion molecules in the knockout mice (Duncan G 1999) and indicates the role of PECAM-1 as important but not essential. Knockout mice exhibit increased cellular infiltrations and an exaggerated prolonged vascular permeability compared to the wild type mice due to PECAM-1’s role in restoring junctional integrity following inflammation (Graesser D 2002).

The final step of trans-endothelial migration, extravasation, is a rapid process after which the endothelial monolayer remains intact (Engelhardt B 2004). Endothelial cells proactively form microvilli-like projections extending from the luminal cell surface towards adhered leukocytes to form a docking structures rich in ICAM-1, VCAM-1, adapter molecules and actin (Carman C 2003). The expression of PECAM-1 between activated endothelial cells is reduced to increase permeability of the endothelium and
facilitate extravasation (figure 1.3). Proper function of the endothelium is evidently essential in processes such as immune surveillance, lymphocyte homing and leukocyte attraction to inflammatory sites for vascular repair.

As most of these cell surface adhesion molecules are shed by proteolytic cleavage into the circulation upon activation producing 'soluble' versions of each molecule, several studies have measured the soluble forms in serum (Gearing A 1993).

Figure 1.3: Leukocyte migration from blood vessels into tissues. Leukocytes migrate from the lumen of the blood vessels into tissues through the endothelium at low levels under normal immune function and at increased levels in response to injury or infection. Adapted from (Stanner S 2005). Abbreviations: oxLDLs, r2GP1, LP(a), HSPs, collagen, AGE, bacteria or viruses.
The selectins (CD62) are a cellular adhesion molecule family of three lectins which bind to carbohydrate polymers: E-selectin, P-selectin and L-selectin. Each selectin is a single chain transmembrane glycoprotein demonstrating properties similar to C-type lectins due to a related amino terminus and calcium-dependent binding (Cotran 1998), with an affinity for carbohydrate moieties (Parham 2005). All the selectins appear to bind to the P-selectin glycoprotein ligand-1 (PSGL-1), the primary ligand for P- and L-selectin on leukocytes. L-selectin is constitutively expressed on all leukocytes and can bind to endothelial cellular adhesion molecules (CAMs) as well as glycoproteins. It’s activation upregulates the chemokine receptor CXCR4 (also known as fusin) which binds to the endothelial chemokine stromal cell-derived factor-1 (SDRF-1 (Smith C 2008)). Knockout mice deficient in E- and P-selectin develop far fewer arterial lesions than wild type mice (Johnson R 1997; Dong Z 1998; Collins R 2000) reinforcing the role of E- and P-selectin in arterial lesion development. E-selectin is shed into the circulation as a result of proteolytic cleavage of the cell-surface expressed molecules as soluble-E-selectin (sE-selectin). sE-selectin is proposed to act as a pro-inflammatory agent through activation of neutrophils (Ruchaud-Sparagano M 1998) and E-selectin, ICAM-1 and VCAM-1, are generally accepted to be associated with the pathogenesis of atherosclerosis and are predictors of clinical events (Hope S 2003).

Chemokines are small (8 to 14kDa) chemo-attractant proteins that, along with selectins, participate in leukocyte rolling and influence the action of endothelial cellular adhesion molecules. Since chemokine gradients in the blood would be washed away by blood flow, leukocytes tend to respond to chemokines immobilised on endothelial cell
surfaces. Chemokines are known to alter the avidity of the cellular adhesion molecules, the integrins, within cells (Murdoch C 2000).

In conclusion, the cellular adhesion molecules play a pivotal role in the movement of molecules from the vascular lumen through the endothelium, a vital process associated with inflammatory events, atherosclerosis and cardiovascular disease.

1.4 Pathogenesis of atherosclerosis

Certain stimuli can cause the endothelium to become overly activated and overthrow its homeostatic balance (Cipollone F 2007) which may result in chronic inflammation (Kumar V 2003; Majno G 2004). Little is known about the causes of, and mechanisms behind, chronic inflammation (Medzhitov R 2008) which occurs in a range of pathologies including type II diabetes and CVD (Ross R 1999) but are thought to be associated with malfunctions of underlying tissue i.e. homeostatic imbalance of physiological systems not directly related to host defence or tissue repair (Medzhitov R 2008) leading to and/or characterised by endothelial dysfunction.

Atherosclerosis, responsible for the underlying pathogenesis of most CVD, was previously thought of as solely due to the accumulation of lipid within the coronary arteries (Osterud B 2003) but is now understood to be an inflammatory disease. Endothelial dysfunction is one of the earliest detectable features in the development of atherosclerosis and CVD (Moller D 2005).
CHD is characterized by coronary ostial stenosis, the narrowing of the mouths of the coronary arteries, causing a short fall in the essential nutrients supplied to the heart. The narrowing is caused by an accumulation of lipids and also leukocytes, mediators and structural components which eventually form a plaque within the wall of the vessel. If the plaque becomes unstable, which can occur due to the partial degradation of the fibrous cap covering it, the cap can rupture, releasing its contents into the blood stream as a bolus, which can partially or totally block the vessel, preventing blood flow and leading to myocardial ischemia or stroke depending on the site of the plaque (figure 1.4 (Libby P 2002)).

Figure 1.4: The progression of atherosclerosis. The reversible fatty streak builds up into an atherosclerotic plaque which reduces the functional area of the lumen and can rupture at any stage of the plaque progression leading to occlusion.
Animal models of atherosclerosis, primarily rat and mouse knockouts, have given much valuable information on the role of many modulators of atherogenesis. Animal models show that signs of inflammation occur together with lipid accumulation (Libby P 2002) and show strong immunological components in every stage of atherogenesis, from fatty streak formation to plaque rupture. The predominant cells involved in atherogenesis appear to be monocytes, platelets and T lymphocytes from the circulation and endothelial cells and smooth muscle cells in the artery wall, which interact in various ways with LDL (Osterud B 2003).

1.4.1 Triggers of atherosclerosis

The precipitating event or trigger of atherosclerosis is not yet fully understood. Shear stress, however, has an important effect on the endothelium and areas of the endothelium exposed to disturbed flow, rather than laminar flow, are polygonal in shape with an ill-defined orientation, as opposed to the ellipsoid shape and defined orientation. Areas associated with branches or curvature show increased permeability to macromolecules and are more commonly associated with cardiovascular plaques than are other areas (Gimbrone M 1999) thus suggesting non-laminar flow as a trigger of plaque formation.

Specific processes allow the movement of LDL and oxidised LDL (oxLDL) into the vessel intima by activation of the endothelium. These processes include local injury, generation of ROS or the presence of pathogens. Once inside the intima, non-oxidated LDL undergoes oxidative modification to form oxLDL which causes the formation of biologically active lipids such as lipid hydroperoxides, lysophospholipids, oxidised
isoprostanes and carbonyl compounds. These active lipids remain localised in the lipid fraction of the atheroma. Small dense LDL (sdLDL) has been demonstrated as a risk marker for the development of CAD in Westernised countries and Japan (Krauss R 1982; Hirano T 2003). Very low density lipoproteins (vLDL) can also undergo oxidation and, like LDL, have atherogenic potential (Libby P 2002). There is evidence that βvLDL may itself activate inflammation in the endothelium (Dichtl W 1999).

The actions of the activated lipids and oxLDL are diverse and result in inflammation and activation of the endothelium. Modified lipids mediate the expression of adhesion markers, chemokines and inflammatory cytokines (Libby P 2002). oxLDL is taken up by macrophages which subsequently become foam cells. Inside the foam cell, proven in mice, oxLDL can upregulate genes for monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF-1) (Shi W 2000) to activate and attract more monocytes to the area. In addition, the apoprotein moieties of lipoproteins can undergo modifications inside the arterial wall to become antigenic and can elicit responses from circulating T lymphocytes (Stemme S 1995).

The exact causes of LDL oxidation are unclear but possibilities include metal ions (Cu^{2+} and Fe^{3+}) superoxide radicals or haem-containing compounds within the arterial wall. It has been hypothesised that specific factors influenced by lifestyle, including hyperlipidaemia, hypertension, smoking and diabetes, share a common pathological mechanism in which stimuli that mediate intracellular oxidative signals are generated. The signals affect gene transcription, resulting in an increased production of reactive oxygen species (ROS (Kunsch C 1999)). The ROS, along with their target molecules are
able to increase the expression of adhesion molecules, cytokines and other proinflammatory mediators. Since protective, antioxidant mechanisms such as superoxide dismutase, glutathione peroxidise and catalase are thrown out of balance by the high proportion of oxidative species the result is a state of oxidative stress and inflammation. Exposure of endothelial cells to superoxide or hydrogen peroxide radicals is also associated with their apoptosis (Dimmeler S 2000).

1.4.2 Progression of atherosclerosis

Once the endothelium is activated by infection or injury the progression of atherosclerosis is mediated by several important factors (figure 1.5). The most active adhesive markers associated with atherosclerosis in mice appear to be P-Selectin, VCAM-1 and MCP-1 while ICAM-1 appears to have a lesser role as demonstrated in ApoE mice knockouts with P-Selectin or ICAM-1 deficiency (Manka D 2001). P-Selectin deficiency inhibits neo-intima formation by 94% whereas ICAM-1 deficiency gives no protection against plaque formation. The role of P-selectin, together with its ligand PSGL-1 thus appears to be more important in atherosclerosis than ICAM-1. ICAM-1, however, is still upregulated in lesion-prone areas of the aorta in early stages of plaque formation. This is coupled with high recruitment of monocytes and T-lymphocytes and so ICAM-1 does appear to have some involvement in leukocyte adhesion in CVD.

There is evidence for the role of VCAM-1 as well as its ligand VLA-4 in early atheromas (Alon R 1995). VCAM-1 particularly binds to the leukocytes found in early plaques (including monocytes and T cells) and its importance in plaque development has been demonstrated in VCAM-1 defective mice that have interrupted lesion development.
compared to wild type mice. The role of VCAM-1 in human atherosclerotic plaques is, however, less certain.

Figure 1.5: Mediators in atherosclerosis. The roles of the key adhesion molecules in the attachment and transendothelial migration of circulating leukocytes. Abbreviations: LFA leukocyte function associated antigen-1, VLA-4 very late acting antigen-4. Adapted from (Stanner S 2005).

Monocytes differentiate into macrophages in response to local signals such as macrophage colony stimulating factor (M-CSF). The macrophages are normally protected from ingesting cholesterol by various mechanisms but are able to take up oxLDL via their scavenger receptors. Scavenger receptors able to bind to oxLDL include SR-A, SR-B, CD36, CD68 and LOX-1. The cholesterol filled macrophages are then known as foam
cells due to the foamy appearance of their fatty contents. Activated foam cells secrete the cytokines TNF-α, IL-1β, IL-8 and M-CSF which alter LDL migration patterns by enhancing LDL and become engorged with cholesterol.

IL-8 can induce the firm attachment of rolling monocytes suggesting an important role in the monocyte-endothelium interaction of extravasation (Gerszten R 1999). IL-8 has been discovered at high levels as both cellular and extracellular deposits within the connective tissue matrix of the human arterial atherosclerotic wall (Rus H 1996) with macrophages identified as the main source of this IL-8 (Apostolopoulos J 1996). High levels of IL-8 have also been detected in foam cells extracted from human atherosclerotic tissues (Liu Y 1997). IL-8 has been determined as an independent predictor of cardiovascular events in individuals with stable coronary artery disease (Inoue T 2008). However, a large trial which identified high levels of IL-8 as preceding coronary artery disease found it did not qualify as an independent risk marker after adjustment for other related risk markers (Herder C 2006). Oxidised LDL has been shown to induce IL-8 and MCP-1 secretion in cultured endothelial cells (Dje N'Guessan P 2009). Further to this, levels of such stimulated production of IL-8 and MCP-1 were reduced on subsequent treatment with statins (Geisel J 2003). MCP-1, which binds to the chemokine receptor CCR2, seems to be the main chemokine responsible for monocyte migration across intimal lesions (Boring L 1998) and is known to be highly expressed in human lesions. Furthermore, levels of IL-8 and MCP-1 were found to be significantly higher in obese individuals than in non-obese controls, and have been shown to be associated with obesity related parameters including homeostasis model assessment (HOMA score), body mass index (BMI) and waist circumference, suggesting IL-8 as a potential marker linking
obesity with complications including atherosclerosis and diabetes (Kim C 2006). This cumulative evidence has led researchers to pose increased IL-8 levels as a marker of CVD risk.

In addition to monocytes, lymphocytes also migrate to plaques. Proximal T lymphocytes are activated by antigen presenting cells, e.g. dendritic cells and macrophages, which present local antigens to them. Local production of IL-12, IL-15 and IL-18 results in the differentiation of Th1 cells which produce IFN-γ, TNF-α and CD40L. These mediators propagate further damage by activating macrophages and endothelial cells to produce more inflammatory mediators (Hansson G 2006) amplifying the pro-inflammatory milieu.

Other mediators that relate to events in cardiovascular disease are clotting factors such as von Willebrand factor (vWF), tissue plasminogen activator-1 (tPA-1) and platelet activating factor. As discussed in 1.2, the vital balance between these factors maintains haemostasis. vWF has an important role in platelet function and its atherogenic role has been widely investigated in LDLR/vWF mouse knockouts. After being fed a low-fat chow diet, the vWF knockout mice had a higher decrease in rolling than the non-vWF knockouts (Osterud B 2003). This suggests that vWF may contribute to plaque formation by accumulating platelets to the local area. CRP, a commonly used marker for inflammation and CVD, may also have active involvement in atherogenesis as suggested by a study on LDL uptake (Zwaka T 2001) in which LDL uptake was mediated by CRP and so LDL-CRP complexes could be taken up by the CD32 Fc receptor which binds CRP.
Extravasated plasma proteins are deposited within the subendothelial space and form matrices onto which the leukocyte integrins can bind (Pober J 2007). If the activation persists, fatty streaks develop into mature plaques with the release of cholesterol into the lipid core on macrophage apoptosis. These plaques consist of a core region of foam cells/activated inflammatory macrophages and extracellular lipid droplets, surrounded by a cap of smooth muscle cells and collagen rich matrix (figure 1.6).

![Figure 1.6: Atherosclerotic plaque progression.](image)

Extravasated macrophages partially phagocytose oxidised low density lipoprotein cholesterol, becoming engorged 'foam cells' which undergo apoptosis, releasing their cholesterol contents into the lipid core of the atherosclerotic plaque, Adapted from (Stanner S 2005).

Collagen, produced by the smooth muscle cells, gives strength and structure to the plaque to prevent rupture. Platelet derived growth factor (PGF) and transforming growth...
factor (TGFβ) increase the rate of collagen production. Endothelial MMPs and T cell derived IFN-γ counteract this effect by degrading collagen and extracellular matrix in the fibrous cap or causing the smooth muscle cells to produce less collagen (IFN-γ (Cipollone F 2007)). Continued activation causes the plaque to grow, occluding progressively more of the cross sectional area of the vessel lumen. As the fibrous cap loses structural integrity, particularly at the shoulder regions where the circumferential stress is highest, it becomes weaker and more sensitive to damage and prone to rupture. Plaque rupture releases pro-thrombotic material into the blood, which causes not only a block in the blood flow due to the bolus released itself, but can also cause sudden platelet aggregation and thrombosis at the site of damage (figure 1.7). This is exacerbated by elevated fibrinogen concentrations in the blood and can lead to further, often terminal, occlusion due to myocardial infarction or stroke, depending on the site of the damage. This atherogenesis is the underlying pathogenesis of CVD.
Figure 1.7: Atherosclerotic plaque rupture. On rupture of the fibrous cap the contents of the atherosclerotic plaque are released into the vessel lumen, attracting platelets and potentially causing occlusion. Adapted from (Stanner S 2005).

1.4.3 Co-morbidities

Influences on the endothelium that lead to atherosclerosis often include dyslipidemia, proinflammatory cytokines released from adipocytes, vasoconstrictor hormones, hyperglycaemia and bacterial products. The vasoconstrictor hormone angiotensin II present in high amounts in hypertension, elicits the production of superoxide from endothelial and smooth muscle cells. It can also increase the expression of pro-inflammatory IL-6 and MCP-1 in smooth muscle cells and VCAM-1 in the endothelium (Libby P 2002).
Diabetes, characterized by hyperglycaemia, results in the glycation of macromolecules to form advanced glycation end products (AGE) that bind to their receptors (RAGE) to raise the production of pro-inflammatory cytokines and their pathways in the endothelium. Obesity contributes in this way also since it is strongly linked with diabetes. It is also characterized by high levels of circulating vLDL and LDL as well as higher activation of adipocytes that synthesise pro-inflammatory cytokines such as TNF-α and IL-6.

1.5 Risk markers

CVD is a complex multifactorial disease with both modifiable and non-modifiable risk markers involved separately and in interaction with each other, in development and progression. Lifestyle factors play a major role in CVD, with high blood pressure and smoking being the strongest predictors of CVD, however, these do not account for every case of the disease (figure 1.8).

Non-modifiable risk markers include sex, with rates of CVD in men significantly higher than those in pre-menopausal women. Oestrogen is the dominant protective factor with more than twice the incidence of CHD in men than women below the age of 60 years (Nathan L 1997). These rates, however, are approximately equalised after the menopause, possibly due to a protective effect of oestrogen or the increase in central obesity (a significant risk marker) in women post-menopause. Ethnicity is also a significant risk factor with individuals originating from the Indian subcontinent at
50% increased risk of CHD (and a 40% increased risk of diabetes – another CVD risk marker) compared to those of European origin, and African-Caribbeans in the UK at a significantly increased risk of stroke compared to Caucasians. A certain proportion of this ethnicity effect may be due to modifiable risk markers such as diet. Family history remains a very significant independent risk marker for arteriosclerosis and CHD even when all other known risk markers are controlled for (Goldbourt U 1986).

Age is positively associated with risk of CVD and stroke (Christie D 1981; Herman B 1982) and as the population as a whole ages, so the prevalence of CVD
increases. Early lesions of the aorta are commonly seen within the first decade of life in humans, followed by lesions in the coronary artery in the second decade and cerebral artery in the third or fourth decade.

Type II diabetes, otherwise known as non-insulin dependent diabetes mellitus, is a disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency. Individuals with type II diabetes have a two- to four-fold greater risk of death from CVD than non-diabetic individuals (Haffner S 1998; Turner R 1998). NO-dependent endothelial dysfunction, impaired vascular responses to exogenous NO donors (Williams S 1996) and enhanced responsiveness to vasoconstrictor agents (Cardillo C 2002) are common features of type II diabetes. The availability of NO (Giugliano D 1997) and endothelium dependent vasodilation (Williams S 1998) are impaired by the hyperglycaemia that can result from a high refined grain diet. One study showed a lower risk of both CVD and type II diabetes in individuals who consumed diets high in whole grains (Jensen M 2004) while another showed the substitution of dietary whole grains with a different source of carbohydrates, potatoes, increased the risk of both CVD and type II diabetes (Halton T 2006). CVD and type II diabetes are intrinsically linked through very similar risk markers; approximately 50% of diabetic patients already have established CVD at the time of diagnosis (Blaschke F 2006) and the progression of CVD precedes the development of type II diabetes, with common features including NO-dependent endothelial dysfunction and impaired vascular responses to NO donors (Williams S 1996). Insulin is a polypeptide hormone secreted by the beta cells of the healthy pancreas. Its primary function is to control levels of glucose within the blood. The level of insulin in the blood is usually directly affected by the level of blood glucose.
but can also be regulated by other factors including intestinal hormones, other beta cell secretory hormones and neural influences. Levels of these blood constituents can be altered by changes in lifestyle factors including diet, exercise and prescription of statins. Insulin resistance, associated with high fasting circulating concentrations of insulin (Laakso M 1993), is a known risk factor for both type II diabetes and CVD (Reaven G 1995; Hsueh W 1998; Ruige J 1998).

Individuals of lower socioeconomic status are at increased risk of CVD than those of higher socioeconomic status but this is intrinsically linked to modifiable factors such as lower education level and poor diet which are all risk markers in themselves. One study showed that low neighbourhood education and low neighbourhood income were independent predictors of incident coronary heart disease, even after adjustment for other risk markers (Sundquist K 2004) and another that socioeconomic status is an independent risk marker for re-admission to hospital for heart failure (Philbin E 2001).

Overweight and obesity are risk markers in themselves and also highly correlated to other independent risk markers including type II diabetes, hypercholesterolemia and hypertension. A body mass index (BMI) of greater than 25kg/m² confers a greater risk of CVD than does a lower BMI. The Framingham Heart Study found the risk of heart failure increased by 5% in men and 7% in women per 1kg/m² increase in BMI, with obese participants (BMI ≥ 30kg/m²) having double the risk of heart failure compared to those with a BMI of 20 to 25kg/m² (Kenchaiah S 2002). Body weight is a known risk marker for CVD and many other associated diseases (e.g. type II diabetes (Bogardus C 1985)). The prevalence of obesity, and that of its associated morbidity, in both the developed and
the developing world has reached epidemic levels, increasing by over 75% worldwide since 1980 (Flegal K 2002). A 2001 audit by the National Audit Office, London, found that obesity, a known risk marker for CVD, cost the National Health Service over £0.5 billion per annum (Bourne J 2001). One meta-analysis found the relative risk among individuals with the highest BMI compared to those with the lowest BMIs to be 1.57 for death due to CHD and 1.48 for death due to CVD (McGee D 2005). The same meta-analysis found that obesity increases CHD mortality by approximately 50% in women and approximately 60% in men.

The prevalence of obesity is usually highest in the lower socio-economic groups (Jimenez-Cruz 2005) who generally have diets lower in nutrients than those of higher socio-economic groups (Ricciuto L 2006). This is highly attributable to Westernised diets which tend to be high in fat and refined carbohydrates (Schulze 2006). Some evidence has shown that the type of carbohydrates in the diet can directly affect bodyweight and obesity risk (Ma Y 2004) with low glycaemic index carbohydrate diets, high in whole grains, starches, inulin and oligofructose, having beneficial effects on body weight (Spieth L E 2000), body composition and appetite. Overweight and obese individuals also have higher than normal levels of the cytokines IL-6, IL-8 and TNF-α. (Esposito K 2003). These cytokines are involved in many processes associated with CVD and inflammation including oxidative stress and the expression of adhesion molecules. They are also known to reduce NO production (Haffner S 2003) (please see below). This evidence provides a causal link between obesity and CVD.
Further to absolute adipose tissue mass, the physiological distribution pattern of the adipose tissue also impacts on risk of CVD. Large waists (greater than 80cm/32inches for women and 94cm/37inches for men), or high waist to hip ratios (greater than 0.8 for women or 0.95 for men) showing greater central adiposity or an ‘apple shape’ are associated with greater risk than storing excess weight on the hips and thighs, or the ‘pear shape’ (Larsson B 1984). This is thought to be due to the greater metabolic activity of the visceral adipose tissue, stored around the vital organs, with facilitated delivery of free fatty acids to the liver. A 10cm increase in waist circumference was associated with a 19% higher rate of heart failure events in women and a 30% higher rate in men (Levitan E 2009).

Hypertension, a blood pressure of greater than 140/90mmHg, increases the risk of both haemorrhagic and ischemic stroke, induces endothelial dysfunction, exacerbates the atherosclerotic process and contributes to the instability of the atherosclerotic plaque. A meta-analysis involving 56,000 vascular-associated deaths associated each 20mmHg difference in usual systolic blood pressure (equivalent to approximately a 10mmHg difference in usual diastolic blood pressure) with a two-fold difference in deaths from CHD, stroke or other vascular-related deaths (Prospective Studies Collaboration 2002). Randomised trials have also associated modest reductions in blood pressure with reduced risk of CHD and stroke mortality and morbidity (Bosch J 2002; PROGRESS Collaborative Group 2003).

Circulating lipids are highly correlated with CVD risk (Stamler J 1986), for example high total cholesterol levels in serum are associated with an increased risk with
the uptake of cholesterol by macrophages at the core of atherosclerotic plaque
development. The reference levels recommended by the Joint British Societies (JBS2)
Guidelines on Prevention of Cardiovascular Diseases in Clinical Practice for individuals
at ‘high risk of developing CVD’ are relevant to the ‘at risk’ population groups
investigated in this research. Recommended ideal total cholesterol concentrations for
adults are <5.0mM followed by mildly high at 5.0 to 6.4mM, moderately high between
6.5 and 7.8mM and very high above 7.8mM. The level recommended for total cholesterol
is <4.0mM (JBS2 2005). The British Hyperlipidaemia Association give the following,
similar, definitions: <5.2mM desirable, 5.2 to 6.4mM borderline, 6.5 to 7.8mM abnormal
and >7.8 high (Betteridge D 1993).

Low density lipoprotein (LDL) is the major transporter of cholesterol in the blood
and plays a pivotal role in the early stages of atherosclerosis. Higher than normal levels
of serum LDL-cholesterol, hypercholesterolaemia, are associated with an increase in
CVD risk (Gordon T 1997) and recommended levels for individuals at high risk of CVD
are < 2.0mM (JBS2 2005). Changes in serum levels, particle size and oxidation status of
LDL cholesterol have all been proposed as instrumental in the pathogenicity of
atherosclerosis and its abnormalities have even been proposed as the single greatest risk
marker for atherosclerosis in Western societies (Navab M 1996). There is a long
established link, from prospective observational epidemiological research, between
circulating LDL cholesterol and the absolute risk of premature coronary deaths (Stamler J
1986). This link is positive and without threshold and was given further weight when
LDL cholesterol was shown to be present in atherosclerotic lesions in amounts directly
proportional to those found in the blood (Smith E 1972). This was the basis of the
‘Cholesterol Hypothesis’ for the positive relationship between serum LDL cholesterol concentration and risk of death from CVD. This was investigated further by Ancel Keys who subsequently developed the ‘Diet-Heart Hypothesis’ as a result of investigations into the relationship between dietary cholesterol, circulating LDL cholesterol and risk of heart disease (Keys A 1970) although this has not stood up to the rigorous testing applied to the Cholesterol Hypothesis. As mentioned previously, oxidised LDL can be taken up by tissue resident macrophages which become engorged foam cells, which can degenerate, releasing their contents into the fatty core of the plaque.

Conversely, low HDL-cholesterol (levels below 1.0mM (0.38g/l)) increase CVD risk (Gordon T 1997), thought to be due to HDL-cholesterol’s ability to transport cholesterol to the liver for excretion. Levels recommended by the National Institute for Health for the general adult population are: <1.03mM low HDL and high risk of CHD, 1.03 to 1.55mM medium level and >1.55mM high level considered optimal for CHD protection. Recommended levels for those at high risk of developing CVD are ≥ 1.0mM (JBS2 2005). Clinical trials have shown beneficial effects on risk markers for CHD on the reduction of vLDL and LDL (Assman G 1999). Lipoprotein-a has also been proposed as a promoter early and advanced atherosclerosis (Kronenberg F 1999) although results are conflicting.

Circulating TAG levels were found to be an independent risk marker even when HDL cholesterol levels were corrected for (Hokanson J 1996) and TAGs have been suggested as being able to modify the potential atherogenicity of lipoproteins. Epidemiological data suggest a positive relationship between fasting triglyceride levels
and CHD (Ginsberg H 1997) at levels greater than 2.3 mM (2.0 g/l). This may be due to the strong inverse relationship with HDL cholesterol or perhaps a more subtle alteration in lipid metabolism. Levels are defined as follows: <1.69 mM ideal; 1.69 to 2.25 mM borderline high; 2.26 to 5.63 mM high; >5.64 extremely high.

The relevant literature cites concentrations of non-esterified fatty acids (NEFA) within the ranges of 0.44 ± 0.17 mM (Pedrini M 2006) and 0.5 ± 0.08 mM in those with type II diabetes (Smit J 2008). The notion of a potential role of NEFA in atherosclerosis and CVD is gaining support (Steinberg H 2002; Semenkovic C 2004) and elevated NEFA levels have been proposed as influencing insulin secretion, thus contributing to the development of type II diabetes (Chalkey S 1998). A possible mechanism of action is activation of the pro-inflammatory NFκB pathway in endothelial cells and associated stimulation of mononuclear cells to increase production of inflammatory cytokines, with the accompanied generation of ROS (Shoelson S 2006). Indeed, direct intervention with five fold increases of intravenous NEFA have been shown to induce endothelial dysfunction, induce inflammation, impair vascular reactivity and increase circulating levels of soluble cellular adhesion molecules (Tripathy D 2003). The direct relevance of this work is unclear as levels of NEFA investigated were not within physiologically relevant ranges, with research continuing in this area.

Smoking is a proven correlator for CVD risk, thought to be due to the resultant increases in oxidative stress and the impaired endothelial function. Oxidative stress increases during inflammation, destroys NO and has been suggested to contribute to endothelial dysfunction (Rush J 2005). Cytokines, including IL-8, IFN-γ and TNF-α, are
involved in oxidative stress, affecting the increased expression of adhesion molecules and the reduction of NO production (Haffner S 2003). Levels of these cytokines are increased in inflammation and obesity (Esposito K 2003).

Physical inactivity is a common factor in a Westernised lifestyle and is associated with increased CVD risk. Physically active adults experience lower risk of CVD and CHD (Lee I 2001; Wannamethee S 2002) with one review of 27 cohorts showing sedentary groups to be twice as likely as more active groups to experience CHD (Berlin J 1990). Data from the Nurse's Health Study showed an association between both walking and vigorous exercise, and a 30 to 40% reduction in risk of coronary events (Manson J 1999) and research continues in this area.

Diet affects CVD risk in both obvious and more subtle ways. For example, a diet high in saturated fats is known to increase levels of cholesterol in the blood. Emerging evidence, however, suggests more subtle interactions, including the ability of whole grain diets to reduce risk of CVD. The inflammatory process has been shown to be beneficially affected by diets high in whole grains (Lopez-Garcia E 2004; Nettleton J 2006) and suggested mechanisms responsible for this beneficial effect include a reduction in endothelial activation and improved endothelium dependent vasodilation when compared with the effects of a low whole grain diet.

The modifiable risk markers for CVD provide a possible route for prevention, reversal and treatment of the disease. Figure 1.9 displays a 'round table' of the factors related to diet, obesity, activity and drugs in their contribution to the prevention and
treatment of CVD (Ashwell M 2000). Interventions aimed at reducing risk through manipulation of the factors illustrated below provide a combined approach to tackling this issue. One of the most promising non-invasive methods of treating the modifiable risk markers is posed by the diet, which has positive effects on a range of risk markers, including total cholesterol, LDL cholesterol, HLD cholesterol and TAGs.

Figure 1.9: A round table of dietary and other factors involved in the prevention of coronary heart disease. The contribution of factors related to diet, obesity, activity and drugs to the combined strategy for prevention and treatment of cardiovascular disease. Taken from (Ashwell M 2000).
1.6 Whole grain diets and CVD risk

Epidemiological evidence associates diets high in whole grains with a reduction in risk of cardiovascular disease (CVD) although the mechanisms behind, and reasons for, this association are unknown. Whole cereal kernels, or grains, consist of three distinct sections: the protective outer or bran section, the endosperm making up the bulk of the grain, and the germ. Nutrients are concentrated within the bran (which contains a high proportion of the fibre) and the germ (containing high levels of vitamins and minerals) leaving the energy dense endosperm relatively nutrient poor.

The most commonly accepted definition of a whole grain is that proposed by the American Association of Cereal Chemists (AACC) in 2005. This definition states that ‘whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal components – the starchy endosperm, germ and bran – are present in the same proportions’. To be labelled as ‘whole grain’ a food must contain at least 51% whole grains by weight.

The most commonly consumed grains in the UK are wheat, rice, maize, oats, rye and barley. Currently, grains provide about 30% of total energy in the British adult diet. This value is much greater than that of any other food group (Truswell A 2002). Dietary guidelines recommend increasing the consumption of whole grains in the average UK diet to 3 portions per day (Lang R 2003) although the actual intake is substantially less than this (Anderson J 2003), with certain studies suggesting that whole grain intake is still less than one portion per day (Gregory J 1990; Finch S 1998). One portion of whole grains is defined by the Whole Grains Council (www.wholegrainscouncil.org) as ‘16g of...
whole grain ingredients’ and is equivalent to approximately one slice of whole grain bread, half a whole grain pita bread or half a cup of whole grain rice or pasta. The combined advice, therefore, is for a daily intake of 48g whole grains. This advice is grounded in recent epidemiological research linking whole grains to beneficial effects on cancer (Chatenoud L 1998; Jacobs D 1998; Williams M 2005), type II diabetes (Jensen M 2004; Halton T 2006), obesity (Slavin J 2005), appetite regulation or reduction (Koh-Banerjee P 2003), body composition (Nelson L 1996), myocardial infarction prevention (Martinez-Ortiz J A 2006), insulin resistance (Esposito K 2004) and total mortality from all causes (Steffen L 2003). High intakes of whole grains have also been shown to lower risk of cardiovascular disease (Jacobs D 1998; Liu S 1999; Liu S 2000; Liu S 2003; Steffen L 2003).

Behall et al found that meals high in both oats and barley reduced glycaemic responses compared to a test meal containing glucose, a factor which they related to the high soluble fibre content of these foods (Behall K M 2005). Glucose responses (measured by the area under the curve (AUC)) were reduced after both barley and oats when compared to a test meal containing glucose, while insulin AUCs were reduced significantly by barley but not by oats. Glucagon and leptin responses to a high oat or barley meal did not differ significantly from those to a test meal containing glucose (Behall K M 2005). Extremes of these physiological responses have been linked with elevated risk of CVD.

Whole cereal grains contain important amounts of most nutrients required by the ‘healthy’ human body (Truswell A 2002). The predominant oil in cereals is
polyunsaturated, with 50% being linoleic acid, which may act to lower low-density lipoprotein (LDL) and total plasma cholesterol as seen in the Mediterranean diet (Vincent-Baudry S 2005). Whole grains also contain significant levels of folic acid which reduces plasma homocysteine levels (Bernasconi A 2006) and high homocysteine levels (hyperhomocysteinaemia) are known risk markers for CVD. Whole grains are also rich in antioxidants including vitamins, trace minerals, phenolic acids, lignans, phytoestrogens, flavonoids and anti-nutrients such as phytic acid (Thompson L 1994; Slavin J 2003) and are a better source of selenium and the intracellular antioxidant vitamin E (especially tocotrienols) than are refined grains. Antioxidants delay the onset of, or slow the rate of oxidation of, oxidizable substrates and therefore the formation of harmful free radicals. Oxidative stress caused by these particles has been implicated in cancer (Klaunig J 1998) and CVD risk (Rush J 2005). The antioxidant phenolic acids (e.g. ferulic, canillic and caffeic acid) are found in the outer layers of whole grains and have been associated with potential protection from CVD (Thompson L 1993). Whole grains contain high levels of beneficial phytoestrogens (e.g. isoflavones, lignans and coumestans) and have mediating effects on insulin and glucose responses. Impaired metabolism of these factors is implicated in CVD risk (Ruige J 1998). Whole grains also contain lignins which are converted to enterolactone and enterodiol in the body. Lignins are hormonally active compounds that may protect against hormonally mediated diseases, cancer and atherosclerosis (Adlercreutz H 1990; Adlercreutz H 1997).

Research shows that refined grains do not contain the same levels of bioactive nutrients as whole grains and are not associated with the same health benefits. It is unknown, however, whether the state of the whole grain is a factor in its beneficial
effects. The supposed benefits of whole grains to health are widely accepted with whole grain products, including bread, pasta and cereals being deemed better for health than their refined grain counterparts. The first evidence for this claim on cardiovascular health came in 1977, when a study in UK men showed that those with the highest cereal fibre intake had a 80% lower risk of heart attacks (Morris J 1977). Since then, much similar work has been carried out although most studies are observational to date so no direct cause and effect can be determined. Studies show that individuals with a higher than average whole grain intake or approximately 48g/d (as defined as the recommended intake by the Whole Grains Council) have a significantly reduced risk of atherosclerotic CVD than those with the lowest intake. This has been associated with whole grain’s ability to reduce several of the major risk markers for atherosclerosis: high LDL cholesterol, low HDL cholesterol, high serum TAGs, hypertension, type II diabetes and obesity (Anderson J 2003). The average adult in the USA and Western Europe has less than one serving of whole grains per day which might partly explain why CVD is so common in these geographical areas (Cleveland L 2000). One study showed a lower risk of both CVD and type II diabetes in individuals who consumed diets high in whole grains (Jensen M 2004) while another showed the substitution of dietary whole grains with a different source of carbohydrates, potatoes, increased the risk of both CVD and type II diabetes (Halton T 2006).

Whole grains consist of three major components: the husky, protective outer layer of bran which is very rich in dietary fibre and designed to protect the grain; the high starch, simple carbohydrate rich endosperm designed to feed the growing plant; and the nutrient rich germ which will become the new plant (figure 1.10). The refining process
removes both the bran and a proportion of the germ, resulting in an increase in the relative starch concentration coupled with a loss of dietary fibre, vitamins, minerals, lignins, phytoestrogens, phenolic compounds and phytic acid. These components, many of which have proven health benefits, appear to work synergistically to produce an effect greater than that of the sum of their parts.

**Figure 1.10: A whole grain kernel showing the major component parts.** Major components include: the protective bran outer coating, the starchy simply carbohydrate rich endosperm and the vitamin rich germ (embryo) Adapted from (Slavin J 2004).

