GENETIC DETERMINANTS OF OXIDATIVE STRESS IN DIABETES MELLITUS

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To Susan, Rebecca and Thomas

'Genes load the gun, but the environment pulls the trigger'

Dr Elliot Joslin, 1921

ABSTRACT

Increased oxidative stress has been implicated in the pathogenesis of atherosclerosis and coronary heart disease, and is a key feature of diabetes mellitus. Increased oxidative stress has numerous adverse effects on the vascular system, including the altered expression of cell adhesion molecules, induction of pro-inflammatory mediators and more specifically the oxidation of low density lipoprotein (LDL) to form oxidised LDL (Ox-LDL). As well as measuring the total degree of oxidative stress in plasma, specific measures may also be recorded, such as the degree of LDL-oxidation.

This thesis focuses on the association between plasma markers of oxidative stress and LDL-oxidation with other biochemical intermediate risk factors and common gene variants, in subjects with diabetes mellitus. Analysis focused on three candidate genes:- a cellular anti-oxidant, glutathione-s-transferase; a plasma lipoprotein, apolipoprotein E; and a mitochondrial protein, uncoupling protein-2 (UCP2). The effect of common variants in these genes was explored in relation to plasma markers of oxidative stress, along with gene-environment interaction in the pro-oxidant environment of cigarette smoking. Initially a cohort of approximately 1000 subjects with diabetes, were recruited from the diabetes clinic at University College London Hospitals. Routine biochemical and clinical data was gathered, as well as plasma and blood for DNA extraction.

Further *in vitro* functional studies were performed in respect to the UCP2 gene, to further our understanding of the role of this gene in the generation of oxidative stress and in the pathogenesis of coronary heart disease.

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DECLARATION

All subjects in UDACS were recruited by myself. I performed all plasma/serum extraction, DNA extraction and biochemical analysis. All genotyping was performed by myself with the exception of the following. Glutathione s-transferase genotyping in the NPHSII study was performed by Mr Jamie Acorn. Within UDACS, this genotyping was performed by Professor Tony Hayek (visiting from The Bruce Rappaport Faculty of Medicine, Haifa, Israel), under my supervision. Sequencing of novel mutations within the apolipoprotein E gene was performed by Mert Sozen, a visiting PhD student from Hacettepe University, Ankara, Turkey under my supervision. Apolipoprotein E phenotypic analysis and LDL particle size was preformed by Dr Muriel Caslake in the laboratories of the department of Vascular Biochemistry in the University of Glasgow. The composition of this thesis is entirely my own work. The data herein and that arising from UDACS have been presented and published as follows.

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CHAPTER ONE

INTRODUCTION

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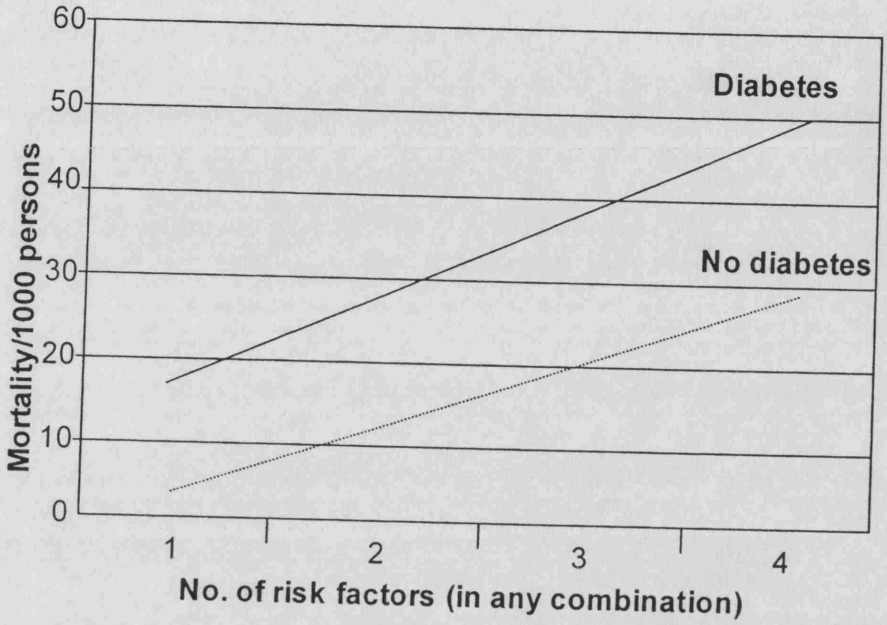
1.1 Diabetes Mellitus and Coronary heart disease