Although evidence shows a link between increased whole grain intake and reduction in risk of CVD, the parts of the whole grain responsible for this relationship are still unknown. For example, phytochemicals in whole grains have also been suggested as
being responsible for reducing the risk of CVD through their hormonal effect on HDL levels (Thompson L 1993). One study reported a significant rise in total plasma cholesterol in subjects, following a high wheat fibre diet (Stasse-Wolthuis M 1979). It is possible in such cases that the HDL was increased even though total cholesterol was not reduced (O'Moore R 1978; Lindegarde F 1984). This would still have beneficial effects on CVD risk.

1.6.1 Dietary fibre

Whole grains contain high levels of both dietary starch and fibre. Starch is a polysaccharide comprised primarily of amylose and amylopectin. The relative ratios of these component polysaccharides in a food influence the rate of starch digestion and insulin and glucose responses (Behall KM 1988) with consequent effects on satiety and further intake (Anderson G 2003). The gel-forming properties of certain soluble dietary fibres (e.g. guar gum, pectin and mucilages) reduce postprandial glycemia by delaying gastric emptying (Nuttall F 1993). Even those without gel-forming properties appear to control food intake to a certain extent thus affecting risk of overweight and associated morbidities. A meta-analysis investigating evidence for a relationship between whole grain intake and CVD found that cereal fibre intake was non-significantly (risk ratio of 0.9, 95% CI 0.8, 1.01) negatively associated with CHD but did not account for the protection from atherosclerotic CVD afforded by whole grain intake (Anderson J 2000).

The term inulin describes a heterogenous blend of fructose polymers, known as the fructans, composed of mainly linear chains of 6 to >60 fructose units (Gibson G 1994) with a terminal sucrose molecule. Inulin is widely distributed in the storage
carbohydrates of approximately 36,000 plant species (Carpita N 1989), which equates to approximately 15% of flowering plant species (Hendry G 1993). As a soluble dietary fibre (Roberfroid M 1993), inulin is resistant to digestion in the upper gastrointestinal tract (Knudsen K 1995) and thus reaches the large intestine intact (Niness K 1999). This stimulates the growth of intestinal bifidobacteria, resulting in the production of SCFAs, lactic acid and gasses such as H₂, CO₂ and CH₄ (Roberfroid M 1993). The average European diet includes approximately 3 to 10g inulin per day (van Loo J 1995) with the most significant sources including wheat, onions, bananas, garlic and chicory with most commercially available inulin being synthesised from sucrose or extracted from chicory (Niness K 1999).

Inulin is used to fortify foods with fibre. As its caloric content is approximately 1kcal/g (Roberfroid M 1993) it is also used to improve flavour and texture of low calorie foods and low-fat foods as alternatives to sugar and fat. Consumption of soluble fibres, including inulin, oligofructose and β-glucose, has been suggested as lowering total and LDL cholesterol (Davidson M 1999). In a review of 77 investigations into the effects of soluble fibre, 68 studies showed a reduction in total cholesterol (Glore S 1994). Of the 49 studies reviewed which investigated LDL cholesterol, 41 showed a reduction due to the high soluble fibre diets. Due to its use as a hypocaloric substitute for both sugar and sweetener and its reputed lipid lowering actions, inulin is recommended for those at high risk of CVD.

Several factors effect the digestibility of a starch including the cooking and cooling processes employed, associations between the starch and other components in the
food and the type and size of the starch granules themselves (Stephen A 1994). The structure of foods also affects the blood glucose and insulin responses to that food (Bjorck I 1994). As soluble starch binds water it has the capacity to lower the energy density of the diet (Roberts S 2002). The type of starch used in the diet may have important metabolic consequences in normal and diabetic rats (Lerer-Metzger M 1996). Starches can be altered and then added to whole grain products in addition to those already present.

Intervention studies have shown that a high-starch regimen causes a decrease in energy intake with no apparent differences in effects between soluble and insoluble starch (Howarth N 2001). Bread made with 50% buckwheat groats induce a significantly higher satiety score than a reference white wheat bread (Skrabanja V 2001), a property attributed to its high levels of insoluble starch. The addition of starch to foods is recommended to increase their ‘filling powers’ and reduce appetite (Holt S 2001). Neither 75g preloads of high-amylose cornstarch or 75g of high-amylopectin cornstarch, however, in the form of sweetened drinks were shown to affect food intake one hour after ingestion (Anderson G 2002). It is possible that this effect is due to the medium, as liquids tend to be less satiating than solids.

As high starch foods increase distension in the stomach they may increase sensations of fullness (Roberts S 2002). In rats a wheat starch load causes a significant reduction in dinner size after the third load (Bensaid A 2002). This study also showed that meal duration was not modified by a carbohydrate load, but was shortened by a 35% protein load. Intake of dietary fibre was found to be inversely related to serum
concentrations of C-reactive protein, a known marker of inflammation (King D 2003; Ajani U 2004) and the National Health and Nutrition Examination Survey in the US determined dietary fibre to be independently and inversely associated with circulating CRP, in a study with approximately 4000 participants (Ajani U 2004).

Ma et al (2004) showed that the type of carbohydrate in the diet may be related to body weight. Additional dietary fibre has been found to reduce body weight to a greater extent than the reduction of fat, although the effects have been found to be additive (Roberts S 2002). Weight loss has been found to be more than three times as great in subjects on a lower-fat, higher-fibre diet than in subjects on a low-fat diet alone (Yao M 2001). Body weight reduction rate was significantly increased when feeding a high fibre diet compared with a control diet (Howarth N 2001) suggesting that the effects of fibre on energy intake do translate to reductions in body weight.

Behall et al found that meals high in both oats and barley reduced glycemic responses compared to a test meal containing glucose, a factor which they related to the high soluble fibre content of these foods (Behall KM 2005). Glucose responses (as shown by the ‘area under the curve’) were reduced after both barley and oats when compared to a test meal containing glucose and insulin AUCs were reduced significantly by barley but not by oats.

In a study by Holt et al mean satiety index scores for high-fibre and protein-and-fibre-rich breads (containing 33.5 and 19.9g of fibre respectively) were significantly higher than those for reference and fruit breads (containing 1.8 and 10.2g of fibre
respectively (Holt S 2001)). These high fibre breads were also associated with lower energy intakes for the rest of the day. The levels of fibre used in this study could realistically be added to foods in practice to potentially control satiety and energy intake, to maintain or reduce body weight (as a marker of CVD risk) as appropriate.

Both oat bran and wheat bran had the same effect on stool weight, even though oat bran contains mostly soluble fibre and wheat bran contains mostly insoluble fibre (Chen H 1998), thus suggesting that the laxation properties of a fibre can not be determined by its solubility. The source of the fibre appears to be important in determining its effects on cholesterol levels. One study reported a reduction in total plasma cholesterol after feeding with bran from hard red spring wheat but not after feeding with ordinary soft white wheat bran (Munzo J 1979) thus warranting further investigations into different sources of fibre.

1.6.2 Refined grain foods

Many of the nutrients known to be beneficial or essential for health, specifically heart health (Truswell A 2002), including the vitamins (e.g. riboflavin, niacin and thiamine), minerals (e.g. iron potassium and copper), fibre, phytonutrients and essential fatty acids, are concentrated within the germ and bran layers as previously stated. Unfortunately, all of the nutrients proposed to be protective to heart health are reduced by the refining process, leaving an energy dense and nutrient poor, smoother white flour. Therefore, a high proportion of the vitamins and minerals found in whole grains, and credited with their beneficial effects, are not found in refined white flour and its products (Holland B 1991).
Some refined flours and cereals are fortified with vitamins and minerals, usually calcium or iron and several B vitamins (including riboflavin, niacin and folic acid). Although these products often display health claims about their added nutritional benefits, only a small number of the nutrients removed are replaced (Truswell A 2002) and fibre is not generally one of them. It is also not clear whether the addition of components provides a CVD risk reduction benefit equal to that of inclusion of the intact whole grain, or whether the impact of the whole grain is greater than that of the sum of its parts.

High intakes of refined carbohydrate foods cause rapid and extreme swings in both blood glucose and insulin levels and may elevate plasma levels of free fatty acids (Foster-Powell K 2002). These effects can cause hunger and over feeding and are associated with development of CVD and type II diabetes. Hyperglycaemia impairs endothelium dependent vasodilation (Williams S 1998) and reduces the availability of NO (Giugliano D 1997). This results in the unrestrained production of free radicals which are also implicated in the promotion of atherogenesis (Giugliano D 1996; Brownlee M 2001). Therefore, the habitual consumption of refined grains can be associated with CVD risk. Replacement of total and unsaturated fats with refined carbohydrates is unlikely to reduce CVD risk and may even increase this risk in people who are predisposed to insulin resistance (Mozaffarian D 2005). The same study found that diets rich in whole grains and low in refined grains are likely to reduce the risk of CVD.
1.6.3 Whole grain foods

1.6.3.1 Physical states of whole grains

The nutritional effect of a whole grain differs with the sources of these grains. The state in which a whole grain is present in a food has also been shown to determine the affect that food has on insulin response (Wrick K 1983) and other markers of physiological response (Behall KM 2005). When fed at the same dosages, course wheat bran has a greater faecal bulking effect than finely ground wheat bran, suggesting that the particle size effects physiological response. Course bran also delays gastric emptying and accelerates small bowel transit time to a greater extent than finely ground bran (McIntyre A 1997) suggesting the effects are not due to simple composition differences between whole and refined grains. One study showed that particle size of both oats and barley had no significant effect on the glycemic response to the food (Behall KM 2005) but did not investigate the effects of wheat bran. Particle size of wheat bran did not alter the effect on stool weight, but did affect faecal concentrations of butyrate suggesting increased bacterial fermentation with the smaller particles (Jenkins D 1999). Hence, it is important to consider the physical state of the whole grain when conducting research.

1.6.4 Fermentation products of whole grains

Short-chain fatty acids (SCFAs) are the major anions present, at approximately 100 to 200mM/day (Cook S 1998), in the large intestinal lumen of non-ruminant, monogastric mammals. They are produced in the cecum and proximal colon by the anaerobic, bacterial fermentation of, primarily, dietary fibre, which is made up of indigestible carbohydrates, those resistant to breakdown by human salivary and intestinal digestive enzymes, but which are broken down by the intestinal microflora (Sako T 1999; van
SCFAs are two to five-carbon weak acids, the bulk of which are acetate (C2), propionate (C3) and butyrate (C4). Valerate (C5) is the next most abundant SCFA but is present at significantly lower concentrations in the lower intestines. The molar ratio of acetate:propionate:butyrate in the mammalian colonic lumen is approximately 60:20:20 respectively (Cummings J 1979) although values are conflicting. The majority (70 to 90%) of the butyrate is metabolised as the primary fuel of the colonocytes but the acetate and propionate move into the circulation (Roediger W 1980) with circulating levels in μM concentrations (Cummings J 1979). Interestingly, the production of SCFAs depends on the substrate, for example, approximately 5 to 20% insoluble fibre is metabolised to SCFAs whereas this value can be 90% for soluble fibre (Cook S 1998).

The SCFAs are known to have a number of physiological and pathophysiological effects on the intestine including a trophic effect on the lining of the intestine itself (Royall D 1990). They have demonstrated prebiotic effects and improved mineral absorption (Teitelbaum J 2002) in the colon, activation of endothelial cells and involvement in the regulation of lipid metabolism (Kaur N 2002). They have immunomodulatory and anti-inflammatory properties (Kelly-Quagliana K 2003) in mice, deter the colonisation of the lower intestine by pathogens (Royall D 1990) and also aid the immune system as butyrate is one of its substrates (Jenkins D 1999). They are absorbed as nutrients across the intestinal epithelium and can influence various functions of the gastrointestinal tract (Cummings J 1995). The SCFAs are known to affect colonic motility and ion transport (Yajima T 1985; Yajima T 1988; Fukumoto S 2003) and water thus acting as anti-diarrhoeal agents (Ramakrishna B 1993). They are have also been shown to provide 5 to 30% of systemic daily energy requirements (Rombeau J 1990),
improve colonic blood flow (Mortensen F 1990) and reduce ammonia uptake from the lower intestine (Royall D 1990).

The preferred substrate for colonocytes is n-butyrate, which has been shown to reduce the risk of colon cancer by stimulating normal cellular growth and reducing damage to DNA (Roediger W 1990) and antagonizing the hyperproliferation of colonic cells induced by the secondary bile deoxycholate (Scheppach W 1995). The trophic effect of n-butyrate reduces the incidence of ulcerative colitis (Smith J 1998). Propionate causes a direct reduction in hepatic gluconeogenesis and an enhancement in hepatic glycolysis (Roberfroid M 1998). It also works indirectly on the liver to lower plasma fatty acid concentrations which are closely related to gluconeogenesis (Kaur N 2002). They are thought to be responsible for some of the health benefits associated with whole grains. Raised faecal levels of SCFAs can be indicative of a number of pathological states, including defective metabolism within colonocytes (Treem W 1996), increased blood in the colon (Holtug K 1988), malabsorption (Scheppach W 1988), rapid gut transit times (Oufir L 2000) or small bowel bacterial overgrowth (Hoverstad T 1985).

Although SCFAs are the most abundant breakdown products of a high whole grain diet, implicated in the health benefits, they are not the only factors worth consideration. Phytoestrogens, which are found commonly in plants and are structurally similar to mammalian oestrogens, have also been suggested to have protective effects against diseases including CHD, type II diabetes mellitus and certain hormone related cancers (on which most of the previous work has focused).
The most common class of phytoestrogens in the Westernised diet are the lignans which are present in most plant foods. These lignins are not digested by the human gut but are metabolised into the mammalian lignins by the intestinal bacteria of non-ruminant, mono-gastric mammals (Lampe 2003; Rowland I 2003). The two main mammalian lignins produced in the human colon are enterolactone and enterodiol. Enterodiol can also be converted into enterolactone on metabolism by the same bacteria. From this point onwards enterolignins produced in the human gut from plant lignans will be known simply as ‘lignins’.

Lignin precursors are found in a wide variety of plant based foods including seeds, legumes and whole grains with flaxseeds being the richest dietary source. They are classified as phytoestrogens as they mimic some of the effects of oestrogens. The principal source of dietary lignans in the Westernised diet include pinoresinol, lariciresinol, secoisolariciresinol and matairesinol (Kleijn M 2002; Valsta L 2003).

Both serum and urine levels of the lignins can be used as markers of dietary intake of lignin precursors. In a population following an average Westernised diet, plasma concentrations of enterolactone plus enterodiol have been found to be in the range of 10 to 270nM but include a very wide range of enterolactone concentrations from non-detectable to greater than 1μM. The therapeutic use of antibiotics has been associated with lower serum levels of enterolactone (Kilkkinen A 2002) suggesting that intestinal, and therefore dietary intake, levels are reflected in the serum. As data on the lignan content of foods is limited, serum and urinary levels are used as markers of intake and intestinal levels in studies. One pharmacokinetic study which measured plasma and
urinary levels of the mammalian lignins after a single 0.9mg/kg body weight dose of secoisolariciresinol (the principal lignan in flaxseeds) found that at least 40% was available to the body for conversion to enterodiol and enterolactone (Kuijsten A 2005). The concentration of enterodiol in plasma peaked at 73nM an average of 15 hours post-ingestion. The concentration of enterolactone in plasma peaked at 56nM after an average of 20 hours post ingestion. Thus, substantial amounts of ingested plant lignans are available to the human gut flora for fermentation into mammalian lignins. High lignin-precursor feeding studies have shown considerable between and intra-subject variation in both the baseline levels of lignins and the ratios of enterolactone: enterodiol produced on feeding. A previous *in vitro* study focusing on the effects of enterodiol and enterolactone on the breast cancer cell line (MCF-7) both lignins were used at concentrations of 10nM to 1μM using ethanol as the vehicle (Carreau C 2008). This study showed over-lapping yet distinct effects on endogenous gene targets at levels which can be achieved in a high lignan diet.

As stated above, diets rich in plant lignans e.g. fruit, vegetables and whole grains, are consistently associated with a reduction in CVD risk markers. It is still not clear, however, whether lignins themselves confer any cardio-protective effects and no specific mechanisms of action have been proposed.

**1.7 Whole grain and CVD interaction**

Epidemiological evidence from observational studies suggests that diets rich in whole grains are associated with a reduced risk of CVD (Jacobs D 1998; Liu S 1999; Liu S 2000; McKeown N 2001; Anderson J 2003). One prospective cohort study of healthy
male health professionals found a negative association between habitual whole grain intake and CVD occurrence (Jensen M 2004). The authors suggested that the bran component of whole grains could play an important role in this relationship and thus have a clinical application. A further recent study showing a lower risk of CVD in persons who consume diets high in whole grains (Jensen M 2004) also showed a lower risk of type II diabetes. In addition, a meta-analysis of 13 studies compared groups with the highest and lowest whole grain intake, revealing a risk ratio of 0.71 (95% CI 0.48, 0.94) for CHD, showing a reduction in risk of CHD of 29% for those with the highest intake, above three portions per day (Anderson J 2003).

Individuals in the UK who consume the most whole grains have a 29% lower risk of developing CVD than those who consume the least (Anderson J 2003). Three diets have been proposed for the prevention of CVD (Hu F 2002), one of which expressly contained increased whole grain consumption. In spite of all of this research, however, only a few countries have dietary guidelines which specify the recommended quantities of whole grains to be included in the daily diet (Lang R 2003). The probable mechanism for the conferred reduction in risk of CVD associated with whole grains is due to improvements in glycaemic control, plasma lipid profile and reductions in inflammatory factors, associated with several of its constituent parts. Few nutritional intervention trials have investigated the effects of a high whole grain diet on risk markers of CVD directly.

In conclusion, mounting evidence associate diets high in whole grains with a reduced risk of CVD, CHD and other co-morbidities including type II diabetes, obesity and hypertension. Much of this data, however, is observational and controlled dietary
intervention trials are required to determine whether this association with whole grains is causal or a symptom of a wider range of lifestyle factors.

1.8 Hypotheses and objectives

Cardiovascular disease, for which the inflammatory disorder, atherosclerosis, is an underlying cause, is the leading cause of mortality and morbidity in the UK. Strong evidence exists for the relationship between high whole grain diets and lower risk of CVD. Much of the evidence to date, however, is observational suggesting the need for randomised controlled dietary intervention trials to establish whether this relationship is causal or passive. This research, therefore, aimed to investigate the relationship between high whole grain diets and markers of risk of CVD in healthy individuals displaying risk markers for CVD and it was hypothesised that whole grains and their constituents would have beneficial effects on markers of CVD risk both in vivo and in vitro.

The first objective was to investigate the effects of whole intact and whole milled grains, compared to a reference control grains, on circulating haemostatic, inflammatory, lipid and insulin sensitivity markers as an indication of CVD risk in an at risk population. The first objective would be achieved through the Pilot Whole grain Intervention Study in England (Pilot WISE) trial in men (aged 30 to 60 year) and post-menopausal women (both with MBI 25 to 35kg/m²).

The literature poses the effects of fibre, found in high levels in whole grain foods and proven to have beneficial effects on CVD related factors including total and LDL cholesterol and weight loss, as a possible mechanism responsible for beneficial effects of
whole grains on CVD risk markers. Different types of fibre, that is, soluble and insoluble fibre, are known to have differing levels of impact on such markers.

The second objective, therefore, was to investigate the effects of both insoluble and soluble fibre, represented in this research by wheat fibre and inulin respectively, on circulating haemostatic, inflammatory, lipid and insulin sensitivity risk markers of CVD in at risk men. The markers investigated were as stated in the first objective and participants included men aged 30 to 60 years with BMI scores of 25 to 35kg/m² (n = 10). This was to be achieved through the Fibre and Inulin Trial in Male Adults (FITMA).

Nutrients interact with metabolic pathways at numerous levels with multiple targets. The range of metabolites present in the metabolome at any time is a reflection of the metabolism at that point in time. Increasing use of ‘-omics’ technologies in nutritional research now allows more detailed research of the influence of specific dietary changes on such physiological processes. Metabolomics, the most recent of the ‘-omics’ technologies, is increasingly being applied to dietary intervention studies to determine effects of specific diets, or dietary components, on the metabolome (Rezzi S 2007; Lodge J 2009). Using metabolomics allows for research to investigate global changes in metabolite profiles within biological samples and interest in this area is turning toward nutritional research (Gibney M 2005; Rezzi S 2007). It possible to investigate the interactions of specific individual nutrients or whole diet interventions with the metabolic pathways and the metabolome, or to identify biomarkers for dietary exposure. Such metabolomics’ technology uses an analytical platform, in this case mass spectrometry, to analyse given biological samples with multivariate statistical analysis to visualise
differences between identified sample groups. To date, metabolomics technology has been applied to a number of nutritional studies with good effect (Rezzi S 2007).

As such, the third objective was to explore the effects of diets high in whole intact and milled grains, wheat fibre and inulin on circulating metabolite profiles when compared to a reference control refined grain, via an untargeted HPLC/MS based metabolomics analysis with the potential for investigation of a biomarker for the dietary intake of such products.

At the molecular level, fibre is fermented anaerobically by the colonic microflora to short chain fatty acids (SCFAs) and other factors. The SCFAs, the most abundant of which are acetate, propionate and butyrate, are known to have anti-inflammatory effects and to be present in the circulation. These factors are, therefore, posed as mediating the beneficial effects of whole grains of CVD risk.

The fourth and final objective was to investigate the potential mechanisms of action of whole grains and their constituents and fermentation products at the cellular level by exploring the effects of fermentation products of whole grain constituents SCFAs, on markers of vascular health and dysfunction in vitro. Markers chosen were the secreted inflammatory mediators interleukin-8 and nitric oxide, the haemostatic secreted factors tissue plasminogen activator-1 and von Willebrand factor, and the cell surface adhesion markers intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and platelet-endothelial cell adhesion molecule-1.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Human intervention products

All intervention products used in the Pilot WISE and FITMA trials were a gift from Holgran Ltd.

The Pilot Whole grain Intervention Study in England: the Pilot WISE trial

The three bread roll-like intervention products used in the Pilot WISE trial were: (i) the control product containing only the refined grain matrix as a reference; (ii) the whole intact grain product containing 48g whole intact grains within a refined grain matrix; (iii) the whole milled grain product containing 48g whole but milled grains in a refined grain matrix. The approximate nutritional composition of the products per 100g final product, were: control: 258kcal, 54.1g carbohydrate and 2.7g fibre; whole intact: 293kcal, 60.0g carbohydrate and 5.6g fibre and whole milled: 283kcal, 57.4g carbohydrate and 5.4g fibre. Please see appendix 1A for recipes and complete nutritional composition.

The Fibre and Inulin Trial in Male Adults: the FITMA trial

As in the Pilot WISE trial, the FITMA trial used three bread roll-like intervention products. These were: (i) the control product containing the refined grain matrix only; (ii) the wheat fibre product containing 4.6% by weight VITACEL® wheat fibre WF 600 DV, a white fine-fibrous dietary fibre powder, (JRS, Netherlands) in a refined grain matrix; (iii) the inulin product containing 4.4% Orafti® HPX inulin, a fine white powder high performance chicory inulin (Orafti®, Belgium) in a refined grain bread-roll like matrix.
Approximate nutritional compositions of the products per 100g of the final product were:
control: 256kcal, 50.7g carbohydrate and 2.5g fibre; wheat fibre: 220kcal, 43.4g carbohydrates and 7.7g fibre; inulin: 239kcal, 47.4g carbohydrate and 7.6g fibre. Please see appendix 1B for recipes and complete nutritional composition and appendix 2A and B for wheat fibre and inulin data sheets respectively.

2.1.2 Reagents and kits

Reagents and kits used for sample treatment, isolation and analysis during all parts of the investigation are listed below.

Antibodies

For the IL-8 ELISA, mouse anti-human IL-8 antibody and biotinylated mouse anti-human IL-8 antibody were purchased from Invitrogen, UK. Mouse mouse anti-tPA-1 antibody and peroxidase conjugated sheep anti-human anti-tPA-1 antibody were purchased from Calbiochem, UK and CEDARLANE, UK respectively. For the vWF ELISA, rabbit anti-human vWF and rabbit anti-human vWF-HRP antibody were sourced from DAKO Cytomation, UK. Mouse anti-human-TNF-α and biotinylated mouse anti human-TNF-α were sourced from BD-Pharminogen, UK. Polyclonal rabbit anti-human CRP antibody and polyclonal rabbit anti-human CRP-HRP were sourced from Dakocytomation, UK.

For the fluorescence activated cell scanning (FACS), referred to in further text as flow cytometry, analysis of activation markers on endothelial cells, antibodies were directly conjugated to FITC or phycoerythrin (PE). Anti-PECAM-1-FITC was sourced
from Sigma-Aldrich, UK. Anti-ICAM-1-PE, anti VCAM-1-FITC, mouse IgG1K-FITC and mouse IgG1K-PE were purchased from BD Biosciences, Belgium.

**Chemicals and kits**

All chemicals used were of analytical grade, unless part of a kit.

- Citrate phosphate tablets (Sigma-Aldrich, UK)
- Dimethyl sulphoxide (Sigma-Aldrich, UK)
- Direct HDL cholesterol kit (Randox, UK)
- Griess reagent kit (Invitrogen, UK)
- Horseradish peroxidase conjugated-avidin (Dakocytomation, UK)
- Human insulin specific radio-immuno assay (Millipore, UK)
- Human serum C-reactive protein high control (Dakocytomation, UK)
- Human ultrasensitive cytokine 10-plex panel (Invitrogen, UK)
- IL Test™ cholesterol kit (Instrumentation Laboratory, Italy)
- IL Test™ glucose kit (Instrumentation Laboratory, Italy)
- IL Test™ triacylglycerides kit (Instrumentation Laboratory, Italy)
- Industrial methylated spirit (Merck, UK)
- Interleukin-8 standard (Invitrogen, UK)
- MTT based cell growth determination kit (Sigma-Aldrich, UK)
- Non-esterified fatty acids kit (Randox, UK)
- Novex® HRP chromogenic substrate (TMB, Invitrogen, UK)
- Paraformaldehyde (Sigma-Aldrich, UK)
- Phosphate-buffered saline (PBS) tablets (Invitrogen, UK)
- Polysorbate (Tween®) 20 (Sigma-Aldrich, UK)
Purified human tissue plasminogen activator-1 standard (Calbiochem, UK)
Purified recombinant human interferon-γ standard (National Institute of Biological Standards and Controls, UK)
Purified recombinant human tumour necrosis factor-α standard (National Institute of Biological Standards and Controls, UK)
Quintikine® human sICAM-1/CD54 kit (R&D, UK)
Saline, sterile Macoflex 0.9% sodium chloride solution (Macopharma UK Ltd, UK)
Sodium acetate (Sigma-Aldrich, UK)
Sodium azide (Sigma-Aldrich, UK)
Sodium hypoxanthine, aminopterin and thymidine growth supplement (Invitrogen, UK)
Sodium propionate (Sigma-Aldrich, UK)
Tetramethylbenzidine dihydrochloride (TMB, Sigma-Aldrich, UK)
von Willebrand factor standard (National Institute of Biological Standards and Controls, UK)

Buffering solutions

Carbonate/bicarbonate (BIC) buffer, 0.1M, was prepared from 0.1M anhydrous sodium carbonate (Na₂CO₃) and sodium bicarbonate (NaHCO₃), pH adjusted to 9.6 using concentrated hydrochloric acid. FACS buffer was prepared from phosphate buffered saline (PBS), prepared from tablets as per the manufacturers instructions and supplemented with 1% bovine serum albumin (BSA) and 0.05% sodium azide.
Metabolomics reagents

HPLC grade acetonitrile and methanol, laboratory reagent grade formic acid and analytical grade sodium hydroxide were sourced from Fisher Scientific, UK. Water was purified using an Elga PureLab Ultra system (Elga, UK) and leucine enkephalin (lock mass) was purchased from Sigma-Aldrich, UK.

2.1.3 Cell culture

Cell culture media and chemicals

High glucose Dulbecco’s Modified Eagle Medium (DMEM) with glutamax-1 was sourced from Invitrogen, UK and Microvascular Endothelial Growth Medium (EGM-2) and Roswell Park Memorial Institute (RPMI) media were purchased from Lonza, UK. F-12K medium was sourced from LGC Promochem, UK.

Endothelial Growth Factor (EGF), heparin sodium salt, gelatin and hydrocortisone were purchased from Sigma-Aldrich, UK. Hank’s balanced salt solution, trypsin-EDTA, foetal bovine serum and penicillin/streptomycin were sourced from Invitrogen, UK. The EGM-2 bullet kits containing hydrocortisone, human endothelial growth factor, vascular endothelial growth factor, human fibroblastic growth factor-B, R3-insulin-like growth factor-1, ascorbic acid, gentamicin/amphotericin-b and heparin, were supplied by Lonza, UK. Lonza also provided HEPES buffered saline solution, trypsin and trypsin neutralisation buffer as a reagent kit for use with the EGM-2 media. Endothelial cell growth supplement was purchased from Millipore, UK.
The cell lines HMEC-1 (human microvascular endothelial cell-1) and EA.hy.926 were gifts from the University of Reading and Dr. Green at The University of Surrey respectively. The primary human coronary artery endothelial cells (HCAEC) were purchased from Lonza, UK.

2.2 Human intervention studies

Human studies were carried out in the Clinical Investigation Unit (CIU) of the University of Surrey with medical cover available during all participant attendance. Favourable ethical opinion from the Advisory Committee on Ethics of the University of Surrey was gained for both Pilot WISE (reference EC/2006/89/SBMS) and FITMA (reference EC/2007/73/FHMS) studies. Laboratory analysis was carried out in the Faculty of Health and Medical Sciences (FHMS) at the University of Surrey and St George’s Hospital.

2.2.1 Participant recruitment

Recruitment material for the Pilot WISE and FITMA trials can be found in appendix 3A and B respectively. Participants were recruited using poster advertisements placed in multiple sites on the campus of the University of Surrey and emailed to students and staff. These adverts were also placed in the local press to attract participants from the wider population. Both men and post menopausal women were recruited to the Pilot WISE trial (n = 19). Men only were used in FITMA to maintain homogeneity within a group of only n = 10. Inclusion criteria included a post-menopausal state for women, an age of 30 to 60 years for men and a Body Mass Index (BMI) of 25 to 35 kg/m² (please...
see section 2.2.3.1). A waist circumference greater than 34 inches (86 cm) for men or 31.5 inches (80 cm) for women was included in the posters as an inclusion criteria for the Pilot WISE trial. However, this was not found to be useful as participants were not found to have accurate knowledge of their waists and often did not have the equipment to accurately measure them. Therefore, this was not used as a recruitment criterion in the FITMA trial, although measurements were still taken and recorded. A BMI of greater than 25 kg/m² confers a greater risk of CVD than does a lower BMI (Kenchaiah S 2002), thus BMI was used as an inclusion criterion.

All potential participants completed a previously validated Health and Lifestyle Questionnaire (appendix 4) developed by the researchers for the purpose of this intervention. This was used, with a Food Frequency Questionnaire (FFQ), to screen out ineligible participants, such as habitual high consumers of whole grains. Any reporting a history of type II diabetes, cardiovascular disease or food intolerance were excluded from participation. Further exclusion criteria included:

- drinking more than 21 units of alcohol per week,
- undertaking regular sessions of vigorous exercise or fitness training (over three 30 minute aerobic exercise sessions per week),
- previous or current medical conditions such as heart disease, diabetes, gastrointestinal disorders, liver disease, depression, psychological disorders, eating disorders, drug or alcohol abuse,
- weight-reducing diets or if under dietary restriction,
- regularly including five or more servings of refined or whole grains per day,
• taking any prescription medicines or supplements within the past six months.

The previously validated Dutch Eating Behaviour Questionnaire (DEBQ, appendix 5) was designed in the light of increasing appreciation of the complexity of eating behaviour and the aetiology of obesity (van Strien T 1986). It was used in this research to screen out participants determined to be restrained or disordered eaters, those who display strong emotional or external eating cues and tendencies.

A haemoglobin monitor (201+Hb monitor, HemoCue, Sweden) was used during screening to ensure that any participants presenting symptoms of anaemia (as determined by a circulating haemoglobin level of less than 14g/l) were excluded from the study. Fresh blood was immediately placed onto an auto-analyser compatible cuvette which was inserted into the auto-analyser to give an instant reading of haemoglobin concentration as per manufacturer’s instructions.

2.2.2 Trial design and protocols

Both the Pilot WISE and the FITMA trials required participants to consume a specified amount of bespoke bread roll-like intervention products per day throughout the course of the trial. This delivery method, providing participants with their complete daily requirements in given portions, was aimed to promote compliance and monitoring.

The first intervention of the FITMA trial was preceded by a 14 day ‘run in’ diet, employed to minimise inter-individual variations at baseline. This was not considered necessary for the Pilot WISE trial as the number of participants was much greater, thus
minimising the effects of inter-individual differences without additional requirements for the participants.

2.2.2.1 The Pilot WISE trial protocol

The Pilot WISE trial was a randomised controlled parallel dietary intervention to investigate the effects of whole intact and whole milled grain products compared to a refined grain control product on risk markers of cardiovascular disease. Please see appendix 1A for composition and nutrition information of the intervention products. Participants’ levels of certain CVD risk markers were measured pre- and post-eight week interventions with one of these products. Please see figure 2.1 for a schematic of the time lines involved.
Figure 2.1: Outline and time course of the 8 week Pilot WISE trial. Men and women at risk of cardiovascular disease (men aged 30 – 65 years and post-menopausal women, BMI 25 – 35kg/m²) were screened and completed a 7 day diet diary, followed by the initial study day, the 8 week dietary intervention with one of the three intervention products (whole intact grain, whole milled grain or control product), then repeated the 7 day diet diary and the final study day.

A power calculation on the estimated fall in post-prandial insulin area under the curve of 20% between groups (which would be metabolically significant) with a standard deviation of 20% would give an estimate of 17 per group. As there is no previous study of this type in this area of research it was aimed to recruit 20 per group but with an interim calculation of 12 per group for the purposes of this, the pilot phase of the trial. This calculation was based on work by Frost et al in 1998 also investigating a primary end point of a 20% reduction in post-prandial insulin area under the curve. If the results of the pilot phase suggest a significant change may be achieved with a fully powered study, further participants will be recruited for a Main WISE trial if resources allow. Therefore, a further objective of the Pilot WISE trial was to determine the need for of a
Main and fully powered, WISE trial, by assessing the interim data for the possibility of a significant result.

Screening required a 12 hour fasted, morning visit to the CIU, University of Surrey, for freely given informed consent (see appendices 6 and 7 respectively for the Participant Information Sheet and Consent Form) and a haemoglobin blood test. Participants were asked to consent to GP notification of their participation (see appendix 8 for the GP letter). This safety measure was designed to enable the GP to contact the investigator should they feel participation would not be appropriate. Consented participants were randomly assigned via the online tool www.randomisation.com to one of the three study arms: whole intact grain, whole milled grain or control intervention products, stratified by sex only.

On the initial day of the intervention, day one, participants attended the CIU in the early morning, having consumed a standardised meal of their choice on the previous evening (to be repeated prior to the subsequent study day) chosen from a list of pre-prepared ‘ready meals’ with known macronutrient compositions. Repeating this meal before each study morning ensured standardisation between study days to minimize any last meal effects. Participants then fasted for 12 hours overnight. Participants refrained from strenuous exercise for 24 hours before study initiation. Participants were cannulated (Y-Can 21G with syringe valve, Beldico, Belgium) by a suitably trained individual to allow blood samples to be taken. Two 30ml fasting blood samples (at minus ten minutes and time zero), blood pressure readings and anthropometric measurements were taken. Participants then consumed a standard test breakfast of 250ml of a hot chocolate-like
drink (505.8 kcal, 18.9 g protein, 59.4 g carbohydrate and 21.4 g fat) containing neither whole nor refined grains (please see appendix 9 for further information). Half hourly 10 ml blood samples were then taken for three hours post intake. Participants were offered refreshment and a chance to rest and were free to leave with their first allocation of intervention products. Serum and plasma as appropriate were analysed for tissue-plasminogen activator-1, von-Willebrand factor, soluble intercellular adhesion molecule-1, C-reactive protein, interleukin-8, interleukin-6, full metabolite profiles, total cholesterol, HDL-cholesterol, triglycerides, non-esterified fatty acids, insulin and glucose.

Following this, participants were required to consume two portions daily of their assigned intervention product for the next eight weeks, with the active products providing 48 g whole grains per day (providing three portions daily of 16 g whole grains) as recommended by the Whole Grains Council, (www.wholegrainscouncil.org). The products were made available to the participants regularly and were either distributed at the CIU or delivered to the participants in a timely fashion to promote compliance. Participants were contacted fortnightly during the eight week intervention by a Registered Dietitian to answer any questions and to promote compliance. At the beginning of the eighth week (Day 49), participants were posted a food diary for recording their habitual intake. On the last day of the eight week intervention participants returned to the CIU for the post-intervention assessment day, a repeat of day one. Participants were remunerated for their time and inconvenience and all reasonable expenses incurred as a result of participation in the study were refunded on provision of a valid till receipt.
2.2.2.2 The FITMA trial protocol

The FITMA trial was a randomised controlled cross-over dietary intervention trial of two active dietary reference products, insoluble and soluble fibre, represented for the purposes of this research by wheat fibre and inulin respectively, and a control reference product. The lipid lowering effects of inulin observed in animals followed high doses, the equivalents of which would be unacceptably high in humans, due to the associated adverse gastrointestinal (GI) effects. The dose of 15g/day was chosen following C William’s Review on the lipid-lowering effects of Inulin feeding studies in animals and humans (Williams C 1999) as an optimized level to induce lipid lowering effects but minimize GI side effects. As such, the dose of 15g/day was also used for wheat fibre for the purposes of standardisation.