By the year 2010, the number of people in the world with diabetes will be around 221 million (Amos *et al.* 1997; Orchard 1998), rising to 300 million by the year 2025 (King *et al.* 1998). Coronary heart disease (CHD) and cardiovascular disease (CVD) are the major cause of mortality in patients with diabetes, and hence the cost and clinical implications of the condition are significant (Amos *et al.* 1997; Orchard 1998). Whilst several well-studied environmental and physiological factors are now documented for CHD, diabetes remains one of the 'major risk factors' as determined by the large prospective Framingham study (Castelli *et al.* 1986). Patients with diabetes have a two to threefold increased incidence of CHD (Garcia *et al.* 1974), and those who present in the fourth and fifth decade have a twofold increase in mortality (Panzram 1987). The quantitative importance of diabetes as a CHD risk factor is illustrated by the Multiple Risk Factor Intervention Trial (MRFIT), which examined the effects of three major risk factors (hypertension, smoking, and hypercholesterolaemia) (Stamler *et al.* 1993). This study showed that a patient with type 2 diabetes (T2DM) who is a non-smoker, normotensive and has normal serum cholesterol has the same mortality risk from CHD as a non-diabetic subject who has two of these three risk factors present (figure 1.1).

Figure 1.1: Effect of diabetes, hypertension, elevated cholesterol and smoking on age standardised CHD mortality



Recently, it has been suggested that diabetes may itself be considered a cardiovascular disease. The close association between diabetes (in particular T2DM) and CHD has led to the 'common soil' hypothesis (Stern 1995; Ceriello *et al.* 2004), proposing that T2DM and CHD share common genetic and environmental antecedents. Further support for this hypothesis may be observed in some pharmacological studies aimed at reducing CHD. For example, therapy with statins (WOSCOPS (Freeman *et al.* 2001)), angiotensin-1 converting enzyme inhibitors (HOPE (Yusuf *et al.* 2001), CAPP (Hansson *et al.* 1998)) and angiotensin II type 1-receptor antagonists (LIFE (Dahlof *et al.* 2002)) have all been associated with a 25-30% reduction in the development of T2DM, presumably through an underlying common mechanism.

At the molecular level, atherosclerosis is a multi-step process involving the interaction of many different complex cascades. These include endothelial function, inflammation, coagulation, lipoprotein metabolism and oxidative stress. Considerable 'cross talk' exists between these processes, resulting in a complex 'cause or consequence' phenomenon with positive feedback on one another. Unravelling the impact that these processes have on one another, and in determining CHD risk, is crucial to our understanding of the pathophysiology of atherosclerosis in high risk subjects, such as those with diabetes.

1.2 'Traditional' risk factors for coronary heart disease

The concept of cardiovascular risk evolved from epidemiological studies of CHD conducted in the 1940s and 1950s, such as the Framingham study (Castelli *et al.* 1986; Kannel 2000; Kannel 2000). Using a prospective epidemiological approach it became possible to demonstrate a consistent association of characteristics observed in apparently healthy individuals with the subsequent development of CHD. Whilst the observation of a statistically significant association between a trait and disease does not provide proof of causality or establish a pathophysiological relationship, such observations greatly contribute to our understanding of pathophysiology.