Screening for the FITMA trial required a 12 hour fasted, morning visit to the Clinical Investigation Unit of the University of Surrey, for an information session, freely given informed consent (please see appendices 10 and 11 respectively for the Participant Information Sheet and Consent form) and a haemoglobin blood test. If participants consented, a letter was sent to their GP outlining the study and the implications of participation.

The primary end-point of this study is a statistically significant change in insulin. Therefore, sample size calculations based on a change in insulin have been used. A sample size of 12 per group would have at least 80% power to detect a clinically significant drop in insulin of 20pM (a difference to detect of 25%) between the groups, assuming that the common standard deviation is 16pM or 20% and using a two sample t-
test with a 0.05 two sided significance level. The sample size allowed for 10% of the subjects to be non-evaluable in each treatment group. The estimate of the standard deviation was based on published data for subjects who took part in a Resistant Starch Study of a similar design conducted by Robertson et al (2005). Ten participants successfully completed the trial, thus power was achieved.

Participants were randomly assigned to the wheat fibre, inulin or control group for Stage 1 of the study using the on line randomization tool (www.randomization.com). Each stage lasted for 28 days. After Stage 1 was complete, the participants were assigned to a different intervention group for Stage 2, this process of rotation continued until all participants had completed all three interventions. Between each stage was a 'wash out' stage of an equal length of time, 28 days. Although it was not possible to determine whether the wash out period was effective, as baseline measurements were not taken prior to each successive intervention diet, it was reasoned that any carry over effects of the diet would be removed by following a washout of the same duration as the intervention. Participants were required to consume three servings per day (5g of active ingredient per serving, providing 15g in total per day) of the relevant intervention product.

Throughout the study, participants were supplied with the appropriate intervention products in a timely fashion to promote compliance. Hand over of the products occurred either at the CIU or at a place specified by the participant for delivery of the product. Extra portions were provided to cover any losses reported and participants were asked to return any surplus rolls. This encouraged compliance and alerted the investigators to any
problems with tolerance or missed portions. Products were delivered fresh to the CIU and frozen for storage and deliver to participants. Shelf life, once defrosted was approximately two days. For an outline of the protocol and time-line of events, please see figure 2.2.

![Diagram](image)

**Figure 2.2: Outline and time course of the FITMA protocol.** The 14 day run in diet was followed by each of the three 28 day intervention diets (wheat fibre, inulin and control) in turn, in a randomised order, with the 28 day wash out diets between them. Post-prandial (PP) study days, taking anthropometric measurements (height, body fat %, weight, waist, blood pressure) and blood samples for circulating markers and metabolic profiles, occurred at baseline and post-each intervention treatment.

Participants were asked to limit their whole grain consumption to less than three portions per day for the two week run-in diet prior to commencing the study, minimising
whole grain intake and thus individual variation at baseline. Participants refrained from strenuous exercise for 24 hours before the initiation of the study. On the initial study day, participants attended the CIU in the morning having consumed a standardised meal of their choice (to be repeated the evening before the remaining study days) the evening before and then fasted for 12 hours overnight. Blood pressure and anthropometric measurements were taken. Participants were cannulated (Y-Can 21G with syringe valve, Beldico, Belgium) by a suitably trained individual and two fasting 25ml blood samples were taken at minus 10 minutes and time zero. An oral glucose tolerance test (OGTT) was then carried out over the course of the following three hours (see section 2.2.3.4) in which participants consumed a standard glucose test drink (containing 75g of glucose in water) which did not contain either whole grains or fibre and 5ml blood samples were taken every 15 minutes for two hours post intake, then half hourly for the remaining hour. After this the cannula was removed and participants were offered refreshments and an opportunity to rest. Prepared serum and plasma samples (please see section 2.2.3.3) were stored and later analysed as appropriate for tissue-plasminogen activator-1, von-Willebrand factor, soluble intracellular adhesion molecule-1, C-reactive protein, interleukin-8, interleukin-6, full metabolite profiles total cholesterol, HDL-cholesterol, triglycerides, non-esterified fatty acids, insulin and glucose.

After the OGTT session, participants were given a 28 day supply of the relevant intervention product to consume over the following four weeks. Participants were free to follow their normal lifestyle and diet during this time with the inclusion of three servings of the intervention products per day in the place of their habitual grain products, including bread, pasta and cereals, so as not to affect the overall energy consumption. It
was requested that participants return prior to the beginning of each new intervention diet for a repeat of the baseline study visit but after discussions of the protocol feasibility with potential participants it was felt this would be too much of a burden on participants and would significantly adversely affect recruitment. Therefore, the initial baseline was used for comparison across the intervention diet. At the end of the 28 days, participants attended the CIU for a repeat of the initial study morning followed by an equal length (28 day) wash-out period aimed to remove any of the active component or its effects. Following the wash-out period, each participant began their 28 day intervention on the next product. This was ended with a study day to repeat the measurements taken in the previous study day for comparison. The cycle was then repeated with a 28 day wash out period and the consumption of the final investigational product for a further 28 days.

During the wash-out stages, no intervention products were provided to the participants and they were required to follow their habitual diets. The final study day ended participation. Participants were contacted regularly during the intervention stages by a Registered Dietitian (RD) to ensure any questions they had were answered, to promote compliance and to take a verbal 24 hour recall of diet.

All study mornings were identical in methodology and risk markers measured. All collected food diaries were reviewed by the RD with the participants. Participants were compensated for their time and inconvenience and all reasonable expenses incurred as a result of participation of the study were refunded on provision of a valid till receipt.
2.2.3 Plasma, serum, anthropometric and diet diary collection and storage

2.2.3.1 Anthropometric data collection

Height was measured using a stationary stadiometer. Participants removed their shoes and stood erect looking straight ahead in the horizontal plane and with feet together and knees to the front. Height was measured to the nearest 0.1 cm. Three readings were taken at baseline and averaged and no significant changes in height were assumed to occur over the course of the trial. The participant’s height was entered into the Tanita Bio-Impedance Body Composition Analyser TBF-300 (Tanita Europe BV, Netherlands) to allow for further anthropometric analysis.

Total body mass (kg) and body fat composition as a percentage of total body mass was measured and calculated using a Tanita analyser (the same instrument was used for each measurement on each study day) following the standardised procedures fully described in the literature. Participants were asked to urinate immediately prior to the measurement to remove excess body water which can adversely affect measurements. Participants wore light clothing, with empty pockets and no shoes or belts, wearing similar clothes on each study day to maintain consistency between measurements. An allowance was made of 1.5 kg for clothes’ weight. Participants removed their socks and shoes and were asked to step onto the foot plates of the Tanita analyser ensuring equal weight distribution between the feet and checking trousers were not beneath the heels thus reducing contact area. Measurements were taken to the nearest 0.1 kg. The Tanita analyser also calculated BMI, via the following equation:

\[
\text{BMI} = \frac{\text{weight (kg)}}{\text{height (m}^2\text{)}}
\]
A BMI of 25 to 30kg/m² (defined as ‘over-weight’) was a pre-requisite for both studies. Waist and hip measurements were taken at the navel and widest point around the buttocks respectively by an RD to the nearest 0.5cm using a flexible tape measure, with participants standing erect as for height measurements, with the arms relaxed at the side of the body and the participant facing straight ahead.

2.2.3.2 Dietary intake

Habitual dietary intakes were recorded using previously validated three or seven day diet diaries. The RD explained diary completion to participants and analysed the completed diaries using WinDiets (WinDiets Research, 2005, Scotland) to determine the effect of the interventions on the habitual dietary intake of the participants and monitor compliance. Compliance was further monitored by giving each participant an exact number of surplus servings of each intervention product than required for the study period and asking them to return any remaining unused with discrepancies being counted and recorded. Participants were also asked on each visit if they had consumed all, or missed any, of the portions.

2.2.3.3 Blood collection

Venous blood was drawn from an intravenous cannula placed in the antecubital vein in the forearm of participants by a suitably trained phlebotomist. Blood was taken into the following specific BD vacutainer® tubes: EDTA (dipotassium ethylenediaminetetraacetic acid, K₂EDTA) tubes for total cholesterol, LDL, TAGs, NEFA and insulin determination in plasma; SST™ tubes for determination of
inflammatory markers and metabolomic profile in serum; fluoride tubes (containing sodium fluoride and potassium oxalate) for determination of glucose.

SST™ tubes were inverted three times, incubated at room temperature for 30 minutes and centrifuged at 1750 x g for 10 minutes at 4°C. All other tubes were inverted three times and were centrifuged immediately at 1750 x g for 10 minutes at 4°C. Supernatants, (serum and plasma) were removed using Pasteur pipettes and aliquoted into 2ml microtubes for storage (at -20°C for plasma samples and -80°C for serum samples) for future analysis as appropriate.

2.2.3.4 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) is a provocation test used by the World Health Organisation (WHO) in the primary diagnosis of diabetes, by examining the efficiency of the body at processing glucose. Participants were prepared for the OGTT as stated by WHO guidelines for adults as follows:

- three days unrestricted carbohydrate rich diet and activity,
- no medication on the day of the test,
- 12 hours fasted,
- no smoking,
- glucose load of 75g in 300 to 400ml water.

Blood samples (5ml) were taken for baseline at minus 10 minutes and time zero. Further 5ml blood samples were taken at 15 minute intervals up to, and including, 120
minutes, then every 30 minutes for one hour. Glucose concentration in plasma was the
determined on an iLab 650 and the method outlined in section 2.2.4.2.

2.2.4 Circulating markers of vascular health

Markers chosen for analysis were as follows. The haemostatic factors tissue
plasminogen activator-1 because of its critical role in the immune response to injury and
infection within the vasculature (Kumar V 2003; Majno G 2004) and its relationship with
increased CHD risk (Gram J 2000), and von Willebrand factor due to its role in blood
coagulation and its proven status as a risk marker for CHD (Meade T 1994; Thompson S
1995). The inflammatory factor soluble intercellular adhesion molecule-1 was
investigated because of its role in leukocyte adhesion to the endothelium (Yang L 2005).
IL-6 was chosen because of its involvement of the proliferation and maturation of
granulocytes which is upregulated during the immune response and its regulation of CRP
production (Pepys M 2003); IL-8 was investigated as it is responsible for the activation
and chemoattraction of neutrophils to the site of inflammation. CRP was investigated due
to its role in inflammation via the induction of ICAM-1 and VCAM-1 (Pasceri V 2000)
and its suggest role as a risk marker for CVD (Ridker P 2000) and CHD (Koenig W
1999; Danesh J 1998). The circulating lipid profiles of total, HDL and LDL cholesterol,
and TAG were investigated because of their proven associations with CHD (Gordon T
1997, Assman G 1999 and Hokanson J 1996 respectively). NEFA levels have also been
proposed as relating to CVD risk and were investigated as such (Semenkovic C 2004).
Markers of insulin sensitivity, fasting and post-prandial insulin and glucose, were
investigated as insulin resistance is a known risk marker for CVD (Hsueh W 1998; Ruige
J 1998).
2.2.4.1 Haemostasis

Tissue plasminogen activator-1

An in-house ELISA was used to measure circulating levels of tissue plasminogen activator-1 (tPA-1) in serum samples. The 96 well, flat bottomed NUNC Immunolon ELISA plates were coated with 50µl/well mouse mouse anti human-tPA-1 at 0.83µg/ml in 0.1M Na₂HPO₄ pH 9.0 and incubated overnight at 4°C. Plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T), blocked with 150µl/well PBS containing 2% bovine serum albumin (PBS-BSA) and incubated for 60 minutes at room temperature. Plates were washed four times with PBS-T containing 0.2% BSA (PBS-T-BSA) then 50µl/well standards (diluted in PBS-T-BSA) over a range of 30 to 0.01ng/ml or samples (diluted 1/2 in PBS-T-BSA) were added in triplicate. Plates were incubated overnight at 4°C and washed four times with PBS-T-BSA. Fifty µl/well 1.0µg/ml peroxidase conjugated sheep anti-human tPA-1 antibody (diluted in PBS-T-BSA) was added and plates were incubated for 60 minutes at room temperature. Plates were washed four times with PBS-T-BSA and 50µl/well 2.0µg/ml horseradish peroxidase conjugated-avidin (avidin-HRP) diluted in PBS-T-BSA was added and plates were incubated for 30 minutes at room temperature and washed three times in PBS-T, twice in PBS and twice in citrate phosphate buffer. Tetramethylbenzidine (TMB, 0.1mg/ml in 0.05M citrate phosphate buffer, pH5.0) at 50µl/well was added to allow colour to develop, followed by the addition of 12.5µl/well 2M H₂SO₄ to stop the reaction and the absorbance of the solution was read at 450nm on a BioTek® ELx800 microplate reader (BioTek®, USA). The inter-assay coefficient of variance for this in-house ELISA is routinely 8-10% (personal communication, Dr Bodman-Smith).
von Willebrand factor

An in-house ELISA was used to measure circulating levels of von Willebrand factor (vWF) in serum samples. ELISA plates were coated with 50μl/well 2.0μg/ml rabbit anti-human vWF in 0.1M BIC buffer and incubated overnight at 4°C. Plates were washed four times with PBS-T, blocked with 150μl/well PBS-BSA and incubated for 60 minutes at room temperature. Plates were washed four times with PBS-T-BSA and 50μl/well standards over the range of 1.0 to 0.001IU/ml in PBS-T-BSA or samples (diluted 1/2 in PBS-T-BSA) were added in triplicate. Plates were incubated overnight at 4°C. Plates were washed four times with PBS-T-BSA then 50μl/well 0.125μl/ml rabbit anti-human-vWF-HRP (in PBS-T-BSA) was added and plates were incubated for 60 minutes at room temperature. Plates were washed three times in PBS-T, twice in PBS and twice in citrate phosphate buffer (pH5.0). TMB (50μl/well) was added to each well followed by 12.5μl/well 2M H₂SO₄ and the absorbance of the wells was read at 450nm. The inter-assay coefficient of variance for this in-house ELISA is routinely 6-8% and quality controls of known concentration were included (personal communication, Dr Bodman-Smith).

2.2.4.2 Markers of inflammation

Soluble intracellular adhesion molecule-1

Levels of circulating soluble intracellular adhesion molecule-1 (sICAM-1) in participants’ serum were quantified using a Quintikine® Human sICAM-1/CD54 Immunoassay kit according to the manufacturer’s instructions. Briefly, 100μl sICAM-1 horseradish peroxidase conjugated anti-sICAM-1 monoclonal antibody was added to mouse monoclonal anti-sICAM-1 antibody coated wells. One-hundred μl/well standard,
control (provided lyophilised recombinant human sICAM-1 in buffer with preservatives) or serum sample (diluted 1/20) was added and the plate was incubated at room temperature on an orbital shaker (GFL 3005 Orbital Shaker) at 500rpm for 90 minutes. Wells were aspirated and washed four times with 400µl wash buffer. Two-hundred µl/well of the reaction substrate, a 1:1 mixture of stabilized hydrogen peroxide and stabilized tetramethylbenzidine (acting as a chromogen) was added. The plate was protected from light and incubated at room temperature for 30 minutes. Fifty µl/well of 2M sulphuric acid was added. The intensity of each well was read at 450 nm (reference filter 540 nm).

**Interleukin-6 and interleukin-8**

Serum interleukin-6 (IL-6) and interleukin-8 (IL-8) concentrations were determined using the human ultrasensitive cytokine 10-plex panel for GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ and TNF-α according to manufacturer’s instructions. Briefly, 200µl/well working wash solution was used to pre-wet plates (30 seconds at room temperature) and was removed by aspiration. Twenty-five µl/well protein-specific capture antibody conjugated bead solution, with beads of defined spectral properties, was added. From this stage, plates were protected from light. Plates were washed twice with working wash solution as above. One hundred µl/well of appropriate standards and samples (diluted ½ with assay diluent) was added and incubated for two hours at room temperature on an orbital shaker (500 to 600rpm). The liquid was aspirated and the plates washed twice. One hundred µl/well biotinylated detector antibody was added and incubated for one hour at room temperature on an orbital shaker (500 to 600 rpm). The liquid was aspirated and the plate was washed twice. One hundred µl/well
streptavidin-RPE was added and incubated for 30 minutes at room temperature on an orbital shaker (500 to 600 rpm). After three further washes, 100μl/well working wash was added and plates were read on a Luminex® 100™ instrument.

C-reactive protein

An in-house high sensitivity ELISA was used to measure circulating levels of CRP in serum samples. ELISA plates were coated with 50μl/well rabbit mouse anti-human-CRP at 2μg/ml in 0.1M BIC buffer and incubated overnight at 4°C. Plates were washed four times with PBS-T, blocked with 150μl/well PBS-BSA and incubated for 60 minutes at room temperature. Plates were washed four times with PBS-T then 50μl/well standards (diluted in PBS-T-BSA) over a range of 0.3 to 0.00001μg/ml or samples (diluted 1/100 in PBS-T-BSA) were added in triplicate. Plates were incubated for 60 minutes at 37°C and washed four times with PBS-T. Fifty μl/well 0.325μg/ml peroxidase conjugated rabbit anti-human CRP antibody (diluted in PBS-T-BSA) was added and plates were incubated for 60 minutes at 37°C. Plates were washed three times with PBS-T, once in PBS and twice in citrate phosphate buffer. TMB (50μl/well) was added, followed by 12.5μl/well 2M H₂SO₄ and the absorbance was read at 450nm. The inter-assay coefficient of variance for this in-house ELISA is routinely 6 to 10% (personal communication, Dr Bodman-Smith).

2.2.5 Metabolomics

Fifty μl defrosted serum samples (stored at -80°C) were vortexed for two minutes with 200μl chilled (-80°C) methanol and incubated at 4°C for 30 minutes to precipitate
proteins. Samples were centrifuged at 1800 x g for 10 minutes and supernatants were transferred to high pressure liquid chromatography (HPLC) vials for analysis.

Chromatography was performed on a Waters Acquity UPLC™ system (Waters, UK) using an Acquity BEH C-18 column (2.1 x 30mm, 1.7µm, Waters, UK). The column oven and autosampler temperatures were 40°C and 4°C respectively. The mobile phases were: (A) 0.1% formic acid in water, and (B) acetonitrile with 0.1% formic acid. The linear gradient program was: 100% (A) for 0.5 minutes, a linear increase to 100% (B) over 8.5 minutes, a switch back to 100% (A) and maintenance for one minute. This had a total cycle time of 10 minutes, a flow rate of 600µl/min and an injection volume of 5µl, using partial loop mode.

Mass spectrometry was carried out on a Waters Mircomass QToF Premier™ system (Waters, UK) operating in both positive and negative ion electrospray modes. The source and the desolvation temperatures were 120°C and 450°C respectively, with nebulisation gas set to 800l/h and collision energy set to 4eV. The capillary and cone voltages were: 3.1kV and 30V respectively in positive ionisation mode, and 3.0kV and 35V respectively in negative ionisation mode. A LockSpray™ interface was used to ensure mass accuracy. Leucine-enkephalin was infused at 200pg/µl at a flow rate of 10µl/min.

Data was visualized and analysed using Waters MassLynx (V 4.1) and Waters MarkerLynx (4.1). Background ions identified in associated blank injections were excluded. The mass and retention time windows were set at 0.05 Da and 0.1m
respectively. Multivariate analysis was performed and visualized using SIMCA-P (V12, Umetrics, Sweden). The dataset was ‘autofit’ and visualized by principal component analysis (PCA) to highlight any differences between groups. Further analysis was performed to visualise discrimination between the sample groups. Six and four group partial least squares discrimination analyses (PLS-DA) were used for the Pilot WISE and FITMA trials respectively to further investigate discrimination between groups. Where appropriate, a PLS-DA loadings plot and a variable importance on projection (VIP) plot of the first component were used to determine any discriminatory species between groups. Any such species were investigated, through their single ion chromatograms (SIC) and mass spectra, to remove false positives and identify the parent ions of any adducts. The signal intensities of those species found to be true ions were analysed outside the analysis platform for significant differences and for any species found to be significantly different between groups, elemental composition analysis was performed using Masslynx. Such ions were then identified putitatively using the metabolite databases (Human Metabolite Database, www.hmdb.ca; METLIN, metlin.scripps.edu; KEGG, www.kegg.com).

2.2.6 Measurement of circulating markers of CVD risk

As a reflection of CVD risk, participant’s blood was analysed at baseline and after each intervention treatment for a number of circulating risk markers.
2.2.6.1 Circulating lipids

Total cholesterol

Plasma total cholesterol concentrations were measured using an iLab 650 auto-analyser and an IL Test™ cholesterol kit (Instrumentation Laboratory, Italy) following manufacturer’s instructions, via end point bichromatic analysis, with measurements taken at primary and blanking wavelengths of 510 and 700nm respectively.

High density lipoprotein cholesterol

Plasma high density lipoprotein (HDL) cholesterol concentrations were measured using an iLab 650 auto-analyser (Instrumentation Laboratory, Italy) using a Randox, UK direct HDL cholesterol kit according to manufacturer’s instructions, via a two step enzymatic colourimetric method read at 600nm.

Triacylglycerides

Plasma triacylglyceride (TAG) concentrations were measured using an iLab 650 auto-analyser using an IL Test™ triacylglyceride kit (both from Instrumentation Laboratory, Italy) via an enzymatic analysis at 510nm.

Low density lipoprotein

Low density lipoprotein (LDL) concentrations were calculated from the measured values of total cholesterol, HDL cholesterol and TAG via the Friedewald equation (Friedewald W 1972):

\[
LDL \text{ cholesterol} = \text{total cholesterol} - (\text{HDL cholesterol} + (0.2 \times \text{TAG}))
\]
Non-esterified fatty acids

Plasma non-esterified fatty acids (NEFA) concentrations were measured via an iLab 650 auto-analyser (Instrumentation Laboratory, Italy) using an Randox, UK, direct non-esterified fatty acid kit according to manufacturer’s instructions, via a colorimetric principal, at 550nm.

2.2.6.2 Markers of insulin sensitivity

Insulin

Insulin concentrations were determined using a human insulin specific radio-immuno assay (RIA) according to the manufacturer’s instructions. Assay buffer was added to the relevant tubes: 300µl to the non-specific binding (NSB) tubes, 200µl to the reference (Bo) tubes and 100µl to the sample tubes. One hundred µl/tube standards and quality controls were aliquoted in duplicate. One hundred µl/tube hydrated $^{125}$-insulin was added followed by 100µl/tube human insulin antibody. All tubes were vortexed, covered and incubated overnight at room temperature. One ml/tube 4°C precipitating reagent was added, tubes were vortexed, incubated for 20 minutes at 4°C and centrifuged at 4°C for 20 minutes at 2500g. Supernatants were decanted and tubes were drained for 30 seconds. Any excess liquid was blotted from the lips of tubes. Samples were analysed and insulin concentrations calculated in a 1470 Wallac Wizzard gamma counter with Multicalc level 4.M automated data reduction procedure software (Wallac International, Finland).

Glucose

Plasma glucose concentrations were measured on an iLab 650 auto-analyser (Instrumentation Laboratory, Milan, Italy) and an assay kit following the manufacturer’s
instructions. The glucose contained within the sample oxidised with oxygen and water in
the presence of the enzyme glucose oxidase resulting in production of gluconic oxide and
hydrogen peroxide. The quinoneimine concentration of the sample was then determined
at 510nm.

**Area under the curve**

The areas under the curve (AUC) of the post-prandial study interventions were
calculated using the trapezoid rule: area = width x (h1 + h2)/2, where h1 = height of side
1 and h2 = height of side 2.

**Insulin and glucose C-max and T-max**

The peak concentrations (C-max) of insulin and glucose reached over each post-
prandial study were calculated as a mean average of each individual’s peak
concentration, within each intervention treatment.

The time taken to reach the peak insulin and glucose concentrations (T-max) over
the course of each post prandial study were calculated as the mean average of each
individual’s time to peak concentration, within each intervention treatment.

**2.2.7 Cell culture methods**

**2.2.7.1 Cell media**

EA.hy.926 cells were cultured in Dulbecco’s Modified Eagle Media (DMEM), a
high glucose media containing glutamax-1, with added 10% heat-inactivated foetal
bovine serum (FBS), penicillin/streptomycin (pen/strep) at 100U/ml and 100µg/ml
respectively and HAT supplement (sodium hypoxanthine 0.1µM, animopterin 0.4µM and
thymidine 16.0µM) a post-fusion selective medium. HMEC-1 cells were cultured in DMEM with added 10% heat-inactivated foetal bovine serum (FBS), penicillin/streptomycin (pen/strep) at 100U/ml and 100µg/ml respectively, 1µg/ml hydrocortisone and 10ng/ml epidermal growth factor. HCAECs were cultured in EGM®-2MV containing the recommended bullet kit additives of: hydrocortisone, endothelial growth factor, vascular endothelial growth factor, fibroblastic growth factor-B, R3-insulin-like growth factor-1, ascorbic acid, heparin and gentamicin/amphotericin-B at the recommended concentrations.

2.2.7.2 Cell culture maintenance

Methods employed were similar for each cell type, with differences outlined below. Cell pellets were frozen down in liquid nitrogen in 90% FBS and 10% dimethyl sulphoxide. Vials were brought up for use by rapid defrosting by immersion in a 37°C water-bath. Using aseptic technique, 37°C media was then added drop-wise to the cells. Cells were plated at the appropriate seeding density in small cell culture flasks.

Cell detachment

Adherent endothelial cells were detached for sub-culture and seeding as follows. Cells were washed with 37°C magnesium and calcium free Hanks’ balanced salt solution. Two ml/25cm² 0.05% trypsin-EDTA in Mg²⁺ and Ca²⁺ free HBSS was added and incubated for the following times by cell type: HMEC-1 2 to 5 minutes, HAAECs 5 to 10 minutes and HCAECs 2 to 6 minutes. Once cells were fully detached from the flask an equal volume of the relevant media was added to halt trypsinisation. Cells were transferred to 50ml tubes and centrifuged at 240 x g at 4°C for 5 minutes. The supernatant
containing the trypsin and media was discarded and cells were resuspended in medium and re-centrifuged as above as a wash step. The supernatant was discarded and cells were resuspended in the relevant fresh culture medium, counted using a modified Neubauer haemocytometer and plated at the required densities.

**Subculture**

EA.hy.926 and HMEC-1 cells were seeded at $10^5$/ml weekly for sub-culture and at $1 \times 10^5$/ml for treatment. HCAECs were seeded at $2.5 \times 10^4$/ml for subculture and at $1 \times 10^5$/ml for treatment. Each cell type was confluent for treatment after a 72 hour incubation.

### 2.2.7.3 Cell treatment

**Reagent preparation**

Tumour necrosis factor-α and interferon-γ were provided as freeze-dried residues of 1ml solutions containing either natural BALL-1 cell derived human tumour necrosis factor-α or human leukocyte derived interferon-γ respectively. The contents of each ampoule, (approximately 46,500IU TNF-α and 3000IU IFN-γ), was dissolved in 0.5ml sterile, distilled water and transferred separately to sterile tubes. Ampoules were rinsed with 0.4ml sterile, distilled water which was also transferred to the relevant tube and the volume of each was made up to 1ml, giving concentrations of 46,500IU/ml TNF-α and 3000IU/ml IFN-γ. These stock solutions were aliquoted and stored at -80°C. Upon use, aliquots were diluted to the required concentrations in the relevant media.
Investigational compounds

Fresh 1M stock solutions of both sodium acetate and sodium propionate were filtered through 0.45μm filters and used at final concentrations of 0.1, 1.0, 10 and 100μM to cells pretreated with TNF-α/IFN-γ for 24 hours.

2.2.7.4 Cell culture analysis methods – cell surface markers

Levels of expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) were determined by fluorescence activated cell scanning (FACS) referred to in future text as flow cytometry. These markers were chosen for analysis because ICAM-1 and VCAM-1 are instrumental in the leukocyte-endothelial cell attachment and PECAM-1 is involved in the endothelial cell-endothelial cell attachment controlling migration out through the endothelium; thus they play important roles in plaque development and altered levels may be associated with risk of CVD. Fresh cells were harvested (as described in section 2.2.7.2) and washed in ice cold FACS buffer. Cells were incubated with the relevant fluorochrome-labeled monoclonal antibody stains and controls (table 1.1) and made up to a final volume of 30μl/stain with ice-cold FACS buffer, protected from light, at room temperature for 45 minutes at 4°C. Cells were washed twice in ice cold FACS buffer and resuspended in 200μl 2% paraformaldehyde. Fixed cells were examined using a Becton Dickinson FACScan flow cytometer and data were analysed using WinMDI 2.8 software (Microsoft). Cell populations were gated by size and granularity.
Table 1: Per well concentration of each antibody stain used for flow cytometry analysis. Cells were stained using fluorescently labeled antibodies for determination of surface levels of the cellular adhesion molecules ICAM-1, VCAM-1 and PECAM-1 via FACS; μg/well are indicated and each antibody was made up to 30μl/well with ice cold FACS buffer.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mg/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained control</td>
<td>-</td>
</tr>
<tr>
<td>Iso-PE</td>
<td>0.6</td>
</tr>
<tr>
<td>ICAM-1-PE</td>
<td>0.6</td>
</tr>
<tr>
<td>Iso-FITC</td>
<td>0.5</td>
</tr>
<tr>
<td>VCAM-1-FITC</td>
<td>0.5</td>
</tr>
<tr>
<td>PECAM-1-FITC</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2.2.7.5 Cell culture analysis methods – secreted haemostatic markers

tPA-1 was investigated because of its role in clot dissolution. It has previously been associated with increased CHD risk (Gram J 2000) and acute myocardial infarction (Thogerson A 1998). vWF is considered an established risk marker for CHD and was investigated as such (Meade T 1994; Thompson S 1995).

Tissue plasminogen activator-1 and von Willebrand factor

Levels of tPA-1 and vWF in media were determined via in-house ELISA as outlined in section 2.2.5.1 above.
2.2.7.6 Cell culture analysis methods – secreted inflammatory markers

IL-8 was investigated because of its role in firm attachment of rolling monocytes to the endothelium (Gerszten R 1999) and as it has been suggested as a possible risk marker for CVD (Herder C 1006) and cardiovascular events (Inoue T 2008). NO is vital in maintaining haemostasis and has a protective effect on the endothelium (Gibbons G 1996); as such it has been suggested that altered levels are associated with CVD risk and so this marker was also investigated.

Interleukin-8

An in-house ELISA was used to measure secreted IL-8 in the cell culture supernatants. Briefly, ELISA plates were coated with 50μl/well 0.5μg/ml mouse anti-human IL-8 antibody in BIC buffer and incubated overnight at 4°C. Plates were washed four times with PBS-T, blocked with 150μl/well PBS-BSA and incubated for 60 minutes at room temperature. Plates were washed four times with PBS-T-BSA. Fifty μl IL-8 standard (diluted in PBS-T-BSA) over a range of 100 to 0.03μg/ml, and samples (diluted 1/2 in PBS-T-BSA) were added in triplicate to the relevant wells. Plates were incubated overnight at 4°C. Plates were washed four times with PBS-T-BSA. Fifty μl/well 0.25μg/ml biotinylated mouse anti-human-IL-8 antibody in PBS-T-BSA was added and plates were incubated for 60 minutes at room temperature followed by four washes with PBS-T-BSA. Fifty μl/well 2.0μg/ml Avidin-HRP in PBS-T-BSA was added and plates were incubated for 30 minutes at room temperature then washed three times in PBS-T, twice in PBS and twice in citrate phosphate buffer. Fifty μl/well 0.1M tetramethylbenzidine (TMB), in citrate phosphate buffer, was added to each well. To stop the reaction 12.5μl/well 2M H₂SO₄ was added and the absorbance of the solution was
read at 450nm. The inter-assay coefficient of variance for this in-house ELISA is routinely 5 to 8% (personal communication, Dr Bodman-Smith).

Nitric oxide

The Griess reagent kit employs the diazotization reaction to spectrophotometrically detect nitrite as a marker of nitric oxide. Samples or standards (150μl), 130μl de-ionised RO water and 20μl Griess reagent (equal volumes of N-(1-naphthyl) ethylenediamine and sulfanilic acid) were incubated in duplicate for 30 minutes in 96 well plates. Spectrophotometric absorbance was measured at 550nm.

2.2.7.7 Cell culture analysis methods – cell viability

Mitochondrial dehydrogenase activity, which reduces 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a purple formazan in active mitochondria, was used to determine cell survival in a colorimetric assay as per manufacturer’s instructions (MTT assay, ViaLight®, Lonza). Cell viability was calculated using the following equation:

\[
\text{Cell viability} = \frac{\text{Absorbance (treated sample)}}{\text{Absorbance (medium treated sample)}} \times 100
\]

2.3 Statistical analysis

The statistical analysis package, GraphPad Prism® version 5.01 (GraphPad, USA), was used for all data and statistical analysis. Due to their biological nature and the small values of n, data were considered to follow a Gaussian distribution for the purposes of analysis. In the Pilot WISE trial, paired Student’s t-tests were used to determine
the significance of any differences within groups, pre- to post-intervention for all correlations. In the FITMA trial, a repeated measures analysis of variance (ANOVA) with a Bonferroni’s multiple comparison post-hoc test was used to determine any significant differences between treatment interventions.

In the *in vitro* work, the nitrite time course was analysed using a Student’s t-test to determine any significant differences between the nitrite levels at each time point and treatment. Two way ANOVA was used to look for any effect of the treatments on the expression and/or secretion of endothelial markers. Statistical significance was assumed if p<0.05.
Chapter 3

The Pilot WISE trial

A pilot randomised controlled parallel dietary intervention trial investigating the effects of high whole intact grain and whole milled grains on risk markers of cardiovascular disease in at risk individuals
3.1 Study rationale and aims

The pathology of CVD involves the inflammatory condition atherosclerosis, the build up of fatty plaques within the artery wall and the dysfunction of the vascular endothelial functions: vasodilation, thrombosis, haemostasis and tissue perfusion (Rush J 2005). Known risk markers include a chronic inflammatory state, insulin resistance and dyslipidaemia. Many CVD risk markers are non-modifiable (e.g. age, sex and ethnicity) but certain factors are modifiable, with dietary interventions posed as a non-invasive method of reducing risk. High whole grain diets have a history of association with reduced CVD risk. Most evidence, however, is observational. The aim, therefore, of the Pilot Whole grain Intervention Study in England (Pilot WISE) trial was to investigate the impact of whole grains by providing a high whole grain diet via a randomised controlled parallel dietary intervention trial and monitoring CVD risk markers for changes. This was assessed in at risk men and post-menopausal women (aged 48.4 ± 11.7 years, BMI 28.8 ± 3.1kg/m²) as their baseline levels of CVD risk markers would be more responsive to an intervention than those of low risk individuals (figure 3.1). The delivery method was unusual as interventions were provided in bespoke bread-like products to be substituted into the diet in the place of the habitually consumed grain products such as bread, cereals and pasta. Eligible, consented participants were randomised in a ratio of 1:1:1, stratified by sex only, to receive an eight week dietary intervention of whole intact or whole milled grain or the refined control grain products. Participants consumed two portions daily (providing 48g whole grain as recommended by the Whole Grains Council) of their intervention product. End points covered a range of markers associated with endothelial function, dysfunction and whole body risk and were measured (fasting and post-prandially) pre- and post-each intervention treatment.

This chapter contains the products of joint research between myself, Laura Tripkovic and Caroline Bodinham.
Figure 3.1: Pilot WISE trial protocol. The Pilot WISE trial was a randomised controlled parallel dietary intervention trial involving participants at risk of CVD (aged 30 – 65 years, BMI 25 – 35 kg/m²). Screening including food frequency questionnaires (FFQ to exclude those with a high intake of whole grains) and Dutch eating behaviour questionnaires (DEBQ) were followed by randomisation to one of three, eight-week intervention treatments of whole intact grain, whole milled grain or control intervention product. Study days involving anthropometric and fasting and post prandial blood measurements were carried out before and after the intervention.
The author worked in equal collaboration with Drs L. Tripkovic and C. Bodinham on the Pilot WISE trial. The author was responsible for one third of all activities including, but not limited to: trial design, authorship of trial paperwork (including protocol, participant information sheets, recruitment paperwork and Trial Master File), informed consent sessions, management of participants’ completion of paperwork including FFQ and Health and Lifestyle Questionnaire, phlebotomy, participant management and care, sample preparation, storage and analysis. The author was not responsible for the collection or analysis of the diet diary data; this was carried out by Dr L. Tripkovic. The author was entirely responsible for all other data analysis presented; data analysis was not carried out in collaboration.

Markers of vascular haemostasis and inflammation such as tissue plasminogen activator-1 (tPA-1) and von Willebrand factor (vWF), soluble intra-cellular adhesion molecule-1 (sICAM-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and C-reactive protein (CRP), have been demonstrated to vary with inflammatory status and CVD pathology and were, therefore, chosen as useful indicators of endothelial health.

Metabolite profiles, as determined via liquid chromatography/mass spectrometry (LC/MS), reflect the current status of the metabolome as a snap-shot in time and, as such, provided a novel approach to determine the effects of whole grain on metabolic pathways and metabolite profiles possibly identifying diet-specific biomarkers.
Levels of the circulating lipids have a well established link with CVD, as do insulin and glucose with respect to type II diabetes, which itself is a strong risk marker for CVD. Thus, these factors provided important surrogate markers for the risk of CVD.

It was hypothesised that levels of risk markers would vary to reflect a lower CVD risk state post-intervention with the active high whole intact and milled grain diets, when compared to the baseline and the control refined grain diet.

3.2 Pilot WISE trial anthropometric and dietary data

Anthropometric data

Anthropometric measurements were taken at the beginning of each study day. No significant differences were seen in any measurements when comparing pre- to post-intervention with any of the three dietary interventions.
<table>
<thead>
<tr>
<th>Control</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age/years</strong></td>
<td>45.9 ± 12.2</td>
<td>45.1 ± 12.2</td>
</tr>
<tr>
<td><strong>Weight/kg</strong></td>
<td>86.5 ± 13.7</td>
<td>86.8 ± 13.4</td>
</tr>
<tr>
<td><strong>BMI/㎏/m²</strong></td>
<td>28.3 ± 3.1</td>
<td>28.5 ± 3.1</td>
</tr>
<tr>
<td><strong>Waist circumference/cm</strong></td>
<td>101.7 ± 8.4</td>
<td>101.4 ± 7.7</td>
</tr>
<tr>
<td><strong>Body fat/% of total body weight</strong></td>
<td>29.6 ± 8.2</td>
<td>29.5 ± 8.1</td>
</tr>
<tr>
<td><strong>Systolic BP/mmHg</strong></td>
<td>120.9 ± 10.5</td>
<td>123.6 ± 13.2</td>
</tr>
<tr>
<td><strong>Diastolic BP/mmHg</strong></td>
<td>71.9 ± 5.6</td>
<td>79.6 ± 6.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Whole Intact Grain</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age/years</strong></td>
<td>51.4 ± 4.8</td>
<td>51.8 ± 4.8</td>
</tr>
<tr>
<td><strong>Weight/kg</strong></td>
<td>90.9 ± 22.2</td>
<td>90.8 ± 22.1</td>
</tr>
<tr>
<td><strong>BMI/㎏/m²</strong></td>
<td>28.9 ± 3.9</td>
<td>28.9 ± 3.8</td>
</tr>
<tr>
<td><strong>Waist circumference/cm</strong></td>
<td>103.6 ± 13.1</td>
<td>101.8 ± 12.6</td>
</tr>
<tr>
<td><strong>Body fat/% of total body weight</strong></td>
<td>32.4 ± 5.6</td>
<td>32.7 ± 6.1</td>
</tr>
<tr>
<td><strong>Systolic BP/mmHg</strong></td>
<td>132.1 ± 7.9</td>
<td>125.4 ± 10.3</td>
</tr>
<tr>
<td><strong>Diastolic BP/mmHg</strong></td>
<td>82.4 ± 9.5</td>
<td>78.4 ± 6.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Whole Milled Grain</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age/years</strong></td>
<td>45.4 ± 16.7</td>
<td>45.7 ± 16.1</td>
</tr>
<tr>
<td><strong>Weight/kg</strong></td>
<td>92.1 ± 17.1</td>
<td>93.7 ± 16.1</td>
</tr>
<tr>
<td><strong>BMI/㎏/m²</strong></td>
<td>29.5 ± 2.8</td>
<td>30.0 ± 2.7</td>
</tr>
<tr>
<td><strong>Waist circumference/cm</strong></td>
<td>102.11 ± 12.0</td>
<td>102.8 ± 8.9</td>
</tr>
<tr>
<td><strong>Body fat/% of total body weight</strong></td>
<td>31.6 ± 6.4</td>
<td>31.0 ± 7.1</td>
</tr>
<tr>
<td><strong>Systolic BP/mmHg</strong></td>
<td>128.7 ± 10.3</td>
<td>129.1 ± 12.3</td>
</tr>
<tr>
<td><strong>Diastolic BP/mmHg</strong></td>
<td>77.0 ± 3.7</td>
<td>72.0 ± 6.2</td>
</tr>
</tbody>
</table>

Table 2: Pilot WISE trial anthropometrics. Age, weight, body mass index (BMI), waist circumference, body fat percentage and systolic and diastolic blood pressure (BP) data are separated to pre- and post- each dietary intervention of control (n = 6), whole intact grain (n = 6) and whole milled grain (n = 5). Data displayed as mean ± standard deviation.

Diet diary data

Seven day diet diary data (post-intervention data including the intervention products) were collected during each intervention. Data was collected and analysed by Dr. L. Tripkovic, University of Surrey. No significant differences were observed either at baseline between the intervention groups or following any of the interventions.
<table>
<thead>
<tr>
<th>Control</th>
<th>Pre-intervention</th>
<th>Post-intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories/kcal</td>
<td>2094.7 ± 378.9</td>
<td>2348.7 ± 450.6</td>
</tr>
<tr>
<td>Fat/g</td>
<td>66.1 ± 15.3</td>
<td>79.7 ± 26.3</td>
</tr>
<tr>
<td>Saturated fatty acids/g</td>
<td>22.3 ± 4.6</td>
<td>28.3 ± 11.7</td>
</tr>
<tr>
<td>Non-starch polysaccharides/g</td>
<td>15.1 ± 5.1</td>
<td>14.6 ± 4.6</td>
</tr>
<tr>
<td>Protein/g</td>
<td>81.2 ± 31.5</td>
<td>84.5 ± 24.6</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids/g</td>
<td>11.4 ± 4.9</td>
<td>12.5 ± 6.2</td>
</tr>
<tr>
<td>Cholesterol/mg</td>
<td>260.3 ± 208.1</td>
<td>237.7 ± 37.6</td>
</tr>
<tr>
<td>Starch/g</td>
<td>102.3 ± 48.1</td>
<td>159.7 ± 45.9</td>
</tr>
<tr>
<td>Carbohydrates/g</td>
<td>274.8 ± 80.1</td>
<td>286.2 ± 49.6</td>
</tr>
<tr>
<td>Sugars/g</td>
<td>118.7 ± 72.0</td>
<td>106.7 ± 35.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Whole intact grain</th>
<th>Pre-intervention</th>
<th>Post-Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories/kcal</td>
<td>1970.5 ± 354.5</td>
<td>2510.0 ± 526.7</td>
</tr>
<tr>
<td>Fat/g</td>
<td>74.7 ± 22.9</td>
<td>74.3 ± 20.5</td>
</tr>
<tr>
<td>Saturated fatty acids/g</td>
<td>26.7 ± 8.2</td>
<td>25.9 ± 7.8</td>
</tr>
<tr>
<td>Non-starch polysaccharides/g</td>
<td>14.1 ± 5.1</td>
<td>23.5 ± 6.5</td>
</tr>
<tr>
<td>Protein/g</td>
<td>73.1 ± 9.4</td>
<td>90.4 ± 12.5</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids/g</td>
<td>10.9 ± 6.9</td>
<td>11.8 ± 7.4</td>
</tr>
<tr>
<td>Cholesterol/mg</td>
<td>214.8 ± 118.0</td>
<td>234.2 ± 117.4</td>
</tr>
<tr>
<td>Starch/g</td>
<td>98.3 ± 21.9</td>
<td>197.5 ± 83.6</td>
</tr>
<tr>
<td>Carbohydrates/g</td>
<td>246.0 ± 46.5</td>
<td>355.1 ± 87.4</td>
</tr>
<tr>
<td>Sugars/g</td>
<td>117.4 ± 40.8</td>
<td>112.5 ± 58</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Whole milled grain</th>
<th>Pre-intervention</th>
<th>Post-Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories/kcal</td>
<td>2674.6 ± 448.2</td>
<td>2393.2 ± 293.9</td>
</tr>
<tr>
<td>Fat/g</td>
<td>108.6 ± 12.3</td>
<td>85.6 ± 22.1</td>
</tr>
<tr>
<td>Saturated fatty acids/g</td>
<td>36.2 ± 4.5</td>
<td>27.4 ± 9.5</td>
</tr>
<tr>
<td>Non-starch polysaccharides/g</td>
<td>18.6 ± 3.5</td>
<td>15.82 ± 7.5</td>
</tr>
<tr>
<td>Protein/g</td>
<td>92.9 ± 26.6</td>
<td>87.6 ± 18</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids/g</td>
<td>17.0 ± 3.1</td>
<td>15.3 ± 5.8</td>
</tr>
<tr>
<td>Cholesterol/mg</td>
<td>226.2 ± 82.3</td>
<td>189.0 ± 66.3</td>
</tr>
<tr>
<td>Starch/g</td>
<td>144.8 ± 98.5</td>
<td>163.8 ± 22.2</td>
</tr>
<tr>
<td>Carbohydrates/g</td>
<td>340.6 ± 77.1</td>
<td>309.8 ± 41.6</td>
</tr>
<tr>
<td>Sugars/g</td>
<td>153.2 ± 38.6</td>
<td>115.0 ± 29.7</td>
</tr>
</tbody>
</table>

Table 3: Pilot WISE trial diet diary data. Seven day diet diary data are separated to pre- and post- each dietary intervention of control (n = 6), whole intact grain (n = 6) and whole milled grain (n = 5). Data displayed as daily mean ± standard deviation. Data were compiled and analysed by Dr L. Tripkovic.
3.3 The effect of whole intact and whole milled grains on serum markers of vascular health

3.3.1 Haemostatic factors

Due to their roles in maintaining haemostasis and their contribution to vascular health, the haemostatic factors selected for analysis were tissue-plasminogen activator-1 (tPA-1) and von-Willebrand factor (vWF).

**Tissue plasminogen activator-1**

Fasting concentrations of the fibrinolytic factor tPA-1 were measured at baseline and after each eight week intervention of control, whole intact grain or whole milled grain products as described in 2.2.4.1. Figure 3.2 shows no significant differences at baseline. The mean tPA-1 concentration after the control diet was 6.31ng/ml compared to 5.04ng/ml before it. The test diets gave slightly lower tPA-1 levels when compared to pre-intervention, with pre-to post-ratios of 7.50:7.03ng/ml for the whole intake grain and 8.25:6.19ng/ml for the whole milled grain. There were, however, no significant differences in fasting total tPA-1 concentration due to any of the interventions when comparing pre- to post-intervention with all mean concentrations in the range of 5.04 to 8.25ng/ml.
Figure 3.2: Fasting tPA-1 concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Fasting tPA-1 concentrations were measured in each participant (n = 18) pre- and post-each 8 week intervention of control (C •), whole intact grain (WIG ■) or whole milled grain (WMG △) products using an in-house ELISA. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

von Willebrand factor

As illustrated in Figure 3.3, no significant differences in concentrations between groups were found at baseline. A trend is displayed in the data, with each intervention reducing the level of vWF. The control diet concentrations average to 11.4IU/ml pre- and 7.8IU/ml post-intervention. The whole intact grain intervention concentrations were 12.7IU/ml pre- and 9.1IU/ml post-intervention and the whole milled grain concentrations were 18.6IU/ML and 10.13IU/ml. There were, however, no significant differences in fasting total vWF concentration due to any of the interventions, when comparing pre- to post-intervention, per treatment intervention.
3.3.2 Inflammatory markers

Due to their role in vascular inflammation and potential contribution to CVD risk, the markers soluble-ICAM-1, IL-8, IL-6 and CRP were selected for measurement.

Soluble intracellular adhesion molecule-1

As alterations in the levels of shedding of, and therefore circulating levels of, adhesion molecules can be indicative of vascular activity, fasting soluble-ICAM-1 concentrations were measured at baseline and after each eight week intervention of control, whole intact grain or whole milled grain products as described in 2.2.4.2. Figure
3.4 shows that mean concentrations of sICAM-1 did not vary significantly at baseline and were not altered significantly due to the intervention.

**Figure 3.4: Fasting sICAM-1 concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product.** Fasting sICAM-1 concentrations were measured in each participant (n = 19) pre- and post-each 8 week intervention of control (C •), whole intact grain (WIG ■) or whole milled grain (WMG △) products using a Quantikine® human sICAM-1 immunoassay kit. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

**Interleukin-8**

Figure 3.5 illustrates an increase in IL-8 concentrations for both the control and whole intact grain diets, at 5.21pg/ml pre- and 7.01pg/ml post-intervention and 7.189pg/ml pre- and 8.2pg/ml post-intervention respectively. The whole milled grain diet shows a very little difference pre- to post-intervention at 7.88 and 7.30pg/ml, with no significant differences.
Figure 3.5: Fasting IL-8 concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Fasting IL-8 concentrations were measured in each participant (n = 17) pre- and post-each 8 week intervention of control (C ●), whole intact grain (WIG ■) or whole milled grain (WMG ▲) products using a human ultrasensitive cytokine 10-plex panel kit on a Luminex® 100. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

Interleukin-6

The pro-inflammatory cytokine interleukin-6 was measured at baseline and after each treatment intervention of control, wheat fibre and inulin. Circulating levels were below the level of detection of the high sensitivity Luminex kit used (data not shown).
C-reactive protein

As a stable, yet sensitive marker of inflammation and a putative risk marker for CVD (Ridker P 1998), fasting levels of CRP were measured. Figure 3.6 shows that all mean values were in the range of 0.465 and 0.915mg/l and there were no significant differences in fasting total CRP concentration due to any of the interventions.

Figure 3.6: Fasting CRP concentrations pre- and post-each 8 week intervention of control, whole intact grain or whole milled grain product. CRP concentrations were measured in each participant (n = 18) pre- and post-each 8 week intervention of control (C •), whole intact (WIG ■) or whole milled grain (WMG ▲) products via high sensitivity in-house ELISA. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

3.4 The effect of whole intact and whole milled grains on metabolite profiles

An untargeted liquid chromatography linked mass spectrometry (LC/MS)-based metabolomics analysis was carried out in order to assess whether the applied dietary intervention rich in whole intact or whole milled grains has an affect on plasma metabolite profiles of high risk men and post-menopausal women.
Figure 3.7: The principal component analysis and partial least squares discriminate analysis score plots illustrating the metabolite profiling data. No significant differences were found for any ions of pre-control ▲, post-control ■, pre-whole intact grain ■, post-whole intact grain ●, pre-whole milled grain ● and post-whole milled grain ●. A) shows the PCA plot with variation of 4.6% and model parameters $R^2 0.102$ and $Q^2 0.0498$. B) shows the PLS-DA score plot with 3.6% variation and model parameters $R^2 0.193$ and $Q^2 0.675$. 
Figure 3.7A above shows a principal component analysis (PCA) score plot of the data with no grouping. Variation was observed to be 4.6% and 3.2% respectively, due to the first 2 principal components. Figure 3.7B illustrates a score plot from a six-group partial least squares discriminate analysis (PLS-DA) model of the data. No clear discrimination was observed between groups. Furthermore, the model parameters $R^2$ (goodness of fit of the model) of 0.193 and $Q^2$ (predictive ability of the model) of 0.675 were poor, demonstrating low discriminatory power and predictive ability. This information suggests that a more extensive analysis of the data was unnecessary and, therefore, no further investigations were carried out.

3.5 The effects of whole intact and whole milled grains on circulating markers of CVD risk

3.5.1 Circulating lipids

Traditional risk markers for CVD include high blood levels of total cholesterol, low density lipoprotein (LDL) cholesterol, triacylglycerides (TAG), non-esterified fatty acids (NEFA) and low levels of high density lipoprotein (HDL) cholesterol. It was, therefore, important to measure levels of these risk markers to investigate the impacts of the intervention products in this study.

Total cholesterol

Fasting total cholesterol concentrations were measured at baseline and after each eight week intervention of control, whole intact grain or whole milled grain products as described in 2.2.6.1. Figure 3.8 illustrates slight differences at baseline between the three groups, none of which were found to be statistically significant. No significant
differences in fasting total cholesterol concentration due to any of the interventions, when
comparing pre- to post-intervention was observed and all mean levels were in the range
of 3.9 to 5.05mM.

Figure 3.8: Fasting total cholesterol concentrations pre- and post-each 8 week
dietary intervention of control, whole intact grain or whole milled grain product.
Fasting total cholesterol was measured in each participant (n = 19) pre- and post-each 8
week intervention of control (C •), whole intact grain (WIG ■) or whole milled grain
(WMG ▲) products using an IL Test™ cholesterol kit on an iLab autoanalyser.
Individual values displayed with mean ± standard error. No significant differences were
found using a paired Student’s t-test.

High density lipoprotein cholesterol

Figure 3.9 shows that the mean HDL cholesterol concentrations varied over a
range of 1.03 to 1.23mM with no significant differences in fasting HDL cholesterol
concentration, when comparing pre- to post-intervention.
Figure 3.9: Fasting HDL cholesterol concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Fasting HDL cholesterol was measured in each participant (n = 19) pre- and post-each 8 week intervention of control (C ●), whole intact grain (WIG ■) or whole milled grain (WMG ▲) products using a direct HDL cholesterol kit on an iLab autoanalyser. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

Triacylglycerides

Baseline levels were not significantly different between groups. Treatments with the control and whole milled grain interventions giving the mean TAG as 1.70 and 1.99mM and 1.23 1.45mM respectively. Figure 3.10 illustrates that there were no significant differences in fasting TAG concentration due to any of the interventions, when comparing pre- to post-intervention.
Figure 3.10: Fasting TAG cholesterol concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Fasting TAG was measured in each participant (n = 15) pre- and post-each 8 week intervention of control (C •), whole intact grain (WIG ■) or whole milled grain (WMG ▲) products using an IL Test™ triacylglycerides kit on an iLab autoanalyser. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

The post prandial TAG response can be altered by dietary changes. To investigate if any such long term effect occurred as a result of the Pilot WISE trial, participant’s TAG concentrations were further investigated by measurement before, and for 180 minutes at 30 minute intervals after, ingestion of a standard breakfast containing neither whole nor refined grains to reflect post-prandial responses as described in 2.2.4.1. This occurred pre- and post-each intervention treatment of control, whole intact grain or whole milled grain products. Areas under the curve (AUC) were calculated for each treatment intervention to determine the overall effect and no significant differences were shown between interventions, or between any intervention and baseline (data not shown).
Low density lipoprotein cholesterol

LDL cholesterol was calculated from the measurements of total cholesterol, HDL cholesterol and triacylglycerides, using the Friedewald equation and no significant differences were determined due to any treatment interventions (data not shown).

Non-esterified fatty acids

Each diet reduced the mean NEFA concentrations but all changes were non-significant. There were no significant differences, however, in fasting NEFA concentration due to any of the interventions, when comparing pre- to post-intervention and no significant differences were observed between the groups at baseline (figure 3.11).

Figure 3.11: Fasting NEFA concentrations pre- and post-each 8 week dietary intervention of control, whole intact or whole milled grain product. Fasting NEFA as measured (n = 15) pre- and post-intervention of control (C ●), whole intact (WIG □) or whole milled grain (WMG ▲) products using an IL Test™ non-esterified fatty acids kit on an iLab autoanalyser. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.
To investigate the impact of the interventions on participants' post-prandial NEFA responses, concentrations were measured before, and for 180 minutes at 30 minute intervals after, ingestion of a standard breakfast to reflect post-prandial responses (figure 3.12). This occurred at baseline and after the eight week interventions of control, whole intact grain or whole milled grain products. Areas under the curve (AUC) were calculated for each treatment intervention. The control intervention product AUC was shown to have very little difference at 55.8 pre- and 50.9 post-intervention. The whole intact grain intervention AUC showed a similar small difference at 48.1 and 45.0 pre- to post-intervention and the whole milled grain intervention AUC also showed little difference at 60.1 and 51.0. None of the differences observed were significantly.

![Figure 3.12: Postprandial NEFA concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Postprandial NEFA concentrations were measured before a standardised test breakfast and at 30 minute intervals for 180 minutes afterwards, to represent post-prandial responses. Fasting was measured in each participant (n = 15) pre- and post-each 8 week intervention of control (Pre-C dashed ●, Post-C full ●), whole intact grain (Pre-WIG dashed ■, Post-WIG block ■) or whole milled grain (Pre-WMG dashed ▲, Post-WMG full ▲) products using an iLab autoanalyser. Mean displayed ± or - standard error.](image-url)
3.5.2 Insulin sensitivity

Raised fasting and post-prandial insulin and glucose levels are associated with insulin resistance and type II diabetes. These factors have long established links to CVD and as such were important to measure in the current study.

Insulin

Fasting insulin concentrations were measured at baseline and after each eight week intervention of control, whole intact grain or whole milled grain products as described in 2.2.6.2. Figure 3.13 illustrates that no significant differences were seen at baseline or following treatment.

![Figure 3.13: Fasting insulin concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product.](image)

Fasting HDL cholesterol was measured in each participant (n = 15) pre- and post-each 8 week intervention of control (C •), whole intact grain (WIG ■) or whole milled grain (WMG ▲) products using a human insulin specific radio-immuno assay. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student's t-test.
To investigate the effects of the intervention treatments on participant’s post-prandial insulin responses, as indicators of insulin resistance and type II diabetes risk, insulin concentrations were measured over the course of 180 minutes after ingestion of a standard breakfast containing neither whole nor refined grains to reflect post-prandial responses (figure 3.14) via a human insulin specific radio-immuno assay. This occurred at baseline and after the eight week treatment interventions. AUC were calculated for each treatment intervention and no significant differences were observed between interventions, or between any intervention and baseline. The control, whole intact and whole milled grain intervention AUC altered from 58,170 to 59,680, from 85,700 to 63,978 and from 114,513 to 111,672 respectively.

Figure 3.14: Postprandial insulin concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Postprandial insulin concentrations were measured before a standardised breakfast and at 30 minute intervals for 180 minutes afterwards. Fasting insulin was measured (n = 16) pre- and post-each 8 week intervention of control (Pre-C dashed •, Post-C full •), whole intact grain (Pre-WIG dashed ■, Post-WIG block ■) or whole milled grain (Pre-WMG dashed ▲, Post-WMG full ▲) products using a human insulin specific radio-immuno assay. Mean displayed ± standard error.
As there were observed differences in both the maximum insulin concentrations reached (C-max), and the times taken to reach maximum concentration (T-max) during the post-prandial response, these data were further investigated. The mean C-max of the control intervention treatment group showed little difference at 659pM pre- and 691pM post-intervention. This was reversed in the whole intact grain group with the mean peak concentration of the group changing from 935 to 544pM altered from 1371 to 1271pM at the mean maximum concentration. None of these differences, however, were found to be statistically significant.

The mean T-max pre- and post- intervention values for the groups were as follows: control varied from 48 to 54 minutes; whole intact grain remaining unchanged at 54 minutes; whole milled grain also unchanged from 50 to 51 minutes. No significant differences were found in insulin T-max when comparing pre- to post-any treatment intervention (data not shown).

Glucose

Fasting glucose concentrations were measured as a marker of insulin sensitivity and, therefore, type II diabetes risk. Figure 3.15 shows that although baseline levels of fasting insulin were not significantly different between intervention groups and there were no significant differences in fasting glucose concentration due to any of the interventions, when comparing pre- to post-intervention. The mean glucose concentrations varied from 4.9 to 5.2mM.
Figure 3.15: Fasting glucose concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product. Fasting glucose was measured in each participant (n = 15) pre- and post-each 8 week intervention of control (C •), whole intact grain (WIG ■) or whole milled grain (WMG ▲) produces using an IL Test™ glucose kit on an iLab autoanalyser. Individual values displayed with mean ± standard error. No significant differences were found using a paired Student’s t-test.

Measurements of glucose concentration were taken at baseline and after the 8 week treatment interventions of control, whole intact grain or whole milled grain products.

The control intervention reaches a similar concentration peak pre- and post-intervention at 7.15 and 7.20mM respectively, with an apparent difference in the times taken to reach the peak concentration. The whole intact grains peaked in concentration at 6.79mM pre-intervention and 6.96mM post-intervention showing very little variation over the intervention and the time taken to reach the peak concentration appears to differ pre- to post-intervention. The whole milled grain group’s mean glucose concentration was reduced slightly from 8.33mM at baseline to 7.49mM after the intervention although
this change was not significant, and again the times taken to reach the peak concentration appear different when comparing pre- to post-intervention. These data suggest potential changes in C- and T-max. AUC were calculated for each treatment intervention. The control intervention AUC decreased from 1029 to 1015, the whole intact grain AUC from 1085 to 1060 and the whole milled from 1033 to 1005. No statistically significant differences were observed for any intervention when comparing pre- to post-intervention treatment.

![Graph](image)

**Figure 3.16: Postprandial glucose concentrations pre- and post-each 8 week dietary intervention of control, whole intact grain or whole milled grain product.** Post-prandial glucose concentrations were measured before a standardised breakfast and at 30 minute intervals for 180 minutes afterwards. Fasting glucose was measured (n = 15) pre- and post-each 8 week intervention of control (Pre-C dashed •, Post-C full •), whole intact grain (Pre-WIG dashed ■, Post-WIG block ■) or whole milled grain (Pre-WMG dashed ▲, Post-WMG full ▲) products using an iLab autoanalyser. Mean displayed ± standard error.

As small differences were seen in both the peak glucose concentration reached over the course of the post-prandial study, and the time taken to reach this peak, these
factors were further investigated. The post-prandial glucose maximum concentration (C-max) reached in the 180 minutes after ingestion of a standardised test breakfast was compared between treatment interventions. C-max was decreased non-significantly in the control and whole milled intervention groups and no significant differences were found when comparing pre- to post-intervention for any treatment group (data not shown).

The time taken to reach the maximum glucose concentration (T-max) reached in the 180 minutes after ingestion of a standardised test breakfast was compared between treatment interventions. The mean glucose T-max for the control intervention group was reduced from 45 to 40 minutes. The whole intact grain group showed a reduction from 54 to 42 minutes and the whole milled grain group showed a reduction from 54 to 48 when comparing pre- to post-intervention mean glucose T-max values. No statistically significant differences, however, were observed for any intervention treatment. Overall, mean values for T-max ranged from 42 to 54 minutes (data not shown).

3.6 Discussion

The primary aim of this research was to investigate the effects of diets high in whole intact grains and whole milled grains on risk markers of CVD in high risk individuals. We hypothesised that markers of CVD risk would be altered to reflect a reduction in risk following dietary interventions with whole intact and whole milled grain intervention products, when compared to the control refined intervention products.
Haemostatic factors

CVD and the underlying pathology, atherosclerosis, are inflammatory disorders reflecting endothelial dysfunction (Rush J 2005). It was key, therefore, to investigate the circulating levels of both haemostatic and inflammatory markers, as a reflection of any such dysfunction. A protective effect of the diets was hypothesised to have been reflected in a reduction in circulating levels of tPA-1 (a pivotal component of the fibrinolytic cascade), in response to reduced levels of coagulation/fibrinolysis. This was observed in 49 overweight or obese men and women (BMI 33.7 ± 0.6kg/m²) aged 50.0 ± 1.1 years, showing a significant reduction (p<0.001) in tPA-1 after 52 week interventions of both very low carbohydrate, high fat diets and isocaloric high carbohydrate, low fat diets, when accompanied by weight loss of 14.9 and 11.5kg respectively (Wycherley T 2010). Levels of circulating tPA-1 in the current research, however, showed no significant differences at baseline or due to the intervention treatments.

With the exception of the control intervention baseline each mean, both pre- and post-intervention, was found to be within the normal range of tPA-1 for healthy adults of 7.77 ± 1.63ng/ml (Gram J 2000). Several groups have shown levels of 9.3 to 10.9ng/ml in individuals with established CVD (Jansson J 1993; Gram J 2000), which are above the mean values of each group in this research, suggesting that baseline levels of tPA-1 may not have been raised enough to reflect a protective response. In support of the current study, a similar high whole grain dietary intervention study using the inflammatory marker plasminogen activator inhibitor-1 (PAI-1), an inhibitor of tPA-1, showed no significant changes after eight weeks of 60g of whole grains per day, or on a further eight week treatment with either 60 or 120g per day (Brownlee I 2010). Work by Andersson et al further supports these results, showing no effect on PAI-1 of a six week high whole
grain intervention (Andersson A 2007). The reduction in tPA-1 observed by Wycherley et al was accompanied by significant reductions in PAI-1 (p<0.01) suggesting that levels of both markers are responsive to such interventions and may be associated to the weight loss. Weight loss also accompanied significant reductions in tPA-1 concentrations in a trial investigating meal replacements (Clifton P 2003). As such, this reduction in tPA-1 could be associated with weight loss rather than the intervention diet, which may explain why no reduction was observed in the current study, as weight loss did not occur.

In this study, the participants' vWF concentrations showed no significant differences at baseline, suggesting a physiological homogeneity between the groups in terms of coagulation status with respect to vWF. Circulating vWF concentrations were reduced after each of the dietary interventions, suggesting a possible alteration in the regulation of pro-coagulants, but differences were not statistically significant. Several reports have shown that levels of circulating vWF in healthy controls vary between 1.2 and 1.3IU/ml (Rumley A 1998; Wiman B 2000; Bongers T 2006). The current values are similar or higher than these values, perhaps suggesting some of the participants, those with the raised levels, may have been at higher risk of CVD, although this is not seen across the entire population.

It was hypothesised that the protective effects of the intervention diets would reduce circulating levels of the pro-coagulant vWF as was observed in 25 healthy males after a 28 day high mono-unsaturated fat diet when compared to a standardised National Cholesterol Education Diet (p<0.001 (Perez-Jimenez F 1999)). Such a response could potentially reflect reduced risk of coagulation associated with CVD, and, as this was not
observed in the current research, there may be a role for an intervention investigating the fat composition of the diets as a method of reducing risk markers.

The current results support those of a similar study by Brownlee et al, in which the effects of high whole grain diets on CVD risk markers showed no significant impacts on another pro-coagulant fibrinogen, in a trial of over 300 participants (Brownlee I 2010). Brownlee’s participants were overweight so would be expected to be likely to reflect a reduction in risk due to the intervention diets, had one occurred. It is possible, therefore, that the interventions do not affect vWF as expected. Interestingly, one study observed significantly lower levels of vWF and fibrinogen after a 12 week diet including 37 ± 4g/day of the novel whole grain Salba (Salvia hispanica L) when compared to a control high wheat fibre diet and a non-significant trend towards a reduction in the alternative coagulation marker factor VIII (Vuksan V 2007) showing that vWF has the potential to be altered by dietary interventions. The portion size was similar between the two studies but the increased intervention length of the Vuksan et al work may account for the differing results. Alternatively, Salba may be more effective than wheat at reducing these markers.

If the dietary interventions were protective as hypothesised, the levels of both tPA-1 and vWF would have been expected to be increased at baseline and to decrease in response the active interventions if the participants were at high risk of CVD. This was not reflected, though, in the results with baseline levels being in the healthy or slightly raised brackets and not changing significantly with the interventions.
Inflammatory factors

As stated previously, CVD and atherosclerosis are inflammatory disorders, thus it was important to investigate circulating inflammatory factors as markers of risk. Similar fasting circulating sICAM-1 levels between intervention groups at baseline suggest physiological homogeneity and thus comparable groups. No significant changes occurred as a result of the interventions. These data are within the normal circulating range found in healthy adults of 115 to 306ng/ml (Glurich I 2002) and suggest that none of the diets had a significant impact on circulating sICAM-1 levels. As ICAM-1 levels are associated with pathogenesis of atherosclerosis (Hope S 2003), a beneficial effect on the endothelium was expected to manifest as a reduction in sICAM-1 levels as observed after a three week high fibre, low fat intervention including physical activity in diabetic men also showing weight loss (Roberts C 2006). Interestingly, the research by Wycherley et al showed a significant reduction (p<0.01) in circulating ICAM-1 following a low carbohydrate diet and weight loss but not following a low fat diet and weight loss. Additionally, although no significant differences were seen in circulating VCAM-1 levels, a significant reduction (p<0.001) was observed in circulating E-selectin levels (Wycherley T 2010) suggesting, again, that weight loss may play a major role in reduction of such circulating factors.

The current results, however, do not reflect such a change and agree with data from another high whole grain dietary intervention trial which showed no changes in circulating levels of ICAM-1 after eight and 16 week diets of 60g whole grain per day (Brownlee I 2010). Further to this, Brownlee et al also observed no significant changes in VCAM-1 and E-selectin, perhaps further suggesting a role for weight loss in alteration of circulating markers.
Other studies have found higher levels of both IL-8 (7.8 ± 3.6 pg/ml) and IL-6 levels in obese subjects (BMI 47.5 ± 9.4kg/m² (Jarrar M 2008)) suggesting that IL-8 and IL-6 levels increase with BMI and, as such, with CVD risk. Levels of circulating IL-8 in patients with CAD were found to be significantly higher than those of controls (3.27pg/ml compared to 2.36pg/ml (Jha H 2009)). As such, levels of IL-8 were hypothesised to fall in response to a cardio-protective intervention such as whole grains. This was observed in ten participants in response to a high conjugated linoleic acid cheese intervention (Sofi F 2009) and twenty healthy participants due to a ten week intervention of bespoke ‘Old Italian Grain’ bread (Sofi F 2010), although this was not observed in the current research.

No significant differences in IL-8 were found. Values were higher than those in the literature, quoted as 5.6 ± 0.6pg/ml in obese subjects (Bruun J 2005) although other research also provides differing values.

Unfortunately, levels of IL-6 were below the limit of detection in this study although as levels of IL-8 and IL-6 have been found to correlate with one another (Jarrar M 2008) it may be assumed that no significant change in IL-8 may be indicative of no significant change in IL-6 although this is speculative. Indeed, IL-6 showed no changes in response to a six week high whole grain dietary intervention (Andersson A 2007). As an inflammatory cytokine, any protective effect of a dietary intervention on CVD risk would be expected to manifest itself as a reduction in circulating IL-6 levels as was shown by the previously mentioned high conjugated-linoleic acid cheese intervention (Sofi F 2009) and a three week very low calorie diet in overweight women, when accompanied by weight loss (Bastard J 2000). These differences in results may, in part,
be due to Bastard et al's participants' circulating levels already being elevated at baseline and, therefore, potentially more responsive to an intervention. The additional intervention of the weight loss may also account for the disagreement between studies as previously suggested.

As a sensitive marker for inflammatory status, and a putative marker for CVD risk (Ridker P 1998), circulating concentrations of CRP were hypothesised to reduce in response to the test diet, as was observed in response to high fatty or lean fish (300g/week) diets over six months in 52 participants (Pot G 2009). Levels of circulating C-reactive protein showed very little variation, with all treatments when compared pre- to post-intervention, covering a range of 0.465 to 0.915mg/l. These data are within the ranges of circulating concentrations of CRP for healthy subjects (1.68 ± 1.42mg/l) and do not reach levels associated with CVD (3.35 ± 1.56mg/l, (Glurich I 2002). These data support work by Brownlee et al who showed, with a far longer intervention period, that no significant differences in circulating CRP levels after eight and 16 week interventions of 60g whole grains per day occurred (Brownlee I 2010) with levels of circulating CRP similar to this study.

These data are further supported by a 12 week high fibre whole grain intervention in type II diabetic patients also showed no significant reduction in circulating CRP, even considering that levels were higher at baseline than in the current research, at 2.6 to 3.1μg/ml (Vuksan V 2007). Andersson et al further support these findings, showing no significant effect on circulating CRP levels after a six week high whole grain intervention (Andersson A 2007) in participants of similar age and BMI values to the current research. As CRP is predominantly produced in the liver under the control of IL-6, longer
interventions may be required to alter its circulating levels than those of products
produced mainly by the endothelium.

As such, the data illustrates that the inflammatory markers sICAM-1, IL-8 and
CRP were not significantly altered by any of the intervention products suggesting that
either changes in levels of the markers investigated were not great enough to be detected
or that the intervention products do not have a significant impact on the endothelium.

**Metabolomics**

Non-targeted liquid chromatography linked mass spectrometry (LC-MS)
metabolomic analysis aimed to identify any significant differences in metabolite profiles
between intervention treatment groups. On analysis of the data, the relatively weak model
parameters ($R^2$ 0.193 and $Q^2$ 0.675) and poor discrimination between sample groups
meant that no further investigation into the data was conducted. These data suggest that
the control, whole intact and milled grain intervention products themselves and the wider
ranging dietary effects of incorporating them into the diet, did not have a significant
overall impact on metabolite profiles (positive ionisation mode).

As this is an emerging research area and few intervention studies have been
completed to date it is difficult to compare the results with those expected, although
limited research has been carried out into the comparison of dietary patterns on the
circulating metabolic profiles (Stella C 2006) and monitoring the metabolism of specific
nutrients and identifying novel metabolic pathways (Zhao X 2009). Indeed, in a very
recent metabolite profiling study, Lankinen *et al* found a significant increase in multiple
proinflammatory lysophosphatidylcholines following a wheat bread diet, suggesting that
dietary carbohydrate modification has the capacity to alter metabolite profiles, particularly with respect to the lysophosphatidylcholines (Lankinen M 2010). These results may differ from the current research as participants in Lankinen et al.'s study were previously diagnosed with metabolic syndrome and were more numerous (n = 10 per leg). The intervention itself was also longer, at 12 weeks.

**Circulating lipids**

With known roles in the pathogenesis of atherosclerosis and as established markers for CVD risk, measurement of the circulating lipid profiles was central to this research. Differences were observed between the groups at baseline but these were not statistically significant. No statistically significant changes were observed in any markers measured or calculated i.e. total cholesterol, HDL cholesterol, TAGs, LDL cholesterol or NEFAs, due to the intervention diets.

Changes in lipid profiles were expected to mimic those of other similar investigations such as those which were observed on an intervention of 123g/day oat bran in 23 mildly hypercholesterolaemic men (Whyte J 1992) and on a four week calorie restricted legume-based diet in 30 obese participants (as accompanied by weight loss (Hermsdorff H 2010)) which both showed significant reductions in total and LDL cholesterol. Similar research showed a significant reduction in total and LDL cholesterol and TAG in response to a four week addition of a wheat whole-grain based dietary product (200g/day) in 31 obese participants with elevated fasting blood glucose (Rave K 2007). Differences between such results and those of the current research may be due to their higher numbers of participants, increased amounts of intervention products, a higher risk profile population group at baseline, including obesity and elevated blood glucose.
and cholesterol levels which would be expected to be more responsive to dietary influence than levels associated with healthy individuals, or the accompanying weight loss observed in their studies.