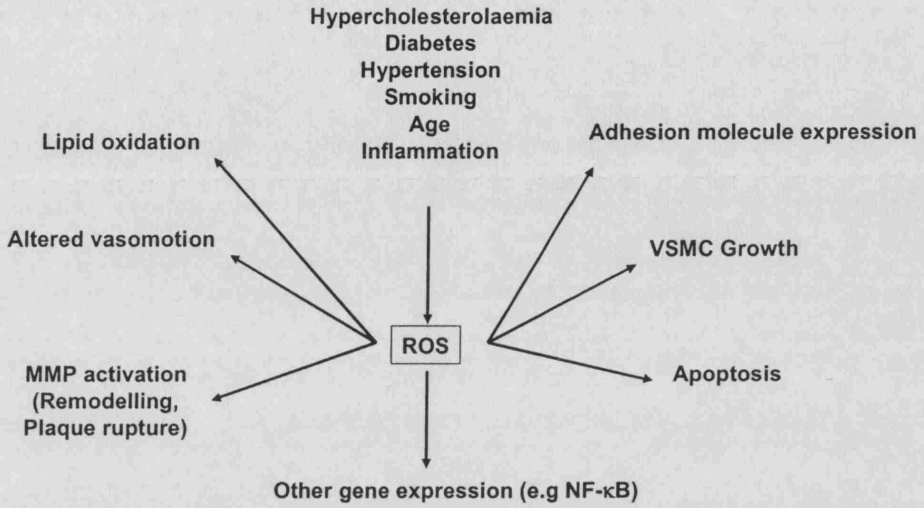
Well-accepted risk factors for the development of CHD include increasing age, high plasma levels of total and low-density lipoprotein (LDL) cholesterol and triglycerides, low levels of high-density lipoprotein cholesterol (HDL), elevated blood pressure, cigarette use, diabetes mellitus, and evidence of left ventricular hypertrophy on electrocardiography (Wilson 1994). Of importance, cardiovascular risk tends to cluster in affected families, where there are a number of identifiable risk factors. This supports the importance of both a shared environment and shared genetic factors (Genest and Cohn 1995; Kannel and Wilson 1995; Kannel 2000; Sakkinen, Wahl *et al.* 2000). Understanding of the importance of many traditional risk-factor measures has expanded over time. For instance, both systolic and diastolic blood pressure levels are associated with the occurrence of CHD, and a gradient of risk increases across the entire range of blood pressure, even if it is below standard treatment thresholds (Ramsay, Williams *et al.* 1999). Over the last 50 years the list of major cardiovascular risk factors has continued to

grow, as our understanding of vascular biology improves. Despite the identification of a variety of potent risk factors for the development of CHD, many of the underlying pathophysiological mechanisms remain unclear. Although patients with CHD commonly have at least one identifiable risk factor (Wilson 1994), many ischaemic events occur in the absence of any of these more classical associations (Futterman *et al.* 1998). For example, in the United States, cholesterol screening fails to identify almost 50% of the 1.3 million individuals who develop a myocardial infarction each year, who have either normal or only moderately increased serum cholesterol concentration (Rifai *et al.* 2001). Furthermore, no more than 25% of the excess CHD risk in diabetes can be accounted for by established risk factors (Pyorala *et al.* 1987). This illustrates the complexity of CHD and CVD, particularly in high risk states such as diabetes, and may explain the inaccuracy of methods such as the Framingham equation and the PROCAM calculation to predict CHD risk in diabetes (Stephens et al in press). Recently, interest has developed in alternative markers, such as plasma markers of oxidative stress, which may be useful in predicting risk.

1.3 The biological relevance of oxidative stress

Considerable interest has developed on the possible role of free radical-mediated damage in many major disorders, in particular CHD, diabetes and cancer (Baynes 1991). Free radicals are atoms or molecules that have one or more unpaired electrons in their atomic structure and are therefore highly reactive. Oxygen is the most ubiquitous of all biologically important chemical species and is a major source of Reactive Oxygen Species (ROS). It has been estimated that between 1-5% of inhaled oxygen becomes an active oxygen species, which is approximately 400,000,000,000,000,000,000,000 molecules/person/day or approximately 25,000,000,000 molecules/cell/day (oral communication by Berk 2003, ADA, New Orleans). Oxidative stress (OS) results from an imbalance between oxidant production (or the formation of ROS) and antioxidant defences (Maritim *et al.* 2003). As illustrated in figure 1.2, increased OS is associated with many of the risk factors implicated in the pathophysiology of atherosclerosis including diabetes, hypercholesterolaemia, renal failure, ageing, hypertension and smoking (Harrison *et al.* 2003). ROS include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$) and the peroxynitrite radical ($OONO^{\cdot-}$). In its most severe form, redox imbalance may result in cell death following widespread macromolecule oxidation, while more subtle changes appear to play a role in modulating a range of signal transduction pathways (Suzuki *et al.* 1997). All molecules are potential targets for ROS (proteins, lipids and DNA), but because of their ubiquitous distribution within cell membranes, and their propensity to contain double bonds, unsaturated lipids are often targeted (Evans *et al.* 2002).