Apart from the baseline mean level for the whole intact grain group at 3.9mM, all mean total cholesterol concentrations were found to be above the recommended 4.0mM (JBS2 2005), with levels spanning the 'ideal' and 'mildly high risk' concentrations for the general population suggesting that the study population was at mildly raised risk of CVD both pre- and post-interventions and, therefore, potentially likely to respond to an intervention such as whole grains. These levels, however, were lower than those observed at baseline in the trials which did show significant reductions (Whyte J 1992; Rave K 2007; Hermsdorff H 2010). Both at baseline and post-intervention, levels of HDL cholesterol were slightly raised above the recommended values for individuals at high risk of CVD (JBS2 2005) showing protective and positive effects. These were reflected, however, in the slightly higher total cholesterol levels. Levels of fasting NEFA concentrations fell within the range cited in the literature, of 0.44 to 0.5mM for healthy and type II diabetic participants respectively (Pedrini M 2006; Smit J 2008) and showed no significant changes, thus suggesting no significant alterations in CVD risk, with respect to NEFA, due to the intervention diets.

Once again, these findings are in support of those of the similar dietary intervention study by Brownlee et al including 316 participants aged 18–65 years with BMI values >25kg/m² who also showed no significant changes in total cholesterol, HDL cholesterol, LDL cholesterol, TAGs or NEFAs in response to eight or 16 week interventions of 60g whole grain per day (Brownlee I 2010). The higher numbers of participants included in Brownlee et al's study reflect higher power of the data and a
broader representation of the population as a whole. Although the age range was greater than in this research it is likely that the group were also at increased CVD risk due to their high BMIs and, therefore, likely higher risk of atherosclerosis progression and insulin resistance. These results also concur with those of Andersson et al who observed no significant differences in total cholesterol, HDL or LDL cholesterol, free fatty acids or TAGs in a randomised controlled cross-over trial investigating the effects of a six week high whole grain intervention (Andersson A 2007). Participants included 30 men and women of similar ages to the current research (35 to 70 years) with similar high BMI values of $28 \pm 2\text{kg/m}^2$ but with previously diagnosed type II diabetes which might suggest an increased CVD risk as hyperglycemia and insulin resistance play pivotal roles in the pathogenesis of atherosclerosis (Neri S 2005) and, therefore, more potential for alterations in the risk markers. Furthermore, Vuksan et al observed no significant changes in total cholesterol, HDL cholesterol, LDL cholesterol or TAGs in response to a 12 week intervention of $37 \pm 4\text{g/day}$ of the novel whole grain Salba ($Salvia hispanica L$), rich in fibre and minerals (Vuksan V 2007). Participants were both men and women, with a higher average age than those of the current research ($64 \pm 8\text{ years}$), with similar BMI values ($28 \pm 4\text{kg/m}^2$) and previously diagnosed type II diabetes. Similar concentrations to those of the current research were found Vuksan et al’s research.

Overall, investigation of the established risk markers, the circulating lipid profiles suggested that the treatment interventions conferred no reduction in risk of CVD. These data suggest that the control, whole intact and whole milled grain interventions had no statistically significant impacts on the circulating lipid profiles of the high risk group investigated in this research.
Fasting and post-prandial serum insulin and glucose concentrations were measured as hyperglycemia and insulin resistance are known to be pivotal in the pathogenesis of atherosclerosis (Neri S 2005) and previous studies have indicated that whole grain dietary interventions may beneficially affect factors reflective of insulin sensitivity, type II diabetes and CVD risk.

These studies include work by Rave et al, who showed a significant reduction in fasting blood glucose and insulin and in the homeostasis model assessment of insulin resistance (HOMA IR) in response to high whole grain-based dietary intervention, accompanied by significant weight loss (Rave K 2007). Participants in Rave et al’s research, however, were obese with elevated fasting glucose levels which are expected to be more responsive to protective interventions such as whole grains than healthy levels and may explain the differences seen between those results and current research, which showed no significant changes in levels of fasting insulin or glucose in response to any of the treatment interventions. Post-prandial effects, as investigated using the area under the curve and although there is a right shift in the curve indicating a beneficial delaying of response, no significant effects of the interventions. This work supports that by Andersson et al who showed no significant differences in insulin sensitivity following a high whole grain diet as determined by a euglycemic hyperinsulinaemic clamp (Andersson A 2007). Brownlee et al’s results also concurred with this study, showing no significant differences in fasting insulin or glucose due to high whole grain interventions, in a randomised controlled dietary intervention trial of the significantly higher number of 316 participants (Brownlee I 2010). Their group used a modified quantitative insulin sensitivity check index as a marker of insulin resistance finding no changes in insulin
sensitivity or resistance. Further support was given by a 12 week study providing 37 ± 4g/day whole grain intervention in type II diabetics showing no significant reduction in fasting insulin or glucose levels of 7.1 to 7.4mM, higher than those observed here (Vuksan V 2007).

A high whole grain, controlled cross-over trial in overweight hyperinsulinaemic adults lasting six weeks, however, did show a significantly lower fasting insulin concentration following a high whole grain diet compared to a control refined grain diet (Pereira M 2002). This may be due to the higher baseline levels of insulin than those of this study, at 169 ± 16.9pM, which may have been more responsive to positive treatment than the levels of this study. In agreement with the current study, Pereira et al showed no significant change in fasting glucose levels after a whole grain intervention when compared to the refined control diet and no significant difference in post prandial insulin AUC.

Overall, this research provides no evidence of whole intact or milled grains having an impact on insulin sensitivity as reflected by fasting and post-prandial concentrations of insulin or glucose.

**Trial limitations**

Certain issues are common to all human intervention trials such as those related to the population groups investigated, compliance and power. The population group investigated in the Pilot WISE trial benefited from including both men and post-menopausal women although, unfortunately, they were not equally represented with 13 men and only six women taking part. Participants were also, through no methodological design, homogenously Caucasian, thus reducing the potential application of the data.
across the general population. Due to the unavoidable restrictions placed upon recruitment, participants were recruited from the University of Surrey and its associates which passively homogenised the group as well educated. Surrey is known to be an affluent area, so inhabitants are likely to be at lower risk for incident CHD than those of average income (Sundquist K 2004). The general population to whom we hope to apply the research would have been better represented had a wider range of socio-economic and educational backgrounds been sampled. Although the number of participants involved was sufficient for the current pilot research, this value limited the power of the data. Involving a higher number of participants in future work would increase the power of the data and also allow for stratification of the data by sex for analysis where relevant.

Pre-menopausal women did not participate as CVD risk is far greater in men and post-menopausal woman than in pre-menopausal women due to the protective effects conferred by their higher oestrogen levels (Nathan L 1997). Participants were chosen as those at increased risk of CVD, as reflected by their age and sex (men and post menopausal women aged 30 to 60 years) and high BMI values (25 to 35 kg/m²) as these factors are indicative of high risk (Christie D 1981, Herman B 1982, Nathan L 1997, Kenachaiah S 2002). Participants were, therefore, expected to present with altered levels of other risk markers (including the inflammatory, haemostatic, lipid and carbohydrate metabolism markers measured) at baseline and thus a greater potential for change due to the interventions.

Unfortunately, this increased risk profile was not observed at baseline, which could account for the lack of change in these markers due to the intervention i.e. if they did not reflect high risk they were unlikely to be possible to reduce. Levels of markers may have been more responsive to interventions in a higher risk population group, for
example, participants who smoked, were older with higher BMIs or who had clinically high blood pressures. Alternatively, clinical screening of participants could have ensured a higher risk population of participants were recruited with, for example, high total cholesterol, TAG or LDL cholesterol or low HDL cholesterol levels. However, this was not viable within the constraints of the trial.

A power calculation for the Pilot WISE trial was based on an estimated fall in post-prandial insulin area under the curve of 20% between groups (which would be metabolically significant) with a standard deviation of 20%. This gave an estimate of 17 per group. Unfortunately, it was not possible to recruit such a high number of participants within the restraints of the Pilot WISE trial. Therefore, the trial is underpowered but, as there were no similar such studies published at the time of the inception of the trial, it was considered that the research question justified the completion of a pilot trial to explore the area. The recent similar trial by Brownlee et al in 2010, the WHOLEheart trial, conducted 316 participants, also failed to show significant changes in the markers measured, including post-prandial area under the curve. This suggests the Pilot WISE trial may not have shown significant differences even if it was fully powered.

Participants reported that trial participation, including the completion of diet diaries, encourage them to more actively contemplate their diets and lifestyles and small numbers of participants reported altering their eating habits as a result of their participation. A control intervention leg was employed to normalise for the direct effect of participation itself but this behaviour change also extended to exercise, which has been shown to cause significant reductions in total and LDL cholesterol, TAG, fasting glucose
and markers of insulin resistance in overweight and obese individuals (Saremi A 2010) suggesting the potential future measurement of physical activity as a marker or for control purposes. Compliance issues are always of note in dietary intervention trials. We aimed to ensure compliance by encouraging our participants and developing a rapport and good working relationships with them. Compliance was also monitored by providing a known number of intervention product portions to the participants and asking them to return any which remained unused. Diet diaries were further used to monitor compliance and phone contact made by a Registered Dietitian reminded the participants to consume their intervention products. Compliance could have been reinforced further by more frequent contact by the research team but such contact was balanced with the negative effects of excess intrusion and demand in the participant’s lives. Participants did comment that they would not have maintained the diets for any longer, suggesting, with other anecdotal information, that the intervention products were not well accepted, which could have negatively affected compliance. Intervention products were included into the participants’ normal diets in the place of regular bread type products thus preventing a significant overall increase in intake and it is possible that the palatability of intervention products was enhanced with fatty additions, thus increasing dietary fat intake. This could, potentially, have had negative impacts on weight and circulating fats, with a resultant increase in atherosclerosis development. Overall, however, compliance was found to be generally good as determined by diet diaries and no significant changes were observed in nutritional intakes (data not shown).

Alternative methods of presenting the intervention products were investigated before trial initiation. Although fibre is now more widely available in the form of drinks, this was not a viable option for the whole intact grains. The unusual delivery method, of
providing the intervention in bespoke, bread roll-like portions, proved minimally invasive to implement. It also enabled thorough monitoring of compliance. Information was collected and reported to the manufacturer on the participants’ preferred methods of preparing the products, the most ingenious of which was incorporating them into a bread and butter pudding, to enable product development for future studies.
Chapter 4

The FITMA trial

A randomised controlled dietary intervention trial investigating the effects of wheat fibre and inulin on risk markers of cardiovascular disease in at risk men
4.1 Study rationale and aims

The pathology of CVD involves the inflammatory condition atherosclerosis and the dysfunction of the vascular endothelial functions: thrombosis, haemostasis and tissue perfusion (Rush J 2005). Known risk markers and co-morbidities for CVD provide possible methods for prevention and treatment and can also act as risk markers for the disease with dietary interventions, including high fibre diets, promoted as non-invasive methods for preventing and treating CVD. Whole grains contain a variety of components which may be responsible for their protective effects and, as such, it is interesting to investigate these components individually. The aim of the FITMA trial, therefore, was to investigate the effects of specific components of whole grains, insoluble and soluble fibre, on various factors reflecting changes in CVD risk status. A cross-over methodology was used to remove inter-individual variations at baseline.

The Fibre and Inulin Trial in Male Adults (FITMA trial, summarised in figure 4.1) was a randomised controlled cross-over dietary intervention trial investigating the effects of insoluble and soluble fibre, represented for the purposes of this research by wheat fibre and inulin respectively, as compared to a control reference product, on risk markers of cardiovascular disease in at risk men, aged 30 to 60 years with body mass index scores of 25 to 35kg/m². Several studies have shown the positive effects of high fibre diets on CVD risk markers, hence their investigation here. The cross-over study design lends high significance to the results and at risk participants were chosen as their levels of risk markers are likely to be more responsive to manipulation than those at lower risk. As participant numbers were limited in this trial, only men were chosen to homogenise the population group. Eligible, consented participants were randomised to an
order of the three intervention products in stratified blocks. Participants attended a post prandial intervention day before their first four week dietary intervention and immediately after each intervention. Four weeks was considered the optimum length of time required to encourage compliance and allow for changes in the markers and metabolites investigated. Interventions were separated by four week washout periods when participants consumed their habitual diets.

Markers of vascular haemostasis (vWF and tPA-1) and vascular inflammation (sICAM-1, IL-8 and CRP) were measured as indicators of vascular health and function which are associated with the underlying pathogenesis of CVD. Metabolomic profiling of participant's serum was carried out to assess any changes in metabolite profiles with high fibre intake and to investigate a possible marker for whole grain intake and, as all metabolomic research is challenged by inter-individual variations, the present study used a relatively homogenous population group to minimise these variations. Established risk markers for CVD were measured, including the levels of the circulating markers of lipid metabolism and insulin resistance as these are associated with CVD risk and type II diabetes risk respectively.

One set of baseline measurements was taken at the beginning of the trial, after the 14 day 'run in' diet and before the first intervention. The data from this baseline were used as the baseline data for comparison to the intervention diets. Order effects were minimised by randomising each participant to a random order of intervention diets, thus preventing any one diet from repeatedly preceding or following another. Carry over
effects were minimised by the wash out period between, and equal to in length, each intervention diet.

The author worked on the FITMA trial in equal collaboration with Dr L. Tripkovic. The author was responsible for one half of all activities including, but not limited to: trial design, authorship of trial paperwork (including protocol, participant information sheets, recruitment paperwork and Trial Master File), informed consent sessions, management of participants’ completion of paperwork including FFQ, Health and Lifestyle Questionnaire and diet diaries, phlebotomy, participant management and care, sample preparation, storage and analysis. The author was not responsible for the collection or analysis of the diet diary data; this was carried out by Dr L Tripkovic. The author was entirely responsible for all data analysis presented – data analysis was not carried out in collaboration.

It was hypothesised that levels of risk markers would vary to reflect a lower CVD risk state post-intervention with the active high fibre diets, when compared to baseline and to the control diets of wheat fibre and inulin.
Figure 4.1: FITMA trial protocol. The FITMA trial: a randomised controlled cross-over dietary intervention trial. Male participants aged 30 – 60 years, BMI 25-35kg/m², following screening and consent, were randomised to the three 28 day interventions of control, wheat fibre and inulin with a 28 day wash out diet between interventions and post prandial study days (PP study days, measuring anthropometrics and circulating markers) at baseline and after each intervention.
4.2 Anthropometric and dietary data

Anthropometric data

Anthropometric measurements were taken from each participant at the beginning of each study day.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Baseline</th>
<th>Control</th>
<th>Wheat fibre</th>
<th>Inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/years</td>
<td>39.8 ± 9.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height/cm</td>
<td>183.1 ± 5.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight/kg</td>
<td>101.6 ± 13.4</td>
<td>101.7 ± 12.9</td>
<td>103.1 ± 12.2</td>
<td>102.0 ± 12.2</td>
</tr>
<tr>
<td>BMI/kg/m²</td>
<td>30.2 ± 2.9</td>
<td>30.7 ± 2.6</td>
<td>30.4 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Body fat/% of total body weight</td>
<td>28.5 ± 4.1</td>
<td>28.6 ± 4.2</td>
<td>29.4 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Waist circumference</td>
<td>106.4 ± 7.6</td>
<td>105.9 ± 7.4</td>
<td>107.0 ± 7.9</td>
<td>105.1 ± 6.3</td>
</tr>
<tr>
<td>Hip circumference</td>
<td>110.9 ± 6.8</td>
<td>111.5 ± 5.8</td>
<td>111.3 ± 5.8</td>
<td>110.4 ± 5.3</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Systolic blood pressure/mmHg</td>
<td>124.8 ± 9.5</td>
<td>121.5 ± 11.2</td>
<td>126.8 ± 8.0</td>
<td>125.2 ± 11.6</td>
</tr>
<tr>
<td>Diastolic blood pressure/mmHg</td>
<td>71.9 ± 9.9</td>
<td>70.9 ± 10.8</td>
<td>76.5 ± 8.7</td>
<td>73.3 ± 12.2</td>
</tr>
</tbody>
</table>

Table 4: FITMA trial anthropometric data. Age, height, weight, body mass index (BMI) body fat percentage, waist and hip circumferences and systolic and diastolic blood pressure were recorded at each study day and waist to hip ratio was calculated. No significant differences were shown in any measurements over the course of the intervention. Data are displayed as mean ± standard deviation, n = 10.

Diet diary data

Three day diet diaries were collected for each participant during each intervention trial. This work, and the analysis of the data, was carried out by Dr. L. Tripkovic.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Control</th>
<th>Wheat Fibre</th>
<th>Inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy/kJ</td>
<td>8563.2 ± 3006.1</td>
<td>12425.6 ± 5198.7</td>
<td>12705.0 ± 4684.2</td>
<td>12534.0 ± 4946.4</td>
</tr>
<tr>
<td>Fat/g</td>
<td>86.7 ± 39.5</td>
<td>84.1 ± 24.7</td>
<td>90.5 ± 34.9</td>
<td>76.0 ± 20.4</td>
</tr>
<tr>
<td>Saturated fat/g</td>
<td>32.4 ± 14.9</td>
<td>32.0 ± 11.3</td>
<td>33.6 ± 16.0</td>
<td>29.5 ± 12.0</td>
</tr>
<tr>
<td>Protein/g</td>
<td>81.7 ± 28.8</td>
<td>118.7 ± 53.9</td>
<td>119.7 ± 63.5</td>
<td>120.6 ± 47.6</td>
</tr>
<tr>
<td>Sodium/g</td>
<td>2449.4 ± 1131.4</td>
<td>2155.0 ± 697.9</td>
<td>2276.4 ± 686.9</td>
<td>2046.1 ± 777.1</td>
</tr>
<tr>
<td>Polyunsaturated fat/g</td>
<td>12.6 ± 7.6</td>
<td>12.0 ± 3.1</td>
<td>15.9 ± 5.1</td>
<td>13.4 ± 3.3</td>
</tr>
<tr>
<td>Water/g</td>
<td>1412.3 ± 203.1</td>
<td>1666.0 ± 614.2</td>
<td>2105.8 ± 1168.4</td>
<td>1495.9 ± 892.4</td>
</tr>
<tr>
<td>Energy/kcal</td>
<td>2041.0 ± 719.7</td>
<td>2945.6 ± 1225.1</td>
<td>3018.6 ± 1112.1</td>
<td>2969.0 ± 1169.6</td>
</tr>
<tr>
<td>Monounsaturated fat/g</td>
<td>28.9 ± 11.5</td>
<td>26.7 ± 8.0</td>
<td>31.3 ± 12.1</td>
<td>23.6 ± 8.4</td>
</tr>
<tr>
<td>Sugars/g</td>
<td>86.4 ± 57.9</td>
<td>95.7 ± 64.9</td>
<td>115.1 ± 118.3</td>
<td>663.3 ± 33.9</td>
</tr>
<tr>
<td>Starch/g</td>
<td>119.4 ± 37.7</td>
<td>320.3 ± 237.8</td>
<td>281.6 ± 200.5</td>
<td>370.2 ± 189.6</td>
</tr>
<tr>
<td>Carbohydrate/g</td>
<td>217.7 ± 85.8</td>
<td>426.5 ± 241.7</td>
<td>407.2 ± 189.2</td>
<td>440.2 ± 211.7</td>
</tr>
</tbody>
</table>

Table 5: FITMA diet diary data. Macro- and micro-nutrient compositions of the participants’ diets were recorded and analysed at base line and within each of the three trial intervention diets. Data are displayed as daily mean ± standard deviation. No significant differences were seen in any nutrients investigated between intervention diets. This work was carried out by Dr. L. Tripkovic.

4.3 The effect of wheat fibre and inulin on serum markers of vascular health

4.3.1 Haemostatic factors

Tissue plasminogen activator-1

In order to investigate whether the wheat fibre, inulin or, indeed, control intervention products could affect endothelial expression of the fibrinolytic factor tPA-1, the soluble levels of this haemostatic marker were assessed in the serum of participants undertaking the FITMA trial as described in 2.2.4.1 (figure 4.2). Each of the diets slightly increased circulating tPA-1 concentrations above the baseline of 2.385 ± 0.65ng/ml but none of the intervention diets significantly altered circulating levels of tPA-1 when compared to baseline or control.
Figure 4.2: Fasting tPA-1 concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting tPA-1 was measured in each participant (n = 10) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using an in-house ELISA. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

von Willebrand factor

Fasting concentrations of the pro-coagulant vWF were measured to investigate whether the intervention products could affect vascular endothelial expression of such factors. Figure 4.3 illustrates a slight reduction in mean fasting vWF concentrations after the wheat fibre and inulin interventions when compared to the control. No differences between any groups, however, were significant and all group mean values fell within the range of 2.49 (wheat fibre) and 3.52 (control).
Figure 4.3: Fasting vWF concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting vWF was measured in each participant (n = 9-10) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using an in-house ELISA. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

4.3.2 Inflammatory markers

Soluble intra-cellular adhesion molecule-1

In order to investigate whether soluble or insoluble fibre, represented by inulin and wheat fibre respectively, could affect endothelial cell shedding of sICAM-1, a marker of vascular inflammation, the soluble levels of this adhesion molecule were assessed in the fasting serum of participants as described in 2.2.4.2. Figure 4.4 illustrates that mean levels of sICAM-1 remained similar throughout the intervention, at 188.5, 180.0, 190.8 and 193.3ng/ml at baseline and after the wheat fibre, inulin and control interventions respectively. None of the intervention diets significantly altered circulating levels of sICAM-1 when compared to baseline or control.
Figure 4.4: Fasting sICAM-1 concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting sICAM-1 was measured in each participant (n = 10) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using a Quintikine® human sICAM-1 immunoassay kit. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

Interleukin-8

Circulating levels of the inflammatory cytokine IL-8 were assessed in the fasted plasma of participants undertaking the FITMA trial. The mean fasting serum concentrations of IL-8 altered from 5.538 pg/ml at baseline to 6.011 pg/ml following the wheat fibre intervention and 6.288 pg/ml following the inulin intervention. The concentration changed to 4.363 pg/ml after the control diet (figure 4.5), however, none of the changes were statistically significant.
Figure 4.5: Fasting IL-8 concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting IL-8 was measured in each participant (n = 7-9) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using a human ultrasensitive cytokine 10-plex kit on a Luminex®100. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

Interleukin-6

Interleukin-6, another potent mediator of inflammation, was also measured as described in 2.2.4.2, at baseline and after each treatment intervention of wheat fibre, inulin and control. Unfortunately, circulating levels were below the limit of detection of the high sensitivity Luminex kit thus all results read as zero.

C-reactive protein

To investigate whether soluble or insoluble fibre, as represented by the inulin and wheat fibre products respectively, could affect circulating levels of CRP, levels of this
marker of inflammation were assessed in the serum of participants undertaking the FITMA trial as described in 2.2.4.2. The mean levels of CRP remained low at 2.001µg/ml at baseline and 1.663, 2.451 and 1.129µg/ml after the control, wheat fibre and inulin interventions respectively, although certain individual values did reach 9.0µg/ml (figure 4.6). No significant differences were seen between any of the treatment interventions or when compared to baseline.

Figure 4.6: Fasting CRP concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting CRP was measured in each participant (n = 10) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) and using an in-house high sensitivity ELISA. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

4.4 The effect of wheat fibre and inulin on metabolomic profiles

Untargeted LC/MS based metabolomics analysis was used in this research to investigate the effects of the interventions on participants’ metabolite profiles to reflect changes to metabolic pathways and potential biomarkers in dietary intake. In total, 2334
ions were detected in positive ionization mode. Principal component analysis (PCA) was unable to discriminate between the four sample groups; baseline, control, wheat fibre and inulin (figure 4.7A). Further investigation of the data using a four-group partial least squares discrimination analysis (PLS-DA) model is shown in figure 4.7B. This PLS-DA model accounted for 5% of the variation between the four different intervention groups and discrimination was observed between the groups. The PLS-DA model parameters $R^2$ (goodness of fit) and $Q^2$ (predictive ability) were 0.363 and 0.715 respectively.
Figure 4.7: PCA and PLS-DA score plots of metabolite profiles in the FITMA trial. Metabolite profiles of sera at baseline ■ and after each dietary intervention of control ◊, wheat fibre ▲ and inulin ● of the FITMA trial, illustrated from A) PCA and B) a four component PLS-DA model ($R^2 = 0.363$ and $Q^2 = 0.715$).

To determine the discriminatory species, the data was further explored through a PLS-DA loadings plot and by a variable importance on projection (VIP) plot of the first
component. The loadings plot showed limited spread of ions, mainly along the principal component 1 axis (figure 4.8).

![Loadings plot](image)

**Figure 4.8: PLS-DA loadings plot of ions detected by LC/MS in the sera of participants in the FITMA trial.** Discriminating ions are those separated away from the origin. Each individual ion is represented by ▲, with mass and retention time.

The VIP plot of the first component (figure 4.9) highlights the most important discriminatory species in descending order.
Figure 4.9: VIP plot illustrating of the major discriminatory species determined participants’ sera in the FITMA trial in descending order. The mass and retention time are given for each of the major discriminatory ion species between the groups of baseline, control, wheat fibre and inulin in the FITMA trial.

Ions highlighted by the VIP and loadings plots are potentially discriminatory between sample groups. These ions were further investigated, by examination of their single ion chromatograms (SIC) and mass spectra, to remove any false positives and determine any adduct formation. Figure 4.10A shows a region of the total ion chromatogram (TIC) of a representative FITMA plasma sample with the major ions and their adducts labeled by retention time and mass to charge ratio. Figure 4.10B, C and D show the extracted SIC of several discriminatory ions (determined from the VIP plot, figure 4.9) with the mass to charge ratio 568.341m/z, 520.34m/z and 524.371m/z respectively, and several related adducts (with lower intensities). Figure 4.11 shows mass spectra of specific ions recognized in the single ion chromatograms in figure 4.10B, C
and D. Figure 4.11B indicates that the signal identified with a mass to charge ratio of 544.3423m/z (figure 4.11B) is, in fact, an adduct of a parent ion with the mass to charge ratio of 520.3439m/z. The signals of those found not to be true ions on examination of their SIC and mass spectra were disregarded, while signals of true ions were statistically analysed for differences between groups.

Figure 4.10: Total ion chromatogram and representative positive ion mode single ion chromatograms from the FITMA trial. A shows the total ion chromatogram as determined by LC/MS. B, C and D show single ion chromatograms of ions of mass to charge ratio 568.341m/z, 544.341m/z and 524.371m/z respectively, with a peak representing each retention time for that specific ion signal.
Figure 4.11: Mass spectra of peaks of discriminatory signals determined in the FITMA trial showing molecular ions and adducts. Mass spectra of ions of mass to charge ratios A 568.34 m/z, B 520.3439 m/z and C 524.37 m/z, as analysed by LC/MS. Peaks shown with mass to charge ratio.

These signals identified as significantly different between groups underwent putative identification. Potential elemental composition analysis was performed using Masslynx. Exact masses were then entered into metabolite databases (Human Metabolite Database, www.hmdb.ca; METLIN, metlin.scripps.edu; KEGG, www.kegg.com) and compared with the possible elemental composition, taking into account adducts from molecular ion analysis, and potential metabolites were assigned. Further confirmatory analysis of the ions was beyond the scope of this research, due to time limitations, and as such, assignments are putative.
A major discriminatory signal was determined at 6.61 minutes, 568.3408m/z. The intensity of this species was significantly reduced by the control diet than at baseline (p<0.05) with the inulin measurement also being higher than the wheat fibre control measurements (p<0.001 and p<0.05 respectively). The best match for this signal from metabolite databases was found to be lysophosphatidylcholine (LysoPC, C22:6), based on the m/z ratio of 567.694 and the formula C\textsubscript{30}H\textsubscript{50}NO\textsubscript{7}P compared to the elemental composition analysis.

![Graph showing signal intensities across treatment interventions](image)

**Figure 4.12:** Signal intensities of the discriminatory signal at 6.61 minutes, 568.3408m/z determined in the FITMA trial. Measurements were taken at baseline and post-each intervention treatment of control, wheat fibre and inulin. Non-targeted LC-MS based metabolomic analysis revealed a discriminatory signal at 6.61 minutes, 568.3408m/z, putatively identified as LysoPC (C18:2) from a comparison of mass. Individual values displayed with mean ± SE where * and ** denote p<0.05 and 0.01 respectively, using a repeated measures ANOVA.
The discriminatory signal at 6.64 minutes, 544.3408m/z was putatively identified by its m/z ratio and chemical formula (C_{26}H_{50}NO_{7}PNa), as a [M + Na^+] adduct of 520.340m/z (C18:2). The intensity of this ion was found to be significantly lower after the control than when compared to the baseline and inulin interventions (p<0.05 and p<0.005 respectively, figure 4.13).

![Figure 4.13: Signal intensities of the discriminatory signal at 6.64 minutes, 544.3408m/z determined in the FITMA trial. Measurements were taken at baseline and post-each intervention treatment of control, wheat fibre and inulin. Non-targeted LC-MS based metabolomic analysis revealed a discriminatory signal at 6.64 minutes, 544.3408m/z, putatively identified as a [M + Na^+] adduct of lysophosphatidylcholine (C18:2). Individual values displayed with mean ± SE where * and *** denote p<0.05 and p<0.005 respectively, using a repeated measures ANOVA.](image)

The third discriminatory signal at 7.61 minutes, 524.3715m/z was putatively identified from the metabolite databases as LysoPC (18:0) C_{26}H_{54}NO_{7}P, with a recorded m/z ratio of 523.3637. The intensity of this signal was significantly higher following all interventions than at baseline (p<0.005, p<0.005 and p<0.01 for control, wheat fibre and inulin respectively).
inulin diets respectively) and significantly higher in the control intervention compared to inulin diet (p<0.05, figure 4.14).

Figure 4.14: Signal intensities of the discriminatory signal at 7.61 minutes, 524.3715m/z determined in the FITMA trial. Measurements were taken at baseline and post-each intervention treatment of control, wheat fibre and inulin. Non-targeted LC-MS based metabolomic analysis revealed a discriminatory signal at 6.61 minutes, 568.3408m/z, putatively identified as an adduct of lysophosphatidylcholine (C18:0). Individual values displayed with mean ± SE, where *, ** and *** represent p<0.05, p<0.01 and p<0.005 respectively, using a repeated measures ANOVA.

4.5 The effect of wheat fibre and inulin on serum markers of CVD risk

4.5.1 Circulating lipids

It was important to determine any changes in lipid profiles occurring as a result of the dietary interventions.
Total cholesterol

Fasting total cholesterol concentrations were measured as described in 2.2.6.1. Figure 4.15 illustrates that the mean of total cholesterol concentration varied between 5.17 mM at baseline and 4.97, 4.88 and 5.15 mM following the control, wheat fibre and inulin interventions respectively and there were no significant differences between treatment interventions, or between the baseline and any intervention.

![Figure 4.15: Fasting total cholesterol concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin.](image)

**Figure 4.15**: Fasting total cholesterol concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting total cholesterol was measured in each participant (n = 10) at baseline (●) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using an IL Test™ cholesterol kit on an iLab autoanalyser. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

High density lipoprotein cholesterol

Fasting HDL cholesterol concentrations were measured as described in 2.2.6.1. No significant differences in fasting HDL concentration were found with baseline levels.
at 1.22mM and levels post each intervention at 1.17, 1.14 and 1.24mM for the control, wheat fibre and inulin interventions respectively (figure 4.16).

Figure 4.16: Fasting HDL cholesterol concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting HDL cholesterol was measured in each participant (n = 9-10) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using a direct HDL cholesterol kit on an iLab autoanalyser. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

**Triacylglycerides**

Fasting TAG concentrations were measured at baseline and after each 28 day intervention as described in 2.2.6.1. Figure 4.17 shows that, although the wheat fibre increased TAG concentrations, there were no significant differences in fasting TAG concentration between treatment interventions, or between the baseline and any intervention.
Figure 4.17: Fasting TAG concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting TAG was measured in each participant (n = 7-8) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using an IL Test™ triacylglycerides kit on an iLab autoanalyser. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

Low density lipoprotein

LDL cholesterol was calculated, as described in 2.2.6.1, from the measurements of total cholesterol, HDL cholesterol and triacylglycerides, using the Friedewald equation (Friedewald W 1972). No significant differences were determined due to any treatment interventions, data not shown.

Non-esterified fatty acids

Fasting NEFA concentrations were measured at baseline and after each 28 day intervention of wheat fibre, inulin and control products, as part of the lipid profile. No
significant differences were observed with mean values at baseline, control, wheat fibre
and inulin at 0.4247, 0.5244, 0.4706 and 0.4783mM respectively (figure 4.18).

Figure 4.18: Fasting NEFA concentrations at baseline and after each 28 day dietary
intervention treatment of control, wheat fibre and inulin. Fasting NEFA
concentrations were measured in each participant (n = 8) at baseline (▲) and after each
28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using
an iLab autoanalyser. Individual values are displayed with mean ± standard error. No
significant differences were found using repeated measures ANOVA.

As post-prandial NEFA responses can change over time in response to changes in
diet, participant’s NEFA concentrations were measured before, and for 120 minutes at 15
minute intervals after, ingestion of a standard glucose test drink (containing 75g of
glucose in water) to reflect post-prandial responses (see figure 4.19). This occurred at
baseline and after each of the 28 day treatment interventions of wheat fibre, inulin and
control products. The control diet has the highest concentrations of NEFA at every time
point so areas under the curve (AUC) were calculated for each treatment intervention to
investigate the physiological NEFA response. The AUC at baseline was 31.19, with
values of 31.59, 26.33 and 24.29 for the control, wheat fibre and inulin treatment interventions respectively. No significant differences were shown between baseline and interventions, or between any interventions.

Figure 4.19: Post-prandial NEFA responses, measured at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Post-prandial (75g glucose in water) NEFA concentrations were measured before a standard 75g glucose drink and at 15 minute intervals for 120 minutes afterwards, to represent post-prandial responses. These post-prandial measurements were taken in each participant (n = 8) at baseline (■) and after each 28 day dietary intervention treatment of control (■), wheat fibre (■) and inulin (■) using an iLab autoanalyser. Means displayed ± standard error.

4.5.2 Insulin sensitivity

Insulin insensitivity or resistance, as suggested by circulating levels of insulin and glucose, are important to measure as surrogate markers for CVD risk as the two conditions are strongly related.
Insulin

Fasting insulin concentrations were measured at baseline and after each 28 day intervention of wheat fibre, inulin and control products as described in 2.2.6.2. Figure 4.20 demonstrates that the wheat fibre and inulin interventions raised the concentration of insulin to 93.25 and 83.47pM respectively compared to the baseline at 79.64pM and the control at 77.07pM, although there were no statistically significant differences.

![Graph showing fasting insulin concentrations](image)

**Figure 4.20:** Fasting insulin concentrations at baseline and after each 28 day dietary intervention treatment of control, wheat fibre and inulin. Fasting insulin concentrations were measured in each participant ($n = 8$) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using a human insulin specific radio-immuno assay. Individual values are displayed with mean ± standard error. No significant differences were found using a repeated measures ANOVA.

Post-prandial insulin responses can alter in response to diet and signify alterations in insulin sensitivity and the ability to process glucose. Insulin concentrations, therefore, were measured before, and at 15 minute intervals for 120 minutes after, ingestion of a standard glucose test drink (containing 75g of glucose in water) to reflect post-prandial
responses (figure 4.21). This occurred at baseline and after each of the 28 day treatment interventions of wheat fibre, inulin and control products. Peak concentrations reached varied between interventions, as did the time taken to reach them. AUC were calculated at baseline and the control, wheat fibre and inulin interventions and found to be 69341, 69577.8, 62807 and 65885.6 respectively. No significant differences were shown between interventions, or between any intervention and baseline.

![Graph showing post-prandial insulin responses](image)

Figure 4.21: Post-prandial insulin responses, measured at baseline and after each 28 day dietary intervention treatment of wheat fibre, inulin and control. Post-prandial (75g glucose in water) insulin concentrations were measured before a standard 75g glucose drink and at 15 minute intervals for 120 minutes afterwards, to represent post-prandial responses. These post-prandial measurements were taken in each participant (n = 8) at baseline (■) and after each 28 day dietary intervention treatment of control (■), wheat fibre (■) and inulin (■) using a human insulin specific radio-immuno assay. Means displayed ± standard error.

As differences in insulin responses were suggested from the post-prandial curves, the peak insulin concentration and the time take to reach this peak post-prandially were further investigated. The mean maximum postprandial insulin concentration (C-max) reached in the 120 minutes after ingestion of 75g glucose in water per treatment intervention group was compared between groups. Although each intervention treatment
reduced the insulin C-max when compared to baseline, no significant differences were found (data not shown). The mode times to maximum insulin concentration (T-max) per group are as follows: baseline 45, wheat fibre 60, inulin 30 and control 60. The mean T-max values for each treatment intervention were compared and although this was increased post-wheat fibre and decreased post-inulin, no significant differences were found (data not shown).

Glucose

As an indication of insulin sensitivity, fasting glucose concentrations were measured described in 2.2.6.2. Although levels increased from 5.325mM at baseline to 5.588, 5.528 and 5.491pM by the control, wheat fibre and inulin interventions respectively, no significant differences were observed between groups (figure 4.22).

![Figure 4.22: Fasting glucose concentrations at baseline and after each 28 day dietary intervention treatment of wheat fibre, inulin and control. Fasting glucose concentrations were measured per participant (n = 8) at baseline (▲) and after each 28 day dietary intervention treatment of control (●), wheat fibre (♦) and inulin (▼) using](image-url)
The physiological ability to remove glucose from the circulation post-prandially is, in part, an indication of responsiveness to insulin. Therefore, participants' glucose concentrations were measured before, and for 120 minutes at 15 minute intervals after, ingestion of a standard glucose test drink (containing 75g of glucose in water) to reflect post-prandial responses (figure 4.23). Measurements occurred at baseline and after each of the interventions of wheat fibre, inulin and control products. The control intervention shows the highest concentrations of glucose beyond 30 minutes and the wheat fibre appears to be very low throughout. AUC were calculated for each treatment intervention to further investigate these differences and were found to be 890, 865.5, 927.3 and 987.9 for the baseline, control, wheat fibre and inulin groups respectively, with significant differences shown between interventions, or between any intervention and baseline.

![Graph showing post-prandial glucose responses](image-url)

**Figure 4.23**: Post-prandial glucose responses, measured at baseline and after each 28 day dietary intervention treatment of wheat fibre, inulin and control. Post-prandial (75g glucose in water) glucose concentrations were measured before a standard 75g glucose drink and at 15 minute intervals for 120 minutes afterwards, to represent...
post-prandial responses. These post-prandial measurements were taken in each participant \( n = 8 \) at baseline (■) and after each 28 day dietary intervention treatment of control (■), wheat fibre (□) and inulin (□) using an iLab autoanalyser. Means displayed ± standard error.

As the maximum glucose concentrations reached and the time taken to reach these peaks varied between intervention groups, these data were further investigated. The mean maximum postprandial glucose concentration (C-max) reached in the 120 minutes after ingestion of 75g glucose in water per treatment intervention group was compared between groups. No significant differences were found between the C-max values between the intervention groups (data not shown). The mode glucose T-max values in minutes reached in the 120 minutes after ingestion of 75g glucose in water per treatment intervention were as follows: baseline 45, wheat fibre 30, inulin 60 and control 60. The mean T-max was compared between groups showing higher values post-control and inulin treatment compared to baseline and post-wheat fibre, but these differences were not significant (data not shown).

4.6 Discussion

The aim of this study was to investigate the effects of wheat fibre and inulin on CVD risk markers in a population group at risk of CVD but without clinical symptoms. Wheat fibre and inulin were representative of insoluble and soluble fibre respectively. Approximately 9 to 17% ingested soluble fibre and 5 to 20% of insoluble fibre is anaerobically fermented to SCFAs in the colon (Cook S 1998). SCFAs have been posed as anti-inflammatory agents and investigations of these products, therefore, may reflect the activity of SCFAs (Tedelind S 2007). It was hypothesised that these high fibre diets would reduce CVD risk as reflected by altered levels of the markers investigated. Since
this study used a cross-over design, it was assumed that any differences seen between each intervention treatment and the baseline occurred as a result of the intervention product. In addition, a metabolomic approach was used to investigate the effects of such diets on the circulating metabolic profiles.

**Haemostatic markers**

As CVD and atherosclerosis are inflammatory disorders reflecting endothelial dysfunction, it was imperative to investigate the circulating levels of both haemostatic and inflammatory markers. Levels of tPA-1 were expected to decrease in response to a protective intervention, as was shown with 52 week interventions of both low carbohydrate and low fat diets when accompanied by significant weight loss, in 49 overweight or obese participants (aged 50.0 ± 1.1 years, BMI 33.7 ± 0.6kg/m², (Wycherley T 2010)) although this was not seen to be the case here. As outlined in chapter 3, this could be because there was no significant impact of the intervention products or because the interventions themselves were not extensive enough. Alternatively, tPA-1 may not a suitable marker although this is unlikely given its high sensitivity and it’s putative role as a CVD risk marker (Ridker P 2000). Weight loss may be the key factor in reduction of circulating tPA-1 levels. Concentrations of tPA-1 were slightly, but not significantly altered by each intervention treatment, when compared to baseline. All mean values varied between 2.39 and 2.69ng/ml which was considerably lower than the range for healthy adults of 7.77 ± 1.63ng/ml, and for adults with proven CVD of 9.3 to 9.67± 1.55ng/ml (Jansson J 1993; Gram J 2000) potentially suggesting that fibrinolysis and clot dissolution were at healthy levels. These data were also below those concentrations of tPA-1 determined in the Pilot WISE study, potentially reflecting a
low level of coagulation and thus protective effects on CVD risk. The participants of the FITMA trial were homogenously male, whereas post-menopausal women were involved in the Pilot WISE trial, which may have altered the levels. Alternatively, the lower tPA-1 levels of the FITMA trial could be due to the participants’ lower average age of 39.8 ± 3.0 years compared to that of the Pilot WISE trial of 48.4 ± 11.7 years. These data were in agreement with similar randomised controlled dietary intervention studies investigating the effects of high whole grain and therefore high fibre diets on markers of cardiovascular risk which found no impact on circulating levels of the tPA-1 inhibitor, PAI-1 (Andersson A 2007; Brownlee I 2010).

tPA-1 and vWF have opposing functions associated with fibrinolysis and coagulation respectively within the coagulation cascade. The literature cites vWF levels of 1.23 ± 0.50IU/ml in healthy controls, rising significantly to 1.47 ± 0.66 U/ml in participants having suffered ischaemic stroke (Bongers T 2006), whereas, other studies have determined healthy levels of vWF as 1.2 to 1.3IU/ml (Rumley A 1998; Wiman B 2000). The circulating levels found in the current research were consistently above both the ‘low’ and ‘high risk’ levels found in the literature, potentially reflecting a high level of coagulation. Levels, therefore, were hypothesised to reduce in response to a protective dietary intervention. This has been reported in the literature where reductions in circulating vWF levels were observed after a 28 day high mono-unsaturated fat diet (p<0.001 (Perez-Jimenez F 1999)) suggesting that modification of the fat content of the diets would have improved responses.

Although concentrations of the pro-coagulant vWF were increased slightly by the control treatment and reduced slightly by the whole intact and whole milled grain
treatments, none of these changes were statistically significant. Concentrations of vWF were hypothesised to reduce after the active diets. Such a response was observed in a study of men with angiographically proven coronary atherosclerosis who consumed a lipid-lowering diet for three years and showed significant reductions in circulating vWF when compared to controls on a control habitual diet (Blann A 1995). These participants also showed reductions in serum total and LDL cholesterol and TAGs, and interestingly their diets were significantly higher in fibre than those of the control group. Another high fibre whole grain dietary intervention study did show significantly lower levels of both vWF and fibrinogen after the intervention diet when compared to a control diet and a non-significant trend towards a reduction in the alternative coagulation marker factor VIII (Vuksan V 2007). These discrepancies may be due to the longer intervention period of 12 weeks in that research, or the increased average age of participants (64 ± 8 years) as age is a further risk marker for CVD.

**Inflammatory factors**

CVD and the underlying pathology, atherosclerosis, are inflammatory disorders, marked by increased circulating concentrations of soluble inflammatory factors such as sICAM-1, IL-8, IL-6 and CRP, thus highlighting these markers for investigation in the current research. Levels of endothelial adhesion molecules were hypothesised to decrease in response to the protective effects of the intervention diets as was observed in response to a very low carbohydrate diet, with concurrent significant reductions in E-selectin and body mass but not VCAM-1 (Wycherley T 2010) and to high fat diets and high exercise interventions with concurrent changes in PAI-1 (Eriksson M 2008).
The concentration of sICAM-1, however, did not alter significantly in response to any of the dietary interventions of the FITMA trial, with levels falling within the normal circulating range found in healthy adults of 115 to 306ng/ml (Glurich I 2002). The results suggest that the intervention diets did not have a significant impact on sICAM-1 levels as a marker of CVD risk, perhaps because they were not significantly raised at baseline and that the addition of weight loss or exercise into the study design may promote reductions in this marker.

Although the control intervention appears to have reduced the concentration of the inflammatory mediator IL-8, the whole intact and whole milled grain products reversed this effect and increased levels slightly beyond those found at baseline. No significant differences, however, were seen between any treatment interventions, or when compared to baseline. Levels of IL-8 in the literature are quoted as 5.6 ± 0.6pg/ml in obese subjects (Bruun J 2005) which correlates with the results of this study. As higher levels of IL-8 have been identified in patients with coronary artery disease (CAD) (Jha H 2009), protective effects of an intervention would be expected to reduce circulating levels of the inflammatory marker IL-8, although this was not seen to be the case. This may be because levels were not significantly raised above those expected when measured at baseline. As outlined in chapter 3, significant reductions in IL-8 concentration were observed due to a ten week intervention of bespoke ‘Old Italian Grain’ bread (Sofi F 2010), perhaps suggesting that the length of the intervention may need to be increased.

Circulating levels of the alternative inflammatory marker IL-6 were undetectable but would have been expected to fall in response to a protective intervention as was observed in response to a very low calorie diet in overweight women, when accompanied
by weight loss (Bastard J 2000). The additional intervention of weight loss could, therefore, aid reduction of this circulating marker. The results of a high fibre whole grain intervention study similar to the current research investigating circulating levels of IL-6 support these results, reporting no significant changes post-intervention (Andersson A 2007).

The current research, although showing inter- and intra-individual differences, as expected with such a highly sensitive and extremely variable marker of inflammation, showed no significant differences in mean concentrations due to the interventions, possibly suggesting a longer intervention period was required. Data were within the ranges of circulating concentrations of CRP for healthy subjects (1.68 ± 1.42mg/l) (Glurich I 2002) which was unexpected. As the population group investigated displayed other risk markers for high CVD risk, it was thought that levels of CRP would also reflect a high risk state. Levels of this sensitive inflammatory marker would be expected to fall in response to cardio-protective interventions such as high fibre diets but this was not seen in the current research, however, perhaps because the levels were not raised at baseline so could not be reduced by a protective intervention. These results are supported by those of Andersson et al who showed no changes in concentrations of circulating CRP in response to high whole grain diets (Andersson A 2007). In further agreement, fasting CRP levels also showed no significant change in response to an eight or 12 week high whole grain diet of 60g/day whole grain (Brownlee I 2010). Dietary supplementation with high fibre whole grains also showed no significant change on circulating CRP levels in type II diabetic patients, even considering that their baseline levels were higher than those of the current study (Vuksan V 2007). Interestingly, data from Ajani et al found a
correlation between fibre in-take and circulating CRP concentrations (Ajani; Ajani U 2004) in disagreement with the current research. That data was from a prospective National Health and Nutrition Survey, though and not from a randomised controlled dietary intervention trial so no cause and effect can be proven.

Metabolomics

The aim of this non-targeted LC/MS based metabolomic investigation was to demonstrate the impact of diets high in wheat fibre or inulin on metabolite profiles and to investigate potential biomarkers for such dietary regimens. PCA could not discriminate between samples when comparing each of the four groups of baseline, control, wheat fibre and inulin intervention products. This is not uncommon in nutritional studies, as PCA may not be sensitive enough to discriminate between groups in which ions display a high degree of variation with only minimal differences between those groups, or between groups where only a small percentage of the total number of ions display differences (Kemsley E 2007). The data was analysed further by establishing a four component PLS-DA model, ($R^2$ and $Q^2$ model parameters of 0.363 and 0.715 respectively), which partially discriminated between sample groups indicating modest dietary-induced changes to the metabolome.

Following confirmatory analysis the intensities of three discriminatory species were found to be statistically significantly different between intervention groups, confirming that the effects on individual species following the dietary interventions were genuine. Assignments were based on the closest matches to parent ions on metabolite databases. This could be further confirmed by comparison of the ion’s mass spectroscopy
behaviour with that of pure standards. Unfortunately, this was not possible within the scope of the current research due to time and financial constraints.

In the current study, significant differences were seen between groups in three lysophosphatidylcholine (lysoPC) species (18:0, 22:6 and 18:2). This may simply reflect an effect of participation in a trial, rather than the intervention products themselves although this is unlikely as changes have been observed between active intervention arms of the trial. The data suggest that dietary supplementation with the control, wheat fibre and inulin products influences phospholipid metabolism, altering circulating concentrations of LysoPCs.

These data agree with the principal metabolomic profiling results of Lankinen et al, who also showed a significant effect on circulating levels of multiple LysoPC species, including (18:0) and (18:2), after a dietary carbohydrate modification including wheat bread. Lankinen et al investigated the metabolic profiles of 20 overweight or obese participants aged 55 ± 6 years with BMI values of 32 ± 3.8kg/m², that is, a similar but higher risk participant population than the FITMA trial investigated. In contrast to the current study, the investigational diet using wheat bread also included oats and potatoes, which could have accounted for the slight differences seen between the LysoPC profiles of the two trials.

LysoPCs are products of phospholipid metabolism and are formed predominantly through the action of either phospholipase A₂ (PLA₂) on biological membranes (Aiyar N 2007), or lecithin cholesterol acyl transferase (LCAT) on lipoproteins, or during
oxidative modification of LDL (Kougias P 2006). LysoPCs have specific actions including a pro-inflammatory activity and the capacity to activate a variety of cell lines (Ojala P 2007). LysoPCs are also known mediators of endothelial function (Kougias P 2006), capable of inducing endothelial dysfunction. They have been evidenced as the major bioactive lipid component of oxLDL, potentially being responsible for certain of oxLDL's inflammatory effects (Aiyar N 2007). They have also been shown to induce platelet activation (Murohara T 2002) and migration of smooth muscle cells (Kohno M 1998) and are, thus, linked to the underlying pathogenesis of CVD, with an increase in circulating levels indicating a pro-inflammatory state. The non-consistent results show inulin significantly increased the concentrations of two of the LysoPC species and reduced the concentrations of one other, with no effect shown to be due to wheat fibre, which is not suggestive of a protective effect.

In conclusion, the intervention diets significantly altered circulating levels of three ion species, putatively defined as the LysoPCs (22:6, 18:0) and (18:2), suggesting, in agreement with previous research, that diets high in wheat fibre or inulin can influence metabolite profiles (Lankinen M 2010). Metabolomics is an emerging technique in nutritional research, showing increasing use (Lodge J 2009) and the current study is one of only a limited number of reports using metabolomics to investigate the impact of dietary modifications in human intervention studies. The current research highlights the potential of metabolomic technology in nutritional research, for example, in defining alternative functions for nutrients and in elucidating how diet influences metabolic pathways, and offers potential for use in determining personalised dietary interventions for the prevention or management of obesity or disease.
To confirm identities of discriminatory ions, pure compounds could be purchased when possible and analysed under identical conditions to that of the plasma samples, including spiking of original samples. Comparison of molecular ion and fragmentation patterns of the standards and the associated peak in original samples, retention time and sample spiking could then confirm the assignments. Further confirmatory analysis of the ions was beyond the scope of this research, due to time limitations, and as such, assignments were putative.

Circulating lipids

Circulating total cholesterol levels have long been associated with atherosclerosis and CVD risk (Stamler J 1986), as posed in the Cholesterol Hypothesis, and thus are central to such research. Fasting circulating total cholesterol levels showed no significant differences either when compared to baseline or between the intervention products. Each group’s mean total cholesterol concentration was above the 4.0mM recommendation for those with heightened risk of CVD, falling within the ‘ideal’ and ‘mildly high ranges’ (JBS2 2005). As total cholesterol is reflective of CVD risk, this reflects an increased risk in the population group investigated. A protective effect of the diets would be expected to manifest as a reduction in total cholesterol. As no significant changes in total cholesterol were found it was assumed that this did not reflect a significant change in CVD risk due to the intervention diets.

Investigation of the fasting circulating levels of the individual components of total cholesterol gives a more detailed reflection of risk. Means of HDL at baseline and after each intervention were above the recommended level of 1.0mM (JBS2 2005), suggesting a protective effect afforded by HDL on CVD risk, however, this could have been a
reflection of the increased total cholesterol levels in this group. The mean levels of fasting TAG at baseline, of 1.68mM, was on the cusp of the ‘ideal’ range of <1.69mM (JBS2 2005) suggesting a slightly increased risk. The mean NEFA concentrations fell within the ranges cited in the literature for healthy adults, of 0.44 ± 0.17mM (Pedrini M 2006), suggesting that the population group under investigation was not at increased risk of CVD according to this marker at baseline or post-interventions. Fasting concentrations of HDL cholesterol and TAG showed no significant changes due to the interventions and as concentrations of LDL cholesterol were calculated from measured concentrations of total and HDL cholesterol and TAG, it was expected, and observed, that no significant differences were determined in LDL concentrations. Concentrations of total and LDL cholesterol, TAG and NEFA were hypothesised to fall in response to protective interventions such as whole grain and circulating HDL levels were hypothesised to rise. Such protective effects in certain lipid markers were shown in previous research in response to a high performance inulin intervention, oat bran, calorie restricted legume-based diet and a wheat whole-grain based dietary product (Whyte J 1992; Letexier D 2003; Rave K 2007; Hermsdorff H 2010). Participants in those interventions, however, displayed a higher risk profile at baseline than those of the current research, including obesity and elevated blood glucose and cholesterol levels which would be expected to be more responsive to dietary influence than levels associated with healthy individuals. Weight loss was also observed and levels of certain intervention products were higher than of the current research, suggesting possible methodological changes for future work (i.e. higher baseline risk profiles, the addition of weight loss to the intervention and the possible need to increase the amount of intervention products used).
Despite finding a significant reduction in plasma TAG levels, Letexier et al found no significant differences in fasting total, HDL or LDL cholesterol levels due to a three week 10g/day high-performance inulin dietary cross-over study in eight healthy participants (Letexier D 2003), thus supporting the current research. Jackson et al investigated the effects of 10g inulin per day for eight weeks on healthy participants with moderately raised total cholesterol and TAG levels (Jackson K 1999). No significant changes were observed in total, HDL or LDL cholesterol despite the moderately raised total cholesterol at baseline. Results did show a significant reduction in circulating free fatty acids after an eight week inulin intervention, but this result was also shown in the control group, suggesting it may have been a result of participation rather than the inulin itself. Concentrations of TAG, however, did show a significant reduction, suggesting that moderately raised levels are potentially more responsive to beneficial interventions such as inulin and that higher risk individuals might be more responsive to such interventions in future research.

A six week high inulin dietary intervention in 25 participants with mild/moderate hypercholesterolaemia aged 30 to 75 years also showed no significant changes in total, HDL or LDL cholesterol or TAGs, despite the baseline hypercholesterolaemia (Davidson M 1999). The inulin, however, was provided naturally contained within commonly available foods, rather than in the controlled delivery system used in the current research.

These results are further supported by other previous dietary interventions which found no change in LDL, HDL or TAG levels in response to high fibre whole grain diets (Andersson A 2007; Vuksan V 2007) or high inulin diets (Davidson M 1999). Brownlee et al also observed no significant changes in total, HDL or LDL cholesterol, TAG or
NEFA in response to a high fibre high whole grain diet in further agreement with this study (Brownlee I 2010).

These data also agree with those of Andersson et al and Brownlee et al who showed no significant changes in levels of circulating NEFA concentrations due to high fibre high whole grain diets, with similar circulating concentrations to the current research (Andersson A 2007; Brownlee I 2010). Further supportive evidence is provided by Letexier et al who showed no significant change in NEFA in response to a six week high inulin diet (Letexier D 2003). (Jackson K 1999).

**Insulin sensitivity**

Fasting and post-prandial insulin and glucose levels were determined as markers of insulin resistance and, therefore, the risk of type II diabetes, a major co-morbidity of CVD. Levels of insulin and glucose were hypothesised to fall in response to the protective diets of wheat fibre and inulin as was shown in overweight hyperinsulinaemic adults after a six week whole grain intervention (Pereira M 2002) and a high fibre diet (Chandalia M 2000) respectively. This was not observed, however, in the current research which showed no significant differences in fasting or post-prandial insulin. These differences between results may have been due to their higher baseline levels of insulin in Pereira’s participants, and the higher mean age and BMI of Chandalia’s participants, which could be more responsive to change than the lower levels seen in the current research. The same study also showed no significant reduction in fasting glucose, in agreement with the current study.

In agreement with these results, circulating insulin and glucose concentrations were found not to change in response to a six week high fibre high whole grain
intervention (Andersson A 2007) or a six week high inulin diet (Letexier D 2003). A further randomised controlled intervention trial, showing lower fasting concentrations of insulin compared to the data shown here, also showed no significant change in response to high whole grain diets (Brownlee I 2010). Brownlee et al also used a qualitative insulin sensitivity check index to further investigate insulin resistance and no significant changes were shown. A high fibre 12 week whole grain intervention in type II diabetics showed no significant reduction in fasting insulin levels (Vuksan V 2007) with baseline levels higher of those found in the current research.

Although Pereira et al observed a significant reduction in fasting insulin after a six week whole grain intervention, no significant changes were observed in insulin AUC (Pereira M 2002). Andersson et al analysed insulin sensitivity using a euglycemic hyperinsulinaemic clamp and showed no change in response to a six week high whole grain intervention (Andersson A 2007).

In conclusion, wheat fibre and inulin have been shown to alter the levels of three LysoPC species but have no further significant impacts on the blood markers investigated in a population group at risk of CVD.

**Trial limitations**

As outlined in chapter 3, certain limitations are common to all human interventions, such as population sampling, population size and compliance. Men only were chosen to participate in the FITMA trial to homogenise the small group involved (n = 10). Participants were also all Caucasian, well educated, university associated Surrey residents. Unfortunately, this limits the relevance of the results to women and the wider
population, with implications for future research. Due to the increased requirements placed on participants in the FITMA trial, it was necessary to limit the number of participants included to 12, with ten participants successfully completing the trial. This was sufficient for the pilot research but would ideally be increased in future work.

Although participants were at risk of CVD, as defined by their sex and age (men aged 30 to 60 years) and high BMI values (25 – 35 kg/m², Christie D 1981, Herman B 1982, Nathan L 1997, Kenachaiah S 2002) they were not at high risk and, as such, a high risk profile was not reflected in their baseline makers measured for change within the study. Had higher levels been present it is likely these would be more responsive to the beneficial interventions, thus suggesting that any similar future work might benefit from involving higher risk participants.

The cross-over design of the study controlled for inter-individual differences and increased the power of the data but required a reduction in intervention length to 28 days compared to the Pilot WISE trial. Increasing the length of the trial may have enhanced changes in risk markers. For example, other studies have shown significant differences in certain circulating risk markers after longer interventions (Jackson K 1999; Letexier D 2003), however, no significant differences were observed in the longer eight week interventions of the Pilot WISE trial.

The FITMA trial required data from ten participants to be fully powered as the original sample size of n=12 allowed for 10% of the subjects to be non-evaluable in each treatment group. This was based on a detecting a clinically significant drop in insulin of 20pM (a difference to detect of 25%) between the groups with at least 80% power,
assuming that the common standard deviation is 16\text{pM} or 20\% and using a two sample t-test with a 0.05 two sided significance level. The estimate of the standard deviation was based on published data for subjects who took part in a Resistant Starch Study of a similar design conducted by Robertson et al (2005). Ten participants successfully completed the trial as so power was achieved.'

Compliance was reported as slightly better in the FITMA than the Pilot WISE trial. This may be due to the shorter intervention periods which, although totalled 84 (3 \times 28) days, were only 28 days long individually. Also, the intervention products used in the FITMA trial were reportedly more palatable than those of the Pilot WISE trial as they were developed with feedback from participants in that study. The delivery system, of providing the bespoke bread roll-like intervention products to be included in the diet, was widely very well accepted and proved very easy to monitor for compliance.

Interestingly, unlike in the Pilot WISE trial, participants did not report altering their habitual dietary intake or exercise as a result of participation. This is potentially due to better general acceptance of the FITMA intervention products which were very similar in appearance and organoleptic qualities to commercially available bread and, therefore, didn’t highlight participation in a dietary intervention trial. Monitoring exercise, or adding a structured exercise protocol for participants, may still be valuable for future work.

It is not possible to conclude, therefore, that either soluble or insoluble fibre, represented in this research by wheat fibre and inulin respectively, actively reduce CVD risk markers in an at risk population.
Chapter 5

The effect of whole grain fermentation products on the vascular endothelium at the cellular level
Endothelial dysfunction is a major player in atherosclerosis, the underlying pathogenesis of CVD, and whole grain diets are associated with decreased risk of this disease. The components of a whole grain diet would never interact with the endothelium under normal physiological circumstances but the breakdown products of such diets could.

Pathology of the vascular endothelium is associated with haemostasis and is reflected by altered levels of the haemostatic molecules, von Willebrand factor and tissue plasminogen activator-1 which play major roles in regulating the clotting and fibrinolytic pathways, respectively. Alterations in the levels of these factors secreted by endothelial cells may, therefore, be indicative of endothelial dysfunction. The cellular adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) are known to vary with inflammation with endothelial cell surface expression of ICAM-1 and VCAM-1 increasing during inflammation and surface levels of PECAM-1 decreasing. In addition, endothelial derived levels of interleukin-8 (IL-8) and nitric oxide (NO) alter during inflammation and have been proposed as markers of the inflammation associated with CVD.

The short chain fatty acids acetate, propionate and butyrate are the products of fermentation of fibres by the colonic micro-flora and have been shown to have anti-inflammatory effects in vitro (Tedelind S 2007). Butyrate is predominantly metabolised by the colonocytes, for which it provides the major source of fuel, but acetate and...
propionate pass into the circulation and thus come into direct contact with the cells of the vascular endothelium.

The SCFAs were made up in RO water to the appropriate concentrations and added to the cell medium. To detect and control for any possible changes caused by the water, both a medium alone and a medium and water control were used. The true control, treated with medium alone, is referred to as ‘Control’ or ‘C’; this is unactivated. All other samples are activated. Of these, the ‘Medium’ or ‘M’ control is treated with medium alone and the ‘Water’ or ‘W’ control is treated with medium and water.

SCFAs occur in the colon at mM concentrations (Tedelind S 2007) but are absorbed to a great extent by the colonocytes associated with enhanced sodium absorption and bicarbonate secretion. Therefore, concentrations ultimately found circulating in the blood and interacting with the endothelial cells are significantly lower than this. Concentrations of propionate were found to range from $5 \pm 4 \, \mu M$ in the peripheral blood to $21 \pm 21 \, \mu M$ in the hepatic blood. Acetate ranged from $70 \pm 41 \, \mu M$ in the peripheral blood to $115 \pm 63 \, \mu M$ in the hepatic blood (Cummings J 1987). Ranges of test SCFAs chosen to represent these levels in the blood were 0.1, 1.0, 10.0 and 100.0μM.

It is thought that the association between high whole grain diets and low levels of CVD may be due to the protective effects of the SCFAs produced as a result of the breakdown of whole grains. The SCFAs acetate, propionate and butyrate have been shown to have anti-inflammatory properties when investigated in vitro (Tedelind S 2007). Therefore, the aim of this research was to investigate the potential anti-inflammatory effect of whole grain fermentation products, the short chain fatty acids sodium acetate and propionate, on endothelial cell function. It was hypothesised that treatment of
endothelial cells with sodium acetate and propionate would act in opposition to activation by TNF-α/IFN-γ, thus reducing markers of dysfunction.

In this chapter, we have investigated the effects of the SCFAs on both a cell line, human microvascular endothelial cells (HMEC-1), and in the more physiologically relevant model, primary human coronary artery endothelial cells (HCAEC).

### 5.2 The effect of SCFAs on endothelial cell secreted inflammatory mediators

#### 5.2.1 Interleukin-8

The concentration of IL-8 secreted by HMEC-1 cells and HCAECs treated with medium alone, TNF-α/IFN-γ and by cells treated with TNF-α/IFN-γ and further treated with sodium acetate or sodium propionate (0.1, 1.0, 10 or 100μM) or with a water control was determined as described in 2.2.1.6.

**HMEC-1**

In HMEC-1 cells, treatment with TNF-α/IFN-γ caused a higher secretion of IL-8 from 5.0 to 11.8pM/ml, however, this difference was not statistically significant. Similarly, the levels of IL-8 secreted by cells treated with the water control were not significantly different from cells treated with medium alone. Treatment of TNF-α/IFN-γ activated cells with acetate at 0.1 and 1.0μM had no impact on the secretion of IL-8 whereas treatment with 10 and 100μM acetate, and all concentrations of propionate investigated, resulted in lower secretion of IL-8 compared to the water control (figure 5.1), however, these differences were not significant.
Treatment of HMEC-1 cells. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of IL-8 secreted into the supernatant was determined by an in-house ELISA. Data are displayed as mean ± standard deviation of 3 experiments. No significant differences were found using a two way ANOVA test.

HCAEC

Treatment of HCAECs with TNF-α/IFN-γ significantly increased the secretion of IL-8 from 1.2 to 5.0pM/ml (p<0.05). Similarly, the levels of IL-8 secreted by cells treated with the water control were significantly greater compared to cells treated with medium alone to 4.9pM/ml (p<0.05). Treatment of TNF-α/IFN-γ activated cells with acetate gave lower secreted levels of IL-8, but this difference was not significant. Treatment with propionate showed a lower IL-8 secretion at 0.1mM, but no differences at the higher concentrations. No differences were statistically significant when compared to the water control (figure 5.2).
Figure 5.2: IL-8 secreted by HCAECs. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of IL-8 secreted was determined by an in-house ELISA. Data are displayed as mean ± standard deviation of 5-6 experiments where * denotes p<0.05 using a two way ANOVA test.

5.2.2 Nitric oxide

Preliminary work was carried out in the form of a pilot study in EA.hy926 an ‘endothelial like’ cell line derived from the fusion of human umbilical cord endothelial cells and the rat carcinoma cells A549. A time course was run to determine the optimum time to measure nitrite concentration, as a marker of nitric oxide secretion following stimulation with TNF-α/IFN-γ and nitrite was assayed in the supernatants by Griess kit as described in 2.2.7.5. Subsequently, HMEC-1 cells and HCAECs were treated with medium alone, TNF-α/IFN-γ alone or with TNF-α/IFN-γ and sodium acetate or propionate at 0.1, 1.0, 10 or 100μM and the concentration of nitrite was determined.
Treatment of EA.hy926 cells with TNF-α/IFN-γ did not cause a significant alteration in levels of nitrite over 0, 3, 6 or 12 hours when compared to cells treated with medium alone (figure 5.3). Treatment did significantly alter the levels of nitrite at 24 hours (p<0.0105) and 48 hours (p<0.0237) as determined by an unpaired Students t-test. Although the levels detected in the supernatants from the treated cells were higher than the levels in the supernatants from the cells treated with medium alone, at 72 hours, this difference was not statistically significant. It was decided, therefore, that 24 hour incubations would be used for the purposes of these investigations.

![Figure 5.3: Time course of detection of nitrite in EA.hy926 supernatants. Cells were grown to confluency then treated with either medium alone (■) or 100IU/ml TNF-α and 50IU/ml IFN-γ (■) for 0, 3, 6, 12, 24, 48 or 72 hours. The concentration of nitrite was determined by a Griess reagent kit. Data are displayed as mean ± standard deviation of 3-4 experiments, where * denotes p<0.05 using a paired Student’s t-test.](image)

**HMEC-1**

The concentration of nitrite was determined in the supernatants of HMEC-1 cells treated with medium alone, TNF-α/IFN-γ alone and from cells activated with TNF-
α/IFN-γ and further treated with sodium acetate or sodium propionate (0.1, 1.0, 10 or 100μM) or with a water control for 24 hours. Treatment with TNF-α/IFN-γ increased the concentration of nitrite from 1.3 to 3.5μM, however, this increase was not statistically significant. The levels of nitrite in the supernatants of cells treated with the water control were significantly higher at 4.6μM compared to cells treated with media alone (p<0.0042). Treatment of TNF-α/IFN-γ activated cells with acetate or propionate appeared to reduce the levels of nitrite to differing extents when compared to the water control, although this was not statistically significant (figure 5.4).

![Nitrite Concentration](image)

**Figure 5.4: Nitrite secreted by HMEC-1 cells.** Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of nitrite in the supernatant was determined by a Griess reagent kit. Data are displayed as mean ± standard deviation of 3 experiments, where *** denotes p<0.005 using a two way ANOVA test.
HCAEC

The concentration of nitrite secreted by HCAECs was determined on cells treated with medium alone, TNF-α/IFN-γ alone and on cells activated with TNF-α/IFN-γ and further treated with sodium acetate or sodium propionate (0.1, 1.0, 10 or 100μM) or with a water control. All levels of nitrite, however, were below the level of detection (1μM) of the assay (data not shown).

5.3 Haemostatic secreted factors

vWF and tPA-1 carry out opposing functions in coagulation and fibrinolysis respectively, maintaining the balance associated with haemostasis. Both can be secreted by endothelial cells and increased levels have been associated with inflammation and vascular injury, supporting suggested links to CVD risk.

5.3.1 Tissue plasminogen activator-1

The concentration of tPA-1 secreted by HMEC-1 cells and primary HCAECs was determined as described in 2.2.5.1, after treatment with medium alone, TNF-α/IFN-γ alone and by cells activated with TNF-α/IFN-γ and further treated with sodium acetate or sodium propionate (at 0.1, 1.0, 10 or 100μM) or with a water control.

HMEC-1

Treatment of HMEC-1 with TNF-α/IFN-γ did not significantly affect the levels of tPA-1 detected in the supernatants’ subsequent treatment with water also showed no significant effect on secretion level. When treated with acetate or propionate (at the concentrations described above) no significant changes in the levels secreted when
compared to the water control were observed, with levels remaining below 11ng/ml (figure 5.5).

Figure 5.5: tPA-1 secreted by HMEC-1 cells. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of tPA-1 expressed was determined by an in-house ELISA. Data are displayed as mean ± standard deviation of 3 experiments. No significant differences were found using a two way ANOVA test.

HCAEC

Treatment of primary HCAECs with TNF-α/IFN-γ did not significantly affect the levels of tPA-1 secreted, nor did subsequent treatment with water. Treatment with acetate or propionate at the levels indicated below also had no significant impact on secretion of tPA-1 by these cells (figure 5.6) with levels remaining below 4ng/ml.
Figure 5.6: tPA-1 secreted by HCAECs. Cells were grown to confluency over 72 hours then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of tPA-1 secreted was determined by an in-house ELISA. Data is displayed as mean ± standard deviation of 6 experiments. No significant differences were found using a two way ANOVA test.

5.3.2 von Willebrand factor

An in house ELISA, as described in 2.2.5.1, was used to determine the concentration of vWF secreted by HMEC-1 cells and HCAECs on treatment with medium alone, TNF-α/IFN-γ alone and by cells activated with TNF-α/IFN-γ and further treated with sodium acetate or sodium propionate (at 0.1, 1.0, 10 or 100μM) or with a water control.

HMEC-1

Treatment of HMEC-1 cells with TNF-α/IFN-γ and further treatment of TNF-α/IFN-γ activated cells with water, acetate or propionate at the concentrations tested did
not significantly alter the levels of vWF secreted (figure 5.7) which remained consistently below 0.0013IU/ml.

Figure 5.7: vWF expressed by HMEC-1 cells. Cells were grown to confluency then treated with either medium (C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of vWF expressed was determined by an in-house ELISA. Data are displayed as mean ± standard deviation of 3 experiments. No significant differences were found using a two way ANOVA test.

HCAEC

A drop in the level of secreted vWF after treatment of the cells with TNF-α/IFN-γ was observed, however, this was not significant. Treatment with water, acetate or propionate at the concentrations shown (figure 5.8) did not change the levels of secreted vWF, with levels continuously below 0.051IU/ml.
Figure 5.8: vWF expressed by HCAECs. Cells were grown to confluency then treated with either medium (C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The concentration of vWF expressed was determined by an in-house ELISA. Data are displayed as mean ± standard deviation of 4-5 experiments. No significant differences were found using a two way ANOVA test.

5.4 The effect of SCFA on endothelial cell surface markers

The cellular adhesion molecules ICAM-1, VCAM-1 and PECAM-1 are active in the movement of cells through the endothelium and into the atherosclerotic plaques. The percentage of HMEC-1 and HCAEC cells expressing these surface adhesion molecules was determined after treatment with medium alone, TNF-α/IFN-γ alone for 24 hours and on cells activated with TNF-α/IFN-γ and further treated with sodium acetate or sodium propionate (0.1, 1.0, 10 or 100μM) or with a water control for 24 hours by flow cytometry, as described in 2.2.7.4.
5.4.1 Intercellular adhesion molecule-1

HMEC-1

Forty-two percent of cells treated with medium alone expressed ICAM-1 on their surface, which was significantly increased to 76% upon stimulation with TNF-α/IFN-γ (p<0.005) and stimulation with TNF-α/IFN-γ and further treatment with the water control (p<0.01). No significant difference was observed between cells treated with TNF-α/IFN-γ alone or further treated with water. Treatment of the TNF-α/IFN-γ activated cells with either acetate or propionate at any of the concentrations tested did not significantly alter the percentage of surface ICAM-1 expression. The mean fluorescence intensity of staining (MFI) of the cells treated with medium alone was 47.

On treatment with TNF-α/IFN-γ this rose to 272 which was a significant increase (p<0.01, figure 5.9) and 316 following further treatment with water (p<0.005). The MFI of TNF-α/IFN-γ activated cells, subsequently treated with any concentration used of either acetate or propionate, did not alter significantly from that of the water treated cells.
Figure 5.9: ICAM-1 expression on HMEC-1 cells treated with SCFAs. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The percentage of cells expressing (A) and the MFI of staining (B), were determined by flow cytometry. Values are displayed as mean ± standard deviation of 3 experiments, where ** and *** denote p<0.01 and p<0.005 respectively, when compared to cells treated with medium alone, using a two way ANOVA test.
In HCAECs, treatment with TNF-α/IFN-γ increased surface expression of ICAM-1 from 37% (medium treated) to 59% (TNF-α/IFN-γ treated) although this was not statistically significantly. Further treatment of TNF-α/IFN-γ activated cells with water, acetate or propionate showed no significant change in the expression of ICAM-1 compared to the water control. No significant differences were observed in MFI between any groups (figure 5.10).
Figure 5.10: Expression of ICAM-1 by HCAECs treated with SCFAs. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100µM for 24 hours. The percentage of cells expressing ICAM-1(A) and the MFI of staining (B), were determined by flow cytometry. Values displayed as mean ± standard deviation (n = 3). No significant differences were found using a two way ANOVA test.
5.4.2 Vascular cell adhesion molecule-1

HMEC-1

HMEC-1 cells, treated with TNF-α/IFN-γ increased their surface expression of VCAM-1 from 0.9% (medium treated) to 5.19% (TNF-α/IFN-γ treated) although this was not statistically significant. Further treatment of TNF-α/IFN-γ activated cells with water, acetate or propionate showed no significant change in the expression of VCAM-1 compared to the water control. The MFI of cells treated with medium alone rose non-significantly on treatment with TNF-α/IFN-γ (figure 5.11). No significant difference was observed when TNF-α/IFN-γ activated cells were subsequently treated with water or with any concentration used of either acetate or propionate.
Figure 5.11: VCAM-1 expression by HMEC-1 cells treated with SCFAs. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The percentage of cells expressing VCAM-1 (A) and the MFI of staining (B) as determined by flow cytometry. Values displayed as mean ± standard deviation of 3 experiments. No significant differences were found using a two way ANOVA test.
The expression of VCAM-1 by HCAECs varied slightly but remained below 2% following all treatments. Treatment with TNF-α/IFN-γ and subsequent treatment with water, or the range of concentrations of acetate or propionate used, did not significantly alter the percentage of HCAECs expressing VCAM-1. The MFI of cells treated with medium alone was 4.6. This was not significantly altered by treatment with TNF-α/IFN-γ (figure 5.12). No significant difference was observed when TNF-α/IFN-γ activated cells were subsequently treated with water (MFI of 3.7), acetate or propionate.
Figure 5.12: VCAM-1 and expression by HCAECs treated with SCFAs. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The percentage of cells expressing VCAM-1 (A) and the MFI of staining (B) as determined by flow cytometry. Values displayed as mean + standard deviation of 3-5 experiments. No significant differences were found using a two way ANOVA test.
5.4.3 Platelet endothelial cell adhesion molecule-1

HMEC-1

The percentage of HMEC-1 cells expressing PECAM-1 after treatment with medium alone was 11.4%. This was reduced to 8.1% on treatment with TNF-α/IFN-γ but the change was not statistically significant. Subsequent treatment with water, or the various concentrations of acetate and propionate used, did not significantly affect the percentage of cells expressing PECAM-1 (figure 5.13). Treatment of HMEC-1 cells with TNF-α/IFN-γ did not alter the PECAM-1 MFI. Further treatment with water, acetate or propionate also had no impact on MFI.
Figure 5.13: PECAM-1 expression by HMEC-1 cells treated with SCFAs. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The percentage of cells expressing PECAM-1 (A) and the MFI of staining (B) as determined by flow cytometry. Values displayed as mean + standard deviation of 3 experiments. No significant differences were found using a two way ANOVA test.
HCAEC

Treatment with TNF-α/IFN-γ, and subsequent treatment with water, reduced the percentage of cells expressing PECAM-1 from 27.0 to 18.0% and 22.4% respectively but not significantly. Further treatment with acetate or propionate did not significantly affect the percentage of cells expressing PECAM-1 when compared to the water control. The MFI of PECAM-1 staining of HCAECs was significantly reduced from 8.6 to 4.6 on treatment with TNF-α/IFN-γ and to 4.3 on subsequent treatment with water (p<0.0046, figure 5.14). Treatment with acetate or propionate did not significantly alter PECAM-1 MFI when compared to the water control.
Figure 5.14: PECAM-1 expression by HCAECs treated with SCFA. Cells were grown to confluency then treated with either medium (control, C) or 100IU/ml TNF-α and 50IU/ml IFN-γ for 24 hours. TNF-α/IFN-γ cells were then treated with medium alone (M), water (W), or sodium acetate or propionate at 0.1, 1.0, 10 or 100μM for 24 hours. The percentage of cells expressing PECAM-1 (A) and the MFI of staining (B), were determined by flow cytometry. Values displayed as mean ± standard deviation of 3 experiments, where ** denotes p<0.01 using a two way ANOVA test.
5.5 Discussion

The aim of this study was to investigate the effects of the SCFAs sodium acetate and sodium propionate, the fermentation products of whole grains, on markers of vascular function at the cellular level.

Endothelial cell secreted inflammatory mediators

It was hypothesised that the SCFAs would have anti-inflammatory effects based on previous research looking at the effects of acetate, propionate and butyrate on cytokine release from human neutrophils. This showed a decrease in LPS-stimulated TNF-α as a result of incubation with 30mM of each SCFA independently (Tedelind S 2007). Indeed, HMEC-1 cells activated with TNF-α/IFN-γ secreted higher levels of the inflammatory cytokine IL-8 than those not stimulated, although this was not significantly different. This enhanced secretion was maintained upon further incubation of the cells with the water control and levels remained high after treatment with low concentrations of acetate but was lower (non-significantly) upon treatment with higher concentrations of acetate and all concentrations of propionate. As an inflammatory cytokine, levels of IL-8 secretion would have been expected to fall on treatment with protective factors and has been shown to do so by one group looking at the anti-inflammatory effect of the platelet activity inhibitor ticlopidine on the secretion of IL-8 by HUVECs (Hu C 2009).

This response was mirrored in HCAECs which significantly increased IL-8 secretion on treatment with TNF-α/IFN-γ and further treatment with water indicating activation of the cells. Subsequent treatment with the higher concentrations of acetate resulted in a reversal of this higher production (similar to the result in HMEC-1 cells)
however, this was not statistically significant. To determine whether the treatments investigated (especially at the higher concentrations) were affecting cell viability and, therefore, function and production of relevant mediators, an MTT cell viability assay was carried out as outlined in 2.2.7.7. The results indicated no significant effects on cell viability of the compounds investigated suggesting the cells remained viable and in good functional health throughout the treatments and that any changes in IL-8 production might be due to an anti-inflammatory effect of SCFAs. Moon et al showed a significant reduction in IL-8 and MCP-1 on treatment of HUVECs with the anti-inflammatory phytochemical caffeic acid (Moon M 2009) showing that this powerful chemotactic cytokine can be modulated \textit{in vitro} and, as such, may be responsive to beneficial effects of whole grains.

Levels of secreted nitric oxide reflect endothelial cell function and can be measured as the stable end product nitrite in cell culture supernatants. Preliminary work carried out in EA.hy926 cells confirmed an increase of nitrite production following activation with TNF-\(\alpha\) and IFN-\(\gamma\) which was apparent after 24 hours of incubation. This increase was mirrored in the cell culture supernatants of HMEC-1 cells following TNF-\(\alpha\)/IFN-\(\gamma\) activation. A trend towards a reduction on treatment with certain concentrations of SCFAs was observed as was hypothesised, although this was not significant or consistent, which could be due to the low levels detected. Cell culture supernatant levels of NO from HCAECs cultures all remained below the limit of detection.

Little is available in the literature to compare to this work. However, the small molecular weight lignan, Gomisin A, has been shown to increase NO production in both
a time- and concentration-dependent manner, and increase eNOS activity, in unstimulated HCAECs (Park J 2009) reflecting a cardio-protective effect of increased vasorelaxation. The cardio-protective diets would be hypothesised to act via a reduction in the increased levels of NO production from eNOS. However, in this research it is likely that iNOS was stimulated to a greater proportional extent than would occur in vivo. Future work could investigate the mRNA of both eNOS and iNOS to determine the effects within the cell. This has been investigated in work by Park \textit{et al} in researching the effect of Gomisin A in HCAECs (Park J 2009) supporting the need for further investigations.

Further potential markers of interest could include TNF-\(\alpha\) or IL-1, or the inflammatory cytokine monocyte chemoattractant protein-1 (MCP-1), the expression of which was also shown by Hu \textit{et al} to increase significantly on treatment with TNF-\(\alpha\).

\textbf{Haemostatic secreted factors}

The fibrinolytic and pro-coagulant factors circulating in the blood work in a tightly controlled balance to maintain haemostasis. Damage to the endothelium can disturb this balance as many of the factors are produced by endothelial cells. As such, the secretion of these mediators is a good indicator of vascular dysfunction and, potentially, risk of CVD (Folsom A 2001; Jager A 2001).

In this study, no significant differences were seen in levels of tPA-1 secreted from either HMEC-1 cells or HCAECs on activation by TNF-\(\alpha/\text{IFN-}\gamma\) or further treatment with the test interventions. Levels would be expected to increase upon activation and possibly decrease after treatment with protective factors such as SCFAs. The reverse has been...
observed, however, in a study investigating the effects of the proposed inflammatory mediator CRP on bovine aortic endothelial cells. In their report, the authors demonstrated significantly increased expression of PAI-1, the tPA-1 inhibitor, by HCAECs after CRP incubation (Nakakuki T 2005). Decreases in tPA-1 gene expression were also observed in a study by Ulfhammer et al in which TNF-α suppressed tPA-1 gene expression by human umbilical vein endothelial cells after a 24 hour activation (Ulfhammer E 2006). These reports indicate that this factor can be modulated in vitro by inflammatory (and presumably anti-inflammatory mediators). However, its secretion in vitro in response to these factors is complicated and may rely on the secretion of other haemostatic factors such as vWF.

No change was detected in levels of vWF expressed from HMEC-1 cells on activation or on subsequent treatment with the test intervention and the level of secreted vWF was very low. HCAECs, however, secreted higher levels of vWF which decreased (albeit non-significantly) upon stimulation by TNF-α/IFN-γ. This was unexpected, as pro-inflammatory cytokines activate endothelial cells usually resulting in the increased secretion of a variety of endothelial cell products.

More recently, however, several reports have been published using slightly different stimulators of a variety of endothelial cells to secrete haemostatic factors. Other activators such as histamine, ionomycin and interleukin-1β (IL-1β) have been demonstrated to enhance the secretion and activity of vWF, tissue factor (TF), PAI-1 and tPA-1 (Disse J 2009; Lakota K 2009; Knipe L 2010) and may, therefore, be better activators for this type of research. Although no relevant research could be found
investigating the effects of SCFAs in human endothelial cells, the above mentioned report by Lakota *et al* demonstrated a significant increase in the activity of TF (another pivotal factor in thrombosis, able to transform the endothelial cell membrane from an anticoagulant to a pro-coagulant surface following vascular injury) upon incubation with IL-1β. This was subsequently down-regulated with the anti-inflammatory plant extracts in a method similar to that of the current research. This clearly demonstrates the potential for dietary constituents to impact on endothelial production of haemostatic factors involved in atherosclerosis and the effects of SCFAs, therefore, should be followed up to elucidate their mechanisms.

**Surface markers**

The cellular adhesion molecules are key mediators of the extravasation of cells including monocytes and regulation of their expression is involved in the progression of atherosclerosis (Springer T 1994; Yang L 2005). As such, levels of expression of these were measured as described in 2.2.7.4.

The percentage of HMEC-1 cells expressing ICAM-1 and the mean fluorescence intensity (MFI) of ICAM-1 staining were both significantly increased upon activation with TNF-α/IFN-γ when compared to those treated with medium alone. This increased level remained on further treatment with water, acetate or propionate showing no significant differences in activation as a result of the SCFA treatment. An increase in expression of ICAM-1 on activation was as expected, reflecting an inflammatory or activated state in an ‘at risk’ individual. Treatment with the SCFAs would have been expected to reduce these levels, post-activation, if any protective effects were afforded by
them. The percentage of HCAECs expressing ICAM-1 mimicked this reaction although the change on activation by TNF-α/IFN-γ was not significant. The MFI of ICAM-1 staining of HCAECs showed no trends toward change on activation or further treatment, with levels were similar to those of the activated HMEC-1 cells. This might be due to maximal stimulation of cells with treatment of TNF-α/IFN-γ levels of which, although widely used in the literature (approximately 100IU/ml TNF-α and 100IU/ml IFN-γ (Lombardi A 2009; Zapolska-Downar D 2009)), were higher than circulating concentrations in vivo which are widely known to be in the range of 0.55 to 2.82pg/ml. These pleiotropic cytokines are known to interact synergistically to stimulate cells in vitro (Lombardi A 2009). Titration of TNF-α/IFN-γ would be of benefit in future work to produce optimal stimulation. Future work may also benefit from stimulation of such physiologically relevant levels of TNF-α.

In agreement with this data, Miller et al exposed human umbilical vein endothelial cells (HUVECs) to 5mM propionate and showed no significant differences in the levels of ICAM-1 expressed, as determined by flow cytometry (Miller S 2005). They also treated HUVECs with the SCFA butyrate for 24 hours and assessed ICAM-1 expression, interestingly showing a significant increase in expression on treatment with 2.5 and 5.0mM butyrate which was unexpected. Levels of ICAM-1 mRNA and E-selectin were also found to be significantly increased by treatment with butyrate. These cells, however, are not primary cells, were treated with a different SCFA and were not activated with TNF-α, which may account for the differences in results. Zapolska-Downnar et al showed a significant increase in ICAM-1 and VCAM-1 production from HUVECs on treatment with 100IU/ml TNF-α in agreement with the current research.
Their investigation into the effects of propionate on HUVECs showed that pre-treatment with 0.1, 1.0 and 10mM propionate significantly reduced the TNF-α induced expression of ICAM-1 in a dose and time dependent manner (Zapolska-Downar D 2009). These results may differ to those of this research as the methodology was different, with HUVECs being treated first with the propionate and then with TNF-α. The umbilical vein origins of the HUVECs renders them less physiologically relevant with regard to CVD than the HMEC-1 cells or the HCAECs. Zapolska-Downar attributed the observed effects to propionate’s inhibition of NFκ-B, a key transcription factor implicated in the regulation of genes during immune and inflammatory responses and in particular in responses to TNF-α. Other studies on the anti-inflammatory effects of dietary components include Moon et al, who showed a significant increase in ICAM-1 expression by HUVECs on stimulation with TNF-α in agreement with this work. This group, however, also showed a significant reduction in ICAM-1 and E-selectin expression on treatment with caffeic acid (Moon M 2009).

The percentage of HMEC-1 cells and HCAECs expressing VCAM-1 remained below 5% throughout the experiment, without significant changes upon activation or treatment. The VCAM-1 MFI of both cell types showed no significant changes due to activation or treatment, with levels continuously below 12 and lower on the HCAECs than the HMEC-1 cells. As VCAM-1 is directly active in the pathology of atherosclerosis, expression of VCAM-1 would be expected to rise on activation, to mimic an inflammatory state, as would be seen in high CVD risk individuals (O’Brien K 1993). Levels of VCAM-1 were, therefore, hypothesised to increase on activation and to decrease in response to protective compounds such as SCFAs, although this was not
shown in the data. This may be due, however, to the overall low concentrations of VCAM-1 rendering any reductions undetectable or a potential overstimulation by stimulators TNF-α/IFN-γ although this is unlikely as other similar concurrent work carried out in human abdominal aortic endothelial cells not pre-treated with the stimulators TNF-α/IFN-γ also showed no significant effects of the SCFAs (data not shown).

Zapolska-Downar *et al* showed a significant reduction in TNF-α stimulated HUVEC expression of VCAM-1 on pre-treatment with several physiologically relevant concentrations of propionate (Zapolska-Downar D 2009), which may differ from this research for the reasons outlined above for ICAM-1. Treatment of HUVECs with the SCFA, butyrate, did not significantly alter expression of VCAM-1 as determined by flow cytometry (Miller S 2005). Interestingly, TNF-α induced increases in VCAM-1 expression by HUVECs were significantly reduced to near baseline levels by the platelet activity inhibitor ticlopidine proving such a reversal of expression is possible although not reflected in the current research (Hu C 2009). This was also reflected in TNF-α treated HUVECs further treated with caffeic acid (Moon M 2009). VCAM-1 is not constitutively expressed by the endothelium so may not be the most appropriate marker for *in vitro* work, with other potential markers including E- or P-selectin.

As a mediator of various migration steps through distinct domains including trans-endothelial migration and migration through the extracellular matrix, levels of circulating PECAM-1 are inversely related to CVD risk as falling levels allow for increased extravasation, an important step in atherosclerosis (Liao F 1995). HMEC-1
cells showed low levels of PECAM-1 expression when incubated with media alone. Although these levels would have been expected to fall on activation, this was not shown to occur and levels did not alter significantly in response to further treatment with SCFAs. Baseline levels of PECAM-1 were higher in HCAECs with a trend towards a reduction upon activation (which was mirrored by the MFI of staining). Neither, however, were significantly different. Levels were then slightly increased by further treatment with certain concentrations of SCFAs, as would be expected, although these changes were not significant when compared to those treated with media alone.

Treatment with SCFAs had no further impact on levels although a protective effect would have manifested itself rise to levels seen with media alone. This was reflected by Peng et al who observed that tumour conditioned medium induced the expression of PECAM by HUVECs as assessed by flow cytometry (Peng Y 2010). The different cells and activators involved in the current research may account for the differences in findings and may suggest other activators for future research.

Increasing the number of repeats of each experiment carried out would have increased power and reduced the impact of the inter-cell variations associated with using primary cells, which can often grown differently between experiments.

Cells were activated first and then treated with the investigational components. This was to reproduce, as closely as possible, the situation in vivo in which the inflamed endothelium (represented by the activated endothelial cells) comes into contact with the SCFAs produced as a by product of the high levels of whole grains or fibres within the GI tract (or added to the media in vitro). An alternative method would have been to pre-
treat the cells with the SCFAs and then activate, although this method was not thought to replicate the human intervention studies as well as the alternative and so was dismissed in favour of the 'activate then treat' method employed.

In summary, although treatment with TNF-α/IFN-γ did, on occasion, significantly alter levels of markers investigated, the short chain fatty acids did not significantly affect any markers at the concentrations involved. Thus it was concluded that this work can not be used to prove a protective role for SCFAs on the cardiovascular endothelium although the model has been shown to be productive suggesting the potential for future work investigating different types or concentrations of activators and SCFAs.
Chapter 6

General discussion
Cardiovascular disease (CVD) is the single largest cause of mortality and morbidity in the UK and several other Westernised countries (Truswell A 2002). It is a complex and multifactorial disease with both modifiable and non-modifiable causes and several co-morbidities including obesity, type II diabetes and hyperlipidaemia (Bogardus C 1985; Haffner S 1998; Turner R 1998). One popular, non-invasive pro- and re-active intervention method proposed to beneficially impact on risk CVD and of several of these co-morbidities is dietary modification. High whole grain diets, for example, have been shown to have beneficial effects on risk of CVD (Jacobs D 1998; Liu S 1999; Liu S 2000; Liu S 2003; Steffen L 2003), cancer (Chatenoud L 1998; Jacobs D 1998; Williams M 2005), type II diabetes (Jensen M 2004; Halton T 2006), obesity (Slavin J 2005), appetite regulation or reduction (Koh-Banerjee P 2003), body composition (Nelson L 1996), myocardial infarction prevention (Martinez-Ortiz J A 2006), insulin resistance (Esposito K 2004) and total mortality from all causes (Steffen L 2003). Unfortunately, the pathways behind this relationship are currently poorly understood, but one proposed mechanism of action is via their fermentation products, the short chain fatty acids, which have proven to be anti-inflammatory (Tedelind S 2007). The aim of this research, therefore, was to investigate the effects of high whole grain diets on a population displaying risk markers for CVD and to explore this proposed mechanism of action, at both the whole body and the cellular level.

6.1 Human intervention trials

It was hypothesised that the high whole grain or high fibre diets, via mechanisms including the actions of the SCFAs, would beneficially affect markers of CVD risk in a population group displaying increased risk profiles (determined by age, sex and BMI).
The human studies used the novel delivery method of including the intervention compounds within a certain number of portions of bespoke bread roll-like products to promote compliance and thorough monitoring.

Approximately 9 to 17% of whole wheat by weight consists of fibre and it has been suggested that an anti-inflammatory protective effect of whole grains may be conferred by this fibre. Indigestible fibre proceeds through the human small intestine, resisting digestion and, on reaching the bowel, acts as a substrate for bacterial anaerobic fermentation to the short chain fatty acids (SCFAs), acetate, propionate and butyrate (Wong J 2006) in the molar ratio 60:20:20 (Cummings J 1979). The average production is 100 to 200mM/day (Cook S 1998) in the lumen of the colon, from where they are absorbed, by both passive and active transport, over the colonic epithelium (Cook S 1998). The majority (70 to 90%) of the butyrate is metabolised as the primary fuel of the colonocytes but the acetate and propionate move into the circulation (Roediger W 1980).

Butyrate is known to have anti-inflammatory effects both in vivo and in vitro (Tedelind S 2007) but, unfortunately, the mechanisms of action are poorly understood. One proposed mechanism is via an inhibitory effect on NF-κB, a key transcription factor involved in the regulation of genes active in the innate immune system and cell cycle control (Luhrs H 2001). It may be hypothesised, therefore, that the similar SCFAs acetate and propionate can affect NF-κB in the same way, thus providing a possible mechanism of action for their inflammatory effects. Indeed, acetate and propionate have been shown to inhibit the TNF-α-mediated activation of the NF-κB pathway in cells of a human colon adenocarcinoma line (Tedelind S 2007). Further to this, SCFAs have been reported to
promote the following in hepatocytes: increased glucose oxidation, decreased fatty acid release and increased insulin clearance, functions which would promote an enhancement in insulin sensitivity (Venter C 1990; Thorburn A 1993; Frayn K 1996). Interestingly, butyrate has also been reported to inhibit the hepatic synthesis of cholesterol (Jenkins D 1989), which may contribute to the proposed cholesterol lowering effects of foods such as whole grains. As acetate and propionate are proposed to have anti-inflammatory effects similar to those of butyrate (Tedelind S 2007), it was further proposed that they may act on the vascular endothelium to reduce inflammation, thus, cardio-protective effects of whole grains may be due to the anti-inflammatory effects of the SCFAs produced by the fermentation of the fibres in the whole grains.

Further to this, different types of fibres are known to lead to differing amounts of SCFAs. Approximately 5 to 20% insoluble fibre is metabolised to SCFAs whereas this value is up to 90% for soluble fibre (Cook S 1998). As such, it was hypothesised that whole grains would have a protective effect on the endothelium via the actions of SCFAs and that this effect would be greater in response to equal quantities of soluble than insoluble fibres due to the increased production of SCFAs. These changes were not observed in the current research, with no changes being shown in total, HDL or LDL cholesterol, TAGs or NEFAs. As circulating levels of markers were not increased at baseline it is unlikely that such impacts would be reflected in the resultant levels. Clinical screening of participants to select a higher risk profile population group may allow for significant observations in these markers in future work.
Whole grains are also rich in antioxidants including vitamins, trace minerals, non-nutrients (e.g. phenolic acids, lignans and phytoestrogens) and anti-nutrients such as phytic acid (Thompson L 1994). They are also a better source of selenium and the intracellular antioxidant vitamin E (especially tocotrienols) than are refined grains. Antioxidants delay the onset of, or slow the rate of oxidation of, oxidizable substrates and, therefore, the formation of harmful free radicals. Oxidative stress caused by these particles has been implicated in cancer (Klaunig J 1998) and CVD risk (Rush J 2005). The antioxidant phenolic acids (e.g. ferulic, canillic and caffeic acid) are found in the outer layers of whole grains and have also been associated with potential protection from CVD (Thompson L 1993), as have the phytoestrogens (e.g. isoflavones, lignans and coumestans) mediating effects on insulin and glucose responses. Impaired metabolism of these factors is, therefore, implicated in CVD risk.

Adipocytes are known to release inflammatory cytokines, including TNF-α and IL-6, which act on the liver to increase production of CRP and CRP has been shown to activate endothelial cells. Therefore, a reduction in either adipocyte number or activity may pose a potential method for reducing endothelial activation. Weight loss has been shown to reduce circulating levels of tPA-1, IL-6 and circulating lipid (Rave K 2007; Hermsdorff H 2010; Wycherley T 2010) and this reduction in adipocyte inflammatory cytokine release may be a possible causal pathway. No changes in inflammatory markers were observed after the intervention diets. None of the participants in the Pilot WISE or the FITMA trials, however, showed significant weight loss so such potential mechanisms would not have been invoked in the current research as was reflected in the stability of the markers pre- to post-intervention.
Whole grains and dietary fibre, predominantly soluble dietary fibre, has been shown to lower circulating cholesterol and lipid levels (Anderson J 1991) with part of the protective effects of whole grains being proposed as due to their fibre content (Truswell A 2002). This may be, in part, due to the slowing of nutrient absorption from the intestines associated with fibre, resulting in increased loss in the faeces, a function which is also greater for soluble fibres in agreement with their increased lipid lowering capacities (Jenkins D 1985). Although circulating levels of total and LDL cholesterol, TAGs and NEFAs were expected to fall in response to the increased whole grain intake, this was not observed in the Pilot WISE or FITMA trials. Although the population groups were at risk of CVD as determined by their sex, age and BMI, future work could involve clinical screening of participants to ensure a higher baseline risk profile as determined by, for example, hypercholesterolaemia.

Products manufactured predominantly from whole grains tend to have lower glycaemic indices than those produced from refined grains (Foster-Powell K 1995) and, as such, their ingestion attenuates the glucose response by inducing a slightly lower rise in blood glucose than those of higher GI products such as potatoes or refined grain pasta. This may be due to the associated increased fibre bulk, with the physical actions of fibre in the gastro-intestinal tract being a slowing of nutrient absorption, with these actions being greater for soluble than insoluble fibres (Jenkins D 1985). The insulin response is, thus, also attenuated which may result long term in an increase in insulin sensitivity, potentially via an increase in expression of insulin receptors, in insulin resistant individuals (Pereira M 2002). One recent study in healthy young males showed the non-
viscous dietary fibre, resistant starch, not only significantly lowered energy intake following consumption when compared to a placebo but also significantly lowered post-prandial insulin responses (Bodinham C 2010). As such, it was hypothesised that increased intake of dietary whole grains or the soluble or insoluble fibres would reduce fasting levels and attenuate post-prandial responses of both glucose and insulin. No significant changes, however, were observed in fasting levels or post-prandial responses of insulin or glucose in either the Pilot WISE or the FITMA trial which may be due in part to levels being only moderately raised at baseline. Alternatively, such benefits may manifest over a longer period of time than was allowed for in the current research, with the resultant beneficial changes taking longer to become established.

Limitations of research

Further investigations could benefit from including a more responsive population group with raised baseline levels of markers, which may include clinical screening before initiation. Longer intervention periods could also enable a longer term impact of the intervention products although this would have to be considered in conjunction with the possible detrimental impacts of an increased imposition on the participants and subsequent potential effects on compliance. The cross-over methodology used in the FITMA trial provides a more robust design and thus more powerful data although the number of participants used was still low.

Future work could include also measurement of other potential markers which may provide alternative or more comprehensive measurements. These could include VCAM-1, PECAM-1, E-selectin, P-selectin, the tPA-1/PAI-1 complex, fibrinogen,
Hageman factor, MCP-1, TNF-α, IL-1, IL-6 or the activity of the vWF cofactor ristocetin (Meade T 1994; Bongers T 2006; Vuksan V 2007). When combined with physical activity, dietary interventions have been shown to reduce circulating CRP, IL-8, IL-6 and monocyte chemoattractant protein-1 and to increase insulin sensitivity (Bruun J 2005). Weight loss has also been shown to reduce circulating inflammatory markers (Wycherley T 2010) and as such, physical activity and weight loss may be considered for concurrent interventions in future work. Future work could use the other emerging technology of nutrigenomics to further investigate any potential nutrient-gene interactions associated with whole grains and their constituents or fermentation products.

**Metabolomics**

The current study is one of only a limited number of reports using metabolomics to investigate the impact of dietary modifications in human intervention studies and highlights the potential of metabolomic technology in nutritional research in defining alternative functions for nutrients and elucidating how diet influences metabolic pathways.

The Pilot WISE trial found no significant differences in any ion signals in, or between, the groups investigated, which may be due to the low numbers of participants involved or changes in ions not detected by the analysis platform. The FITMA trial putitatively defined three ion species present in significantly different intensities between intervention groups as the lysophosphatidylcholines (LysoPCs 22:6) and (18:0) and (18:2), suggesting, in agreement with previous research, that diets high in wheat fibre or inulin can influence metabolite profiles. Although relevant interventions are very limited.
in the literature, these data concur with those of Lankinen et al, who also showed a diet including wheat bread significantly altered levels of several LysoPC species, including (18:0) and (18:2) (Lankinen M 2010). Future investigations could investigate for any correlations between these LysoPC species and the circulating markers found at the whole body level.

Other research provides evidence of the alkylresorcinols, the phenolic lipids present in the outer layers of wheat, rye and barley grains but few other foods (Ross A 2003), as biomarkers of whole grain intake in blood (Landberg R 2008) and adipose tissue (Jansson E 2010). Their validated use as biomarkers would greatly benefit nutritional research as a method for monitoring dietary intake in the place of the less robust methods such as diet diaries. Unfortunately, although the whole intact and whole milled grain interventions of the Pilot WISE trial and the wheat fibre intervention of the FITMA trial would have been expected to show increased levels compared to baseline, no significant differences in the signals of any alkylresorcinols were observed so the current research can not add further support to this hypothesis. This may be due to analysis of the data on the HPLC/MS in positive mode, where alkylresorcinols are observed in negative mode. Alternatively, it may be due to the short half life of all homologues of alkysresorcinols in the blood (5 hours (van Dam R 2008)) which suggests that circulating concentrations reflect only recent intake. It is also possible that higher intakes of the dietary products may be necessary to induce a significant change in levels. Thus, future work could investigate the effects of shorter term, higher level intakes, analysed in negative mode on the HPLC/MS.
A challenge common to all human metabolomic studies is the inter-individual variations in factors such as habitual dietary intake and metabolic rate, and in metabolite profiles themselves. Standardisation of both the experimental design at inception of the study (Scalbert A 2009) and the dietary intervention itself (Walsh M 2006) is, therefore, important if the impacts of such variations are to be controlled for. In the FITMA study, differences in baseline levels between dietary interventions were controlled for by implementing a cross-over design although this has no impact on inter-individual variation. As mentioned previously, increasing the numbers of participants in the research would have increased the power of the results. No restrictions were placed on the diet to impose standardisation, other than the inclusion of each intervention product, as this is not always practicable in human dietary intervention studies. The researchers felt this would be too much of an imposition on the life-styles of the free living participants and would lead to reduced levels of compliance.

6.2 Cell Work

The aim of this part of the current research was to investigate the effects of the fermentation products, the short chain fatty acids (SCFAs), on endothelial markers of cardiovascular risk expressed or secreted by endothelial cells in vitro. Levels of the markers investigated were hypothesised to increase on TNF-α/IFN-γ activation to reflect the activated endothelium and to decrease towards their resting state on further treatment with the SCFAs. No significant impacts, however, were observed due to the SCFAs on the markers investigated.
The cell types investigated, HMEC-1 and HCAECs, were chosen to reflect the human vascular endothelium and initial work was carried out on the endothelial like cell line HMEC-1 (immortalised with simian virus, SV40). As such, any results from such work may not be directly reflective of those from primary cells or, indeed, the in vivo situation. Investigations were, therefore, also carried out in human abdominal artery endothelial cells, but were ceased in favour of the HCAECs, a more physiologically relevant cell type as atherosclerotic plaques are common in the coronary arteries. The cells used behaved as expected in culture and were within their recommended doubling numbers. No significant impacts were seen in any of the markers investigated and results from both cell types were similar, suggesting immortalisation had no impact on the processes involved in this investigation although other markers, not investigated in the current research, could be affected and reproducibility of results both within and between cell types. As such, future work would be more relevant if carried out solely in HCAECs.

Markers were chosen to cover a range of physiological functions of the endothelium and to reflect those measured in vivo in the Pilot WISE and FITMA trials in so far as was possible. However, the markers chosen could have been unrepresentative of the impacts under investigation. Other potential factors to measure could have included the inflammatory markers E-selectin, PAI-1, IL-6 and MCP-1. TNF-α could also have been measured if an alternative compound was used for cell activation. It is possible that markers could have broken down in the media before measurement, although this is unlikely as other investigations have noted significant changes in several of the markers used here (Moon M 2009; Sen U 2009; Zapolska-Downar D 2009; Peng Y 2010).
The endothelial activators TNF-α and IFN-γ were chosen as suggested by the literature (Hu C 2009; Moon M 2009) and preliminary investigations with EA.hy926 cells supported the literature in showing significant activation of the cells upon TNF-α/IFN-γ treatment. This research used 100IU/ml TNF-α and 50IU/ml IFN-γ to activate cells, thus modelling the stimulated endothelium associated with endothelial dysfunction and chronic inflammatory state associated with disorders such as CVD. Other research has shown significant increases in factors including ICAM-1 and VCAM-1 on stimulation with 100IU TNF-α (Zapolska-Downar D 2009) and various concentrations of TNF-α and IFN-γ (Lombardi A 2009). The concentrations of TNF-α and IFN-γ used were above those normally found in the circulation but were similar to those of previous research. As such, it is possible that the cells were over stimulated and that in future work this level should be reduced to more physiologically relevant levels. A further possible alternative research method could involve pre-treatment with SCFAs and subsequent activation with TNF-α and IFN-γ.

Other potential activators could include fibrinogen, LPS or IL-1. A single time point of 24 hours was chosen for these experiments as other in-house work into similar markers, as well as the EA.hy926 mentioned above, suggested 24 hours as optimum. This time point is also generally widely accepted in the literature. The incubation times with both the activators and the SCFAs may be further investigated to optimise the cells responses to them.

The range of concentrations of the SCFAs were chosen as representative of those relevant physiologically. A broader range of concentrations of the investigational
compounds may provide further evidence of an impact. Indeed, other *in vitro* work has investigated mM concentrations of SCFAs on endothelial cells, showing a significant reduction in the TNF-α induced increased expression of ICAM-1 (Zapolska-Downar D 2009). The current research investigated μM concentrations as these are physiologically relevant (Cummings J 1987). As such, these higher concentrations could account for the differences in results.

Other potential breakdown products posed as influencing health include the lignins, such as enterolactone and enterodiol and other concurrent research has investigated these factors in endothelial cells although no significant alterations were seen in the markers investigated in this research (Kivela A 200). This work, however, was preliminary and involved cells treated with medium alone and treatment with the investigational factors, rather than pre-treatment to activate. Thus, cells represented the resting ‘low risk’ endothelium so would not have been expected to show changes in response to enterolactone or enterodiol. Of course, the breakdown products of whole grain and fibre do not act on the endothelium in isolation and future work could investigate combinations of break down products.

Other similar work investigating the effects of the lignan gomisin A on HCAECs found significant enhancement of eNOS activity, thus, future work could involve iNOS and eNOS mRNA as markers, to further investigate cellular responses. Investigations could be extended to include combinations of such factors, at differing ratios to one another, thus providing a more physiologically relevant model and allowing for compounding effects of the treatments or interactions between them.
Potentially, the treatment factors investigated could have been toxic to the cells chosen. Microscopic examination of the cells throughout the course of the treatments did not reveal a change in apparent confluency, with no significant changes in numbers of dead cells in the media. An investigation into the effects of the treatment interventions on cell viability using an MTT assay showed no significant impact, suggesting that the sodium acetate and propionate had no negative effects on viability of the cells.

Endothelial cells do not exist in isolation in vivo, there are a multitude of factors acting on them, thus limiting the relevance of such work to the whole body. The work must be modelled in vitro, however, as it is not viable to conduct this work in vivo, which is a limitation common to all cell culture models of whole body responses.

Future work could investigate these activators or alternative haemostatic secreted factors including Hageman factor, factor XIII and those of clotting/fibrinolytic pathways. Increased numbers of repeats would add significance to the results and investigation into other potential activators including CRP and/or fibrinogen may reveal interesting data.

6.3 Conclusions

This thesis addressed the impact of diets high in whole grains and their constituents on risk markers of endothelial function and, therefore, cardiovascular disease at both the whole body and the cellular level. The two whole body, randomised controlled human dietary intervention trials yielded interesting results suggesting that, although epidemiological evidence points to a clear correlation between diets high in whole grains,
the links may not be as simple as cause and effect. Metabolomic analysis, indeed, reflected three significantly altered species of lysophosphatidylcholines, two of which were increased after the inulin treatments. LysoPCs are products of phospholipid metabolism often formed during oxidative modification. LysoPCs are pro-inflammatory with the capacity to activate cell lines and induce endothelial dysfunction, thus, a decrease in circulating levels, as was observed in one of the species, may be reflective of a cardio-protective effect, but the increase suggests that more work is required in this area. No significant changes, however, were observed in any of the inflammatory, haemostatic, lipid or insulin sensitivity end points investigated. The observations of the cell work were in agreement with the whole body work, in that no significant impacts of the investigational products were observed on the markers investigated.

Although this research shows no significant effects of whole grains or their constituents on whole body risk markers, observational evidence suggests a link between such diets and a reduced risk profile for CVD. Other research combining such diets with exercise or weight loss have shown cardio-protective results and, therefore, future work investigating the effects of the bespoke products with the inclusion of exercise or weight loss may warrant further work. Similarly, the short chain fatty acids showed no significant impacts on the markers investigated but there is a growing body of evidence to suggest their anti-inflammatory effects which requires further investigation.


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van Laere K (2000). Degradation of structurally different non-digestible oligosaccharides by intestinal bacteria: glycosylhydrolases of Bifidobacterium adolescentis Wageningen, Wageningen University. PhD.


Appendices
Appendices

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Appendix 1

A) The nutritional composition of the whole intact grain (36.0% flour, 27.0% water, 0.6% salt and 0.6% yeast with the addition of 36.0% whole wheat grain kernels) and whole milled grain (34.7% flour, 30.0% water, 0.6% salt and 0.5% yeast with the addition of 36.0% milled whole wheat grain kernels) products, and the control (63.2% flour, 34.8% water, 1.0% salt and 1.0% yeast) product, used in the Pilot WISE trial.

<table>
<thead>
<tr>
<th></th>
<th>Whole Intact</th>
<th>Whole Milled</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>293</td>
<td>283</td>
<td>258</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>1243</td>
<td>1200</td>
<td>1093</td>
</tr>
<tr>
<td>Moisture</td>
<td>21.8</td>
<td>24.5</td>
<td>30.4</td>
</tr>
<tr>
<td>Carbohydrate (of)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugar</td>
<td>1.5</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Starch</td>
<td>58</td>
<td>56</td>
<td>53.1</td>
</tr>
<tr>
<td>Protein</td>
<td>10</td>
<td>9.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Fat (of which)</td>
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<tr>
<td>Sats</td>
<td>0.22</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td>Mono</td>
<td>0.18</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>Polys</td>
<td>0.76</td>
<td>0.74</td>
<td>0.55</td>
</tr>
<tr>
<td>Fibre</td>
<td>5.6</td>
<td>5.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.288</td>
<td>0.278</td>
<td>0.505</td>
</tr>
<tr>
<td>Salt equivalent</td>
<td>0.733</td>
<td>0.708</td>
<td>1.284</td>
</tr>
</tbody>
</table>

B) The nutritional composition of the wheat fibre (51.7% flour, 40.8% water, 1.6% yeast, 0.8% salt and 0.5% ‘Zippy Plus’ with the addition of 4.6% WF 600 DV), inulin (56.4% flour, 36.1% water, 1.7% yeast, 0.9% salt and 0.6% ‘Zippy Plus’ with the addition of 4.4% Orafti HPX Inulin) and control (60.4% flour, 36.2% water, 1.8% yeast, 1.0% salt and 0.6% ‘Zippy Plus’) products used in the FITMA trial.

<table>
<thead>
<tr>
<th></th>
<th>Wheat Fibre</th>
<th>Inulin</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>220</td>
<td>239</td>
<td>256</td>
</tr>
<tr>
<td>Energy (kJ)</td>
<td>932</td>
<td>1015</td>
<td>1088</td>
</tr>
<tr>
<td>Moisture</td>
<td>37.4</td>
<td>32.2</td>
<td>33.1</td>
</tr>
<tr>
<td>Carbohydrate (of)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugar</td>
<td>0.8</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>Starch</td>
<td>42.6</td>
<td>46.5</td>
<td>49.8</td>
</tr>
<tr>
<td>Protein</td>
<td>8.5</td>
<td>9.3</td>
<td>9.9</td>
</tr>
<tr>
<td>Fat (of which)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sats</td>
<td>0.5</td>
<td>0.54</td>
<td>0.58</td>
</tr>
<tr>
<td>Mono</td>
<td>0.07</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Polys</td>
<td>0.38</td>
<td>0.42</td>
<td>0.45</td>
</tr>
<tr>
<td>Fibre</td>
<td>7.7</td>
<td>7.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.415</td>
<td>0.45</td>
<td>0.482</td>
</tr>
<tr>
<td>Salt equivalent</td>
<td>1.057</td>
<td>1.145</td>
<td>1.226</td>
</tr>
</tbody>
</table>
Appendix 2A
Product information sheet
WF 600 DV, VITACEL®

Wheat Fibre
Characteristic
VITACEL® Wheat Fibre WF 600 DV is a white, fine-fibrous dietary fibre, produced by a special process, which stands out for its extremely low dust building. The multi-functional and physiological properties of VITACEL® Wheat Fibre make it an excellent ingredient for a wide range of foodstuffs.

Standard Analysis
- dietary fibre content (acc. to AOAC-method)* min. 96.5 % i. Tr.
- of which: insoluble dietary fibre 94.0 %
- soluble dietary fibre 2.5 %
- loss on drying max. 8 %
- ash max. 3 %
- protein* 0.4 %
- triglyceride* 0.5 %
- fat* 0.2 %
- phytic acid* negative
- gluten* negative
- pH-value (10 % suspension) 6.5 +/- 1.5
- average fibre length 80 μm
- average fibre thickness 20 μm

Composition of Dietary Fibre*
- cellulose 74 %
- hemicellulose 26 %
- lignin max. 0.5 %

Microbiological Analysis
- standard plate count max. $5 \times 10^3$ KBE/g
- yeasts and moulds max. $2 \times 10^2$ KBE/g
- salmonella* in 25 g negative
- aflatoxines* not detectable
  (* typical values)

Heavy Metals*
- lead (Pb) 0.14 ppm
- cadmium (Cd) max. 0.01 ppm (detection limit)
- mercury (Hg) max. 0.01 ppm (detection limit)
- arsenic (As) 0.01 ppm

Pesticides and Fungicides*
- chlorinated hydrocarbons max. 0.002 ppm (detection limit)
Physical Data

- Water binding capacity (AACC-method) min. 4.9 g H2O/g dry. s.
- Oil absorption min. 3.7 g oil/g dry. s.
- aw-value* 0.44
- Calorific value/g* 0.09 kcal resp. 0.39 kJ
- Bulk density (in accordance with DIN 53 468) 200 g/l - 240 g/l
- Fineness 90 % < 70 µm

Sensory Properties

- Appearance: white, fibrous
- Flavor: neutral
- Odor: neutral

Screen Analysis (in accordance with DIN 53 734/air jet sieve)

- > 200 µm max. 5 %
- > 100 µm max. 10 %

Declaration

VITACEL® Wheat fibre WF 600 DV is a foodstuff which can be added to all other foodstuffs, provided that no other special instructions have to be observed due to the composition of these foodstuffs. We recommend a declaration as Wheat Fibre or Wheat Plant Fibre.

Packaging and Storage

- Paper bags ± 20 kgs with foil in-between,
- 960 kg/pallet, measurement (cm): 130 x 90 x 215
- 480 kg/pallet, measurement (cm): 130 x 90 x 120
- Store at room temperature in dry condition. Shelf life: 18 months.

(* typical values)

J. RETTENMAIER & SÖHNE GMBH + CO,
Fibres designed by Nature
Holzmühle 1
D-73494 Rosenberg
Appendix 2B
Product information sheet
Orafti®HPX

Description
Orafti®HPX is a High Performance Inulin. It is a food ingredient consisting of chicory inulin, from which the smaller molecules were removed (patent pending). Compared to the standard Orafti®HP, Orafti®HPX has a higher gel strength in fat substitution applications at higher temperatures.

Chicory inulin is a mixture of oligo- and polysaccharides which are composed of fructose units linked together by β(2-1)linkages. Almost every molecule is terminated by a glucose unit. The total number of fructose or glucose units (= Degree of Polymerisation or DP) of chicory inulin ranges mainly between 2 and 60.

Compositional Specifications
All values expressed on dry matter.
Analytical Methods: see our Technical Brochures.
- Inulin > 99.5 %
- Inulin DP ≥5 ≥99 %
- Glucose + fructose + sucrose ≤0.5 %
- Dry Matter (d.m.) 97 ± 1.5 %
- Carbohydrate content > 99.5 %
- Average DP of the inulin ≥ 23
- Ash (sulphated) < 0.2 %
- Conductivity (15 Brix) < 250 μS
- Heavy Metals Pb, As each < 0.1 mg/kg
- Cd, Hg each < 0.01mg/kg
- pH (10°Brix) 5.0 - 7.0

Microbiological Specifications
All values expressed on dry matter.
Analytical Methods: see our Technical Brochures.
- Mesophilic bacteria - total count max. 1000/g
- Yeasts max. 20/g
- Moulds max. 20/g
- Thermophilic aerobic Spores max. 1000/g
- Anaerobic H2S producing thermophilic spores max. 25/g
- Enterobacteriaceae absent in 1 g
- Bacillus cereus max. 100/g
- Staphilococcus aureus absent in 1 g
- Escherichia coli absent in 1 g
- Clostridium perfringens absent in 1 g
- Clostridium botulinum absent in 1 g
- Salmonella absent in 100 g
- Listeria Absent in 25 g

To the best of our knowledge, this information is reliable but should not be considered as a warranty of any kind. Specifications might be subject to change without notice.
Labeling

All values are average values expressed per 100 g commercial product.
Carbohydrates 0 (971) Gluten absent
Sugars 0 Lactose absent
Dietary Fibre2) 97 Milk/meat/fish/egg components absent
Protein absent Seed/soy components absent
Fat absent Insecticides, pesticides absent
Vitamins and Minerals Negligeable Nuts, nut components absent
Caloric value 3) 97 kcal/407 kJ Colza absent
Broteinheiten 4) 0 Other allergens absent
Enzymatic activity absent
Folat absent
N.D. = Not Detectable N/A = Not Applicable
1) including dietary fibre
2) measured by AOAC Method 997.08
3) based on a caloric value of 1 kcal/g for pure inulin. To be adapted to local regulations.
4) in accordance with German regulations.

Other Information (see also our Technical Brochures)

Aspect Fine white powder
Behaviour Hygroscopic
Taste Neutral, not sweet, without aftertaste
Solubility in water
Wettability in water
1 g/l at 25°C
fairly good
Dispersability in water Requires stirring.
Properties and Applications See our Technical Brochures.
Particle Sizes See document “Particle Sizes”.
Density Approx. 490 ± 40 g/l
Labelling - Ingredients List Inulin
Excessive consumption may cause laxative effects.
Is, like other fine powders, when mixed with air and ignited, capable of causing an explosion.
Packaging Paper bags on pallets, see 'Packaging Sheet Powders'
Optimal storage conditions Cool and dry, in its original airtight packaging.
Maximum durability See packaging (minimum 18 months upon delivery)
Transport conditions According to document 'Transport Conditions'
Irradiation Not irradiated
GMO Not containing GMOs or GMO-derived components.
Not produced using GMO-based technology.
Kosher Certified, Orthodox Union Represented by :
Halal Certified, Halal Feed and Food
Inspection Authority
Plant origin Suitable for vegetarians & vegans

Produced by BENEO-Orafti, Belgium
IF YOU ARE:

- Male, aged 30 – 60 years or a post-menopausal female
- Waist greater than 34 inches (86 cm) for men or 31.5 inches (80 cm) for women
- Willing to alter your diet for 8 weeks
- Willing to give a number of blood samples

If you are interested in being involved in this study or would like more information, please contact:

Laura (bsp3lt@surrey.ac.uk) or Caroline (bsp1cb@surrey.ac.uk)
Nutrition Research Group
School of Biomedical and Life Sciences, The University of Surrey
Mobile: 07817 229246
A Nutrition Study: How does Fibre affect our health?
The FITMA trial

IF YOU ARE:
• An adult, aged 30 - 60 years
• Willing to alter your diet slightly for 20 weeks
• Willing to give a number of blood samples

Compensation will be given for your time and inconvenience. If you are interested in being involved in this study or would like more information, please contact:

Laura (bsp3lt@surrey.ac.uk) or Niky bsp2nm@surrey.ac.uk)
Nutrition Research Group
School of Biomedical and Life Sciences, The University of Surrey
Mobile: 07817 22 92 46, Office: 01483 68 63 93

This study has been given a favourable ethical opinion from the University of Surrey Ethics Committee
### Health and Lifestyle Questionnaire

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

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:

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

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.

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>
</tr>
<tr>
<td>Red/White Wine</td>
<td>Std 175ml</td>
<td>2</td>
</tr>
<tr>
<td>Red/White Wine</td>
<td>Lg. 250ml</td>
<td>3</td>
</tr>
<tr>
<td>Spirits</td>
<td>Std 25ml</td>
<td>1</td>
</tr>
<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>
<td>1.5</td>
</tr>
</tbody>
</table>
Dutch Eating Behaviour Questionnaire (DEBQ)

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>
</tr>
</thead>
<tbody>
<tr>
<td>1. If you have put on weight, do you eat less than you usually do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Do you have a desire to eat when you are irritated?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. If food tastes good to you, do you eat more than you usually do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Do you try and eat less at meal times than you would like to eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Do you have a desire to eat when you have nothing to do?</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6. Do you have a desire to eat when you are fed up?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7. If food smells and looks good, do you eat more than you usually eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. How often do you refuse food or drink offered because you are worried about your weight?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>9. Do you have a desire to eat when you are feeling lonely?</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10. If you see or smell something delicious, do you have a desire to eat it?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Do you watch exactly what you eat?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. Do you have a desire to eat when somebody disappoints you?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. If you have something delicious to eat, do you eat it straight away?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Do you deliberately eat foods that are slimming?</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>15. Do you have a desire to eat when you are cross?</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>16. Do you have a desire to eat when you are expecting something to happen?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>17. If you walk past the baker do you have a desire to buy something delicious?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18. When you have eaten too much, do you eat less than usual on the following days?</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>19. Do you get a desire to eat when you are anxious, worried or tense?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>20. If you walk past a snack bar or café, do you have a desire to buy something delicious?</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>21. Do you deliberately eat less in order not to become heavier?</td>
<td></td>
<td></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>
<td></td>
<td></td>
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<tr>
<td>23. If you see others eating, do you have a desire to eat?</td>
<td></td>
<td></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>
<td></td>
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</tr>
<tr>
<td>26. Can you resist eating delicious food?</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>27. How often in the evening do you try not to eat because you are watching your weight?</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>28. Do you have a desire to eat when you are disappointed?</td>
<td></td>
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<tr>
<td>29. Do you eat more than usual when you see other eating?</td>
<td></td>
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</tr>
<tr>
<td>30. Do you take your weight into account when you eat?</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>31. Do you have a desire to eat when you are emotionally upset?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32. When preparing a meal are you inclined to eat something?</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>33. Do you have a desire to eat when you are bored or restless?</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Appendix 6

Participant Information Sheet – The Pilot WISE trial
The Pilot Whole grain Study in England

Title: A Pilot Study: The influence of whole grain intervention on markers of cardiovascular disease risk in adult males and post-menopausal women.

Investigators: Prof. Gary Frost (Professor of Nutrition, Principle Investigator), Dr Kathryn Hart (Lecturer in Nutrition), Dr John Lodge (Lecturer in Nutritional Biochemistry), Dr Denise Robertson (Lecturer in Nutritional Physiology), Caroline Bodinham (PhD student), Nicola Muirhead (PhD student), Laura Tripkovic (PhD student). Nutrition Research Group, School of Biomedical and Molecular Sciences, University of Surrey.

Contact Names: Prof. Gary Frost on 01483 689384, Nicola Muirhead, Laura Tripkovic or Caroline Bodinham on 07817 229246.

Introduction:
Cardiovascular disease is a serious condition, which is affecting an increasing number of people in the United Kingdom. It is a complex disease that has many causes including dietary, social and genetic factors. Changes to diet and lifestyle factors are implicated in the treatment and prevention of cardiovascular disease.

Evidence from population studies have suggested that diets that are rich in whole grains (>3 servings/day) may help to reduce the risk of cardiovascular disease. This is thought to be due to improvements in blood pressure, glycaemic control (sugar and insulin levels in the blood), blood lipids (including cholesterol), and reductions in inflammatory markers. Whole grains comprise 3 main parts, bran, endosperm and germ, all of which need to be present in the same amounts as when the grain is intact in a product for it to be termed ‘whole grain’.

There are currently no intervention studies that directly compare whole grains versus refined grain in relation to cardiovascular disease. Therefore this study aims to determine whether a diet high in whole grains will beneficially affect markers of cardiovascular disease and appetite, compared to a diet high in an equivalent amount of milled whole grain or refined grain. This will ascertain whether it is the amount or the structure of the grain that is the determining factor for reducing cardiovascular disease risk.

Who is suitable to participate?
• Adult males (aged between 30 – 60 years)
• Post-menopausal females
• Overweight (Body Mass Index 25 - 35kg/m² *)
• Waist circumference greater than 86cm (34inches) for men and 80cm (31.5inches) for women
• Fasting Insulin >60pmol/L
• Not taking any prescription medicines or supplements within the past 6months
• Those who drink no more than 21 units of alcohol per week
• Those who do not regularly undertake vigorous exercise or fitness training (no more than 3 x 30minute aerobic sessions per week)

*To calculate BMI: body weight in kg divided by height in m²
What is the screening process?
The screening involves you attending the Clinical Investigation Unit, in the School of Biomedical and Molecular Sciences (University of Surrey) one morning, having fasted overnight, for a small blood sample (~20ml), which will be used to test for anaemia and fasting insulin levels. The presence of anaemia will exclude you from the study, for health reasons. Height, weight, waist circumference and blood pressure will also be taken. You will also be requested to complete the questionnaires (Health and Lifestyle, Dutch Eating Behaviour and Food Frequency) accompanying this Participant Information Sheet. Individuals with certain medical conditions such as heart disease, diabetes, gastro-intestinal problems (for example coeliac disease, irritable bowel syndrome), liver disease, will be unable to take part in the study. Once the questionnaires are completed and you are happy that the study has been fully explained to you, please complete the consent form.

If you meet all of the screening criteria you will then be invited to take part in the study and requested to complete a 7 day food diary.

What will the study involve?
You will be requested to attend the Clinical Investigation Unit (CIU) for an initial study day, the day prior to the start of the study. You will be requested to attend the CIU having consumed a standardised meal the previous evening and having fasted for 12 hours overnight. It will also be advised that you refrain from strenuous exercise the day before the study. On arrival you will be cannulated which involves inserting a small tube into your vein, this will be gently secured and used to obtain blood samples, safely and painlessly, throughout the duration of the study day. A fasting blood sample (30ml) will be taken along with a questionnaire regarding your appetite and body measurements (e.g. height and weight). Your blood pressure and blood flow (Pulse Wave Velocity - see below) will also be measured. You will then consume a standard breakfast test meal. Following this, half hourly blood samples (10ml) will be taken via the cannula and appetite questionnaires will be completed for three hours after the meal. After two hours the Pulse Wave Velocity will be repeated. After the three hours, you will be offered a pre-weighed meal to consume freely. You will then be free to leave and provided with a food diary to be completed for the rest of the day.

The following day, the 8 week study will begin. You will be required to consume a standardised meal (similar to the previous standardised meal) the evening prior to the study day and then fast for 12 hours overnight. You will also be advised to refrain from strenuous exercise the day before the study. On arrival in the morning, you will be cannulated and an initial 30ml blood sample, body measurements and a questionnaire regarding your appetite will be taken. You will then be required to consume a test meal containing either whole grains or an equivalent amount of milled whole grain or refined grain. Blood samples (10ml) will be obtained via the cannula and questionnaires on appetite will be completed every 30minutes for 3 hours, following the meal. After two hours the Pulse Wave Velocity will be repeated. After 3 hours you will be provided with a pre-weighed meal (pasta based with sauce) and asked to consume as much as you wish. Before leaving the unit you will be provided with a 24 hour ambulatory blood pressure monitor, which you will be shown how to fit and be asked to wear for 24 hours following the study day. After which you are free to leave and will be provided with a food diary to be completed for the remainder of that day.
As part of the study you will be randomly assigned to the diet rich in Whole Intact, Whole Milled grains or refined grain (Control) products, therefore, you need to be prepared to consume these products for the entire 8 weeks of the study. Two servings of the products will be supplied for you to have for each day of the study. You will not need to alter your diet in any other way, please continue to eat your normal diet. You will be contacted regularly by the Dietitian during the study to answer any questions you may have, to reiterate previous advice and obtain a 24 hour recall of your diet.

At the beginning of the eighth week, you will be posted a food diary which you will need to complete for 7 consecutive days. On the last day of the study you will return to CIU for a repeat of the first study day with the addition of the 24 hour ambulatory blood pressure monitor.

*7 Day Diet Diary to be completed at some point between screening and Initial Study Day

**What will be measured in the collected samples?**

All of the collected blood samples will be analysed for plasma glucose, insulin, triglyceride, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol. The samples will also be analysed for inflammatory/clotting markers, markers of adipocyte metabolism, gut hormones involved in appetite and for metabolic breakdown products.
What is Pulse Wave Velocity?
Healthy arteries have elastic properties and evidence shows that as the elasticity of the arteries reduces the risk of developing cardiovascular disease increases. Pulse Wave Velocity (PWV) is a specialised technique designed to measure how the blood flows around the body and therefore can give an indication of the elastic properties of arteries. The method involves a small metal probe being gently placed upon your pulse points on your wrist, neck and the top of your leg (groin area) for a short amount of time (approximately 30 seconds at each point). Measurements will be taken before and after the study to see if the inclusion of whole grains affects the elastic properties of arteries.

How confidential are the results?
The results are strictly confidential to the investigators and each volunteer. Your General Practitioner (GP) will be informed of your participation in the study. If you do not wish us to contact your GP, please indicate this on the consent form you will be asked to complete.

All of the collected data will be kept anonymous and all participants will be allocated a confidential number at the start of the study, which will be used to identify all collected blood samples and subsequent results.

Are you free to withdraw from the study once you have agreed to take part?
Participants are free to withdraw from the study at any time without having to give a reason.

Do you need to modify your diet or activities in any way?
You will be asked to exercise and carry out activities as normal, apart from the evening prior to the study day where you will be asked not to carry out excessive exercise. You will only be required to consume the test foods provided as part of your normal diet and no other dietary changes are required and therefore there should be no additional cost involved for you. The ambulatory blood pressure monitor will be work for 24 hours following the second and third study days. All normal daily activities can be carried out, with care, and the monitor should not restrict movement or function. We do ask that you inform us if you are ill or start/stop taking any prescription medicines during the study period.
As a result of being involved in this study are there any adverse consequences to my health?
Blood samples will be taken via cannula by experienced investigators (trained in venepuncture and first aid) with on-call medical cover throughout the study days. The volume of blood collected over the course of the study is approximately 300ml for the whole study, which is less than a typical blood donation.

As with any increase in fibre intake there maybe an increase in stool frequency.

Although whole grains are beneficial to health no adverse effects should be seen in the refined grain group. If beneficial effects are seen any of the groups, then those who had not consumed that product will be offered it, in equal amounts to those used, after completion of the study.

Will you get payment for taking part in the study?
You will be reimbursed £50 for any inconvenience caused due to the study. Any additional travel costs incurred directly relating to the study will also be reimbursed, on provision of a valid receipt.

What if I have a complaint or concern?
Any complaint or concerns about any aspect of the way you have been dealt with during the course of the study will be addressed. Please contact Prof. Gary Frost (Principal Investigator) on 01483 689384 (email: g.frost@surrey.ac.uk).
Appendix 7

The Pilot WISE trial
A Pilot Study: The influence of whole grain intervention on markers of cardiovascular disease risk in adult males and post-menopausal women.

Consent Form

I have read and understood the information sheet provided relating to the study on the benefits of whole grains on cardiovascular disease. 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 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 £50 and will be reimbursed for any costs incurred directly relating to the study (e.g. travel and extra food costs) on provision of a till receipt.

PTO
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.

**Signatures:**

<table>
<thead>
<tr>
<th>Participant</th>
<th>Principal Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Name</td>
</tr>
<tr>
<td>Signed</td>
<td>Signed</td>
</tr>
<tr>
<td>Date</td>
<td>Date</td>
</tr>
</tbody>
</table>

**Witness**

<table>
<thead>
<tr>
<th>Name</th>
<th>Signed</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I have no objection to my general practitioner (GP) being informed of my participation in the study.

Signed ......................................................................................
Appendix 8

Date:

Dr _______
Address 1
Address 2
Surrey
Post code

Dear Dr _______

Re: Mr. Smith
DOB:
Address:

The individual stated above has agreed to participate in the study ‘A pilot study: The influence of whole grain intervention on markers of cardiovascular disease risk in adult males and post-menopausal women’.

Mr Smith has been selected after the appropriate screening mechanisms. Health, lifestyle and behaviour questionnaires have been completed, of which Mr. Smith indicated that he does not have any significant medical history nor is he currently taking any prescription medications that may unduly influence the outcome of the study.

During the course of the 8 week study, blood lipids, fasting and post-prandial glucose and insulin will be monitored, as well as other anthropometric, metabolic and vascular outcome measures.

If you have any queries regarding the above information, or have additional information regarding Mr. Smith that you feel is relevant to the study, please feel free to contact The Nutrition Research Group on the contact details above.

Yours Sincerely

Laura Tripkovic BSc (hons) RD
(On behalf of The Nutrition Research Group, University of Surrey)
Appendix 9

Pilot WISE trial test breakfast nutritional information

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight (g)</th>
<th>Protein (g)</th>
<th>Carbohydrate (g)</th>
<th>Fat (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed milk</td>
<td>200</td>
<td>6.6</td>
<td>10</td>
<td>0.2</td>
</tr>
<tr>
<td>Marvel dried milk</td>
<td>31</td>
<td>11.2</td>
<td>16.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Olive oil</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Glucose</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Cadbury's drinking chocolate</td>
<td>18</td>
<td>1.1</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>289</strong></td>
<td><strong>18.9</strong></td>
<td><strong>59.4</strong></td>
<td><strong>21.4</strong></td>
</tr>
</tbody>
</table>

Total kcal in 250ml serving: 505.8kcal (75.6kcal from protein, 237.6kcal from carbohydrates and 192.6kcal from fat).
Appendix 10

Participant Information Sheet – FITMA
The Fibre and Inulin Trial in Male Adults

Title: The acute effects of the soluble and insoluble fibres found within whole grain on the markers of cardiovascular disease risk in adult men and women.

Investigators: Prof. Gary Frost (Professor of Nutrition, Principle Investigator), Dr Kathryn Hart (Lecturer in Nutrition), Dr John Lodge (Lecturer in Nutritional Biochemistry), Laura Tripkovic (PhD student), Nicola Muirhead (PhD student). Nutrition Research Group, School of Biomedical and Molecular Sciences, University of Surrey.

Contact Names: Prof. Gary Frost on 01483 689384, Laura Tripkovic or Nicola Muirhead on 07817 229246 or 01483 68 63 93.

Introduction:
Cardiovascular disease is a serious condition, which is affecting an increasing number of people in the United Kingdom. It is a complex disease that has many causes including dietary, social and genetic factors. Changes to diet and lifestyle factors can have a major impact on the treatment and prevention of cardiovascular disease.

When carbohydrates are eaten sugars are released as energy. If an individual is in good health then the body produces a hormone called insulin to help the body use the sugar effectively. However, if an individual is overweight, has a poor diet, and/or exercises infrequently, then the insulin can lose its effectiveness in helping the body use the sugar released from food. The extra sugar then stays in the bloodstream for longer which can lead to many health problems.

Currently, the latest evidence shows that the more soluble fibre (β-glucan - commonly found in barley and oats) and insoluble fibre (cereal fibre – e.g. bran) that is eaten, the more effective insulin becomes in assisting the body to use energy from food. However, questions remain as to how the fibre works and how much we need to eat each day to help keep us healthy.

There are currently no dietary studies that directly compare individual fibre components of whole grains to see the effect they have on the risk of developing cardiovascular disease. Therefore, this study aims to determine whether a diet high in soluble fibre or insoluble fibre will beneficially affect markers of cardiovascular disease, compared to a control diet (i.e. no active ingredient present.) This will ascertain which part of the whole grain (i.e. soluble or insoluble fibre) is responsible for improving particular markers of CVD risk.

Who is suitable to participate?
- Adult males
- Aged 30 to 60 years
- Not taking any prescription medicines or supplements within the past 6 months
- Those who drink no more than 21 units of alcohol per week
- Those who do not regularly undertake vigorous exercise or fitness training (no more than 3 x 30 minute aerobic sessions per week)
What is the screening process?
The screening involves you attending the Clinical Investigation Unit, in the School of Biomedical and Molecular Sciences (University of Surrey) one morning, having fasted overnight, for a small blood sample (10ml) which will be tested for anaemia and certain glucose markers. Height, weight, waist circumference and blood pressure will also be taken. You will also be requested to complete two questionnaires (Health and Lifestyle and Food Frequency). Individuals with certain medical conditions such as heart disease, diabetes, gastro-intestinal problems (for example coeliac disease, irritable bowel syndrome), liver disease or anaemia will be unable to take part in the study.

If you meet all of the screening criteria you will be invited to take part in the study. If you are happy that the study has been fully explained to you (including risks and benefits), you will then be requested to complete the consent form for your involvement in the study.

What will the study involve?
Once the screening phase has been completed successfully and before the study commences you will be required to complete a 2 week wash-out diet at the beginning of the study, during which time you will be asked to refrain from eating any whole grain products. During this time you will also be asked to complete a 3 day diet diary. Dietary advice will be provided throughout this time by the Registered Dietitian via a preliminary one-to-one interview and regular telephone conversations as required.

At the end of this run-in period you will be asked to attend the Clinical Investigation Unit (CIU) for an initial study morning to provide baseline data. Having consumed a standardised meal the previous evening (guidance will be given) you will need to be fasted for 12 hours overnight. We will also ask that you refrain from strenuous exercise, caffeine and alcohol the day before the study. The purpose of each study day is to test your body’s ability to handle sugars (glucose). This will be determined using an Oral Glucose Tolerance Test which is described in detail below.

On arrival at the CIU, measurements of blood pressure, Pulse Wave Velocity, height, weight, body fat percentage and waist circumference will be taken. You will then be cannulated. This involves inserting a small tube into your vein, which will be gently secured and used to obtain blood samples, safely and painlessly, throughout the duration of the study morning. A fasting blood sample of 50ml will then be taken. You will then consume a high glucose drink (containing 75g glucose). Following this, blood samples of 5ml will be taken every 15minutes via the cannula for two hours after the drink. After the two hours, the pulse wave velocity will be repeated and a further two 5ml samples will be taken in the following hour at half hourly intervals. Once the blood samples have been completed you will be offered refreshments and an opportunity to rest. Each study day should last no longer than 4 hours 30 minutes. A blood pressure monitor will then be fitted which is a small, light-weight machine and will be required to be worn for 24hrs after the study morning.

Starting the following day you will be required to consume the food products provided as directed for 28 days. These are to be included in your normal diet in the place of other grain products such as bread or cereals. At regular intervals during this intervention period the Registered Dietitian will contact you by telephone (at a pre-arranged time) to complete a recall of your dietary intake over the last 24 hours. You are free to follow your normal lifestyle and diet during this time. At the end of the 28 days, you will attend the CIU for a repeat of the initial study morning.
On day 3 and day 7 of each intervention period you will be invited to attend the CIU for a fasting blood sample to assess the short-term effects of the fibre you are consuming. These appointments will be fitted around your work schedule and time-commitments and should take no longer than 15 minutes each.

There are three stages to the study, each stage testing a different food product (soluble fibre, insoluble fibre and a control.) You will be provided with the required amounts of food product for 28 days and you will not be told which product you are testing at any particular time as this may inadvertently influence the results. Each stage lasts 28 days, with a study morning at the end, after which there is a break of another 28 days. This is a wash-out period during which you will continue to eat your normal diet but be required to refrain from consuming any whole grain products with ongoing support from a Registered Dietitian. This is an important part of the study, as we need to be sure that each food product does not interfere with the next.

After the initial baseline study morning, you will not be required to attend the CIU at the beginning of the intervention period, only at the end. Therefore, the second and third intervention legs will only involve two very short visits and one longer one each. Please see study diagram for further information.

The study should take no more than a total of 20 weeks to complete and no more than 530ml (equal to 19 fl oz) blood, including screening, will be taken over the 15 weeks. This is just under a pint (570 ml), which is the average amount taken at one time in a blood donation. The average adult body contains 10 to 12 pints of blood. Please see below for the outline of the study design.
Study Plan:
The diagram below shows the plan of the study and what would be involved throughout the study.

Diagram 1

<table>
<thead>
<tr>
<th>Screening</th>
<th>Run-In Diet</th>
<th>Intervention 1</th>
<th>Wash-Out 1</th>
<th>Intervention 2</th>
<th>Wash-Out 2</th>
<th>Intervention 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 days</td>
<td>3</td>
<td>7</td>
<td>28 days</td>
<td>28 days</td>
<td>28 days</td>
<td>28 days</td>
</tr>
</tbody>
</table>

**Blood sample**
- Height, weight, body fat % and waist
- Blood sample
- Blood pressure
- 24 hour blood pressure

**PWV**
- Height, weight, body fat % and waist
- Blood sample
- Blood pressure
- 24 hour blood pressure

**PWV**
- Height, weight, body fat % and waist
- Blood sample
- Blood pressure
- 24 hour blood pressure

**PWV**
- Height, weight, body fat % and waist
- Blood sample
- Blood pressure
- 24 hour blood pressure

Key:
- BS = Blood Sample (20ml)

* - Study Morning for Day 0 only applies for the ‘Intervention 1’ phase.
What will be measured in the collected samples?
All of the collected blood samples will be analysed for plasma glucose, insulin, triglyceride, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol. The samples will also be analysed for inflammatory/clotting markers, markers of fat cell metabolism and for metabolic breakdown products.

Lymphocytes and monocytes will be isolated from blood in order to test lymphocyte proliferation and monocyte production of IL-2 and TNF-α. These are both reliable markers of inflammation.

What is Pulse Wave Velocity?
Healthy arteries have elastic properties and evidence shows that as the elasticity of the arteries reduces the risk of developing cardiovascular disease increases. Pulse Wave Velocity (PWV) is a specialised technique designed to measure the speed of blood flow around the body and therefore can give an indication of the elastic properties of arteries. The method involves a small metal probe being gently placed upon your pulse points on your wrist, neck and the top of your leg (groin area) for a short amount of time (approximately 30 seconds at each point). Measurements will be taken at the beginning of the study to see if the inclusion of the components of whole grains affects the elastic properties of arteries.

How confidential are the results?
The results are strictly confidential to the investigators and each volunteer. Your General Practitioner (GP) will be informed of your participation in the study. If you do not wish us to contact your GP, please indicate this on the consent form you will be asked to complete.

All of the collected data will be kept anonymous and all participants will be allocated a confidential number at the start of the study, which will be used to identify all collected blood samples and subsequent results.

Are you free to withdraw from the study once you have agreed to take part?
Participants are free to withdraw from the study at any time without having to give a reason.

Do you need to modify your diet or activities in any way?
You will be asked to exercise and carry out activities as normal, apart from the evening prior to the study day where you will be asked not to carry out excessive exercise and to eat a standardised meal (which will be arranged with you closer to the time). You will only be required to consume the test foods provided as part of your normal diet and no other dietary changes are required and therefore there should be no additional cost involved for you. We do ask that you inform us if you are ill or start/stop taking any prescription medicines during the study period or wish to donate blood.
As a result of being involved in this study are there any adverse consequences to my health?
Blood samples will be taken via cannula by the experienced investigators Dr Shelagh Hampton, Laura Tripkovic and Nicola Muirhead (trained in venepuncture by Yvonne Vogt, a registered nurse, and first aid). Cannulas will be positioned by Dr Shelagh Hampton with on-call medical cover throughout the study days provided by Dr. John Wright. The volume of blood collected over the course of the study will be no more than 530ml for the whole study (including screening).

Any increase in dietary fibre may cause an increase in stool frequency.

Fibre and its constituents are beneficial to health and therefore no adverse effects are anticipated. By the end of the study all participants would have received identical amounts of all the food products tested. However, if one food product appears to provide significant health benefits, all participants will be advised at the end of the study.

Will you get payment for taking part in the study?
You will be reimbursed £120 for any inconvenience caused due to the study. Any additional travel costs incurred directly relating to the study will also be reimbursed, on provision of a valid receipt.

Ethical study approval:
This study has been given a favourable ethical approval by the University of Surrey Ethics Committee.

What if I have a complaint or concern?
Any complaint or concerns about any aspect of the way you have been dealt with during the course of the study will be addressed. Please contact Prof. Gary Frost (Principal Investigator) on 01483 689384 (email: g.frost@surrey.ac.uk).
Appendix 11

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 and women.

- 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 12

Date:

Dear Dr _______

Re: Mr/Ms. Smith
DOB: 
Address:

The individual stated above has agreed to participate 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 and women’.

Mr/Ms Smith has been selected after the appropriate screening mechanisms. Health, lifestyle and dietary behaviour questionnaires have been completed, of which Mr. Smith indicated that he does not have any significant medical history nor is he currently taking any prescription medications that may unduly influence the outcome of the study.

During the course of the 20 week study, blood lipids, fasting and post-prandial glucose and insulin will be monitored, as well as other anthropometric, metabolic and vascular outcome measures.

The protocol for this study has been given a favourable opinion from the University of Surrey Ethics Committee. If you have any queries regarding the above information, or have additional information regarding Mr. Smith that you feel is relevant to the study, please feel free to contact The Nutrition Research Group on the contact details above.

Yours Sincerely

Laura Tripkovic BSc (hons) RD
(On behalf of Carbohydrate Metabolism Research Group, University of Surrey)