Figure 1.2: Causes & consequences of Oxidative stress



ROS: Reactive oxygen species, MMP: Matrix metalloproteinases, VSMC: Vascular smooth muscle cell

Endogenous antioxidant systems exist to reduce oxidative stress (table 1.1). These include the mitochondrial enzyme superoxide dismutase (SOD), which catalyses the conversion of $O_2^{\cdot-}$ to H_2O_2, which may then be detoxified further in the cytoplasm to form H_2O by the action of glutathione peroxidase (mitochondria), glutathione s-transferase (cytoplasm) or catalase (peroxisomes).

Table 1.1: Endogenous Antioxidants

Vitamins	A, C, E
Enzymes	Glutathione peroxidase, Glutathione reductase, Glutathione s-transferase, Superoxide dismutase, Catalase, Paraoxonase
Minerals	Cu, Zn, Mn, Se
Co-factors	Vitamins B1, B2, B6, B12,
Mitochondrial proteins	Uncoupling proteins
Others	α-lipoic acid, Carotenoids, CoQ10, Bioflavanoids

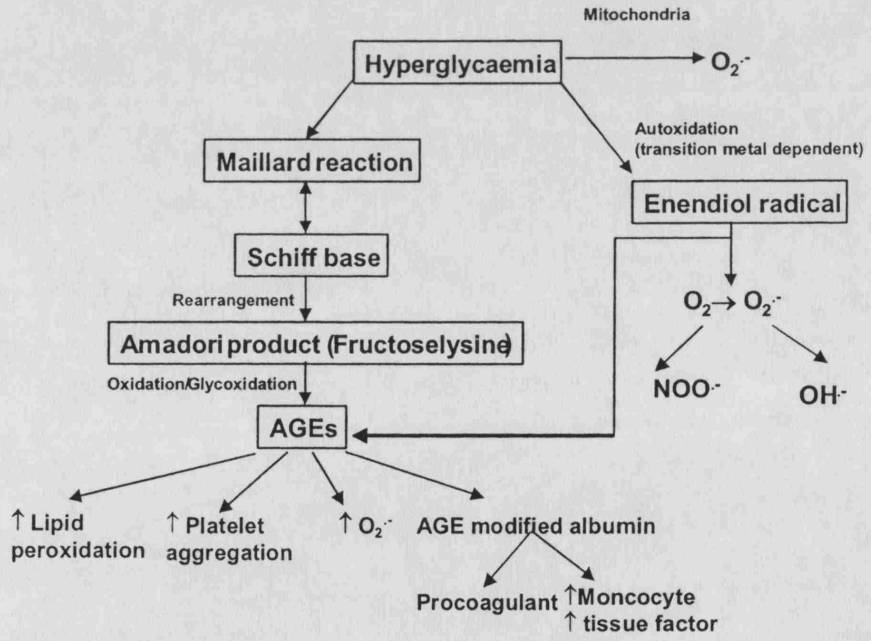
1.3.1 Oxidative stress and diabetes mellitus

Diabetes mellitus has been consistently associated with increased oxidative stress (Davi *et al.* 1999; Cai *et al.* 2000; Brownlee 2001). It is however unclear whether diabetes is a cause or the result of increased oxidative stress. Furthermore, within the cluster of phenotypes that make up diabetes, certain factors, such as obesity may contribute further to increased oxidative stress and subsequent insulin resistance.

Several studies have demonstrated that hyperglycaemia *per se,* is associated with increased oxidative stress and provides a potential 'link' between diabetes and atherosclerosis. This is supported by the observation that plasma from diabetic subjects contains increased levels of end-products of oxidative damage, including thiobarbituric acid reactive substances (TBARS) and lipid peroxides (Nourooz-Zadeh *et al.* 1995). Furthermore, subjects with both type 1 and type 2 diabetes have elevated urinary F_2-isoprostanes compared to healthy controls. Interestingly, in this study, the mean level of

urinary F_2-isoprostanes was identical in both type 1 and type 2 subjects (Davi *et al.* 1999). F_2-isoprostanes are the end-product of free radical attack on cell membrane phospholipids, and as will be discussed later, are often considered to be the 'gold standard' measure of plasma oxidative stress. Further evidence for a direct role of hyperglycaemia in increasing oxidative stress, comes from the observation that with improved glycaemic control, urinary F_2-isoprostane levels decreased significantly. Possible mechanisms (Evans *et al.* 2002; Maritim *et al.* 2003) by which hyperglycaemia may induce ROS formations are shown in figure 1.3.

Figure 1.3: Hyperglycaemia induced oxidative stress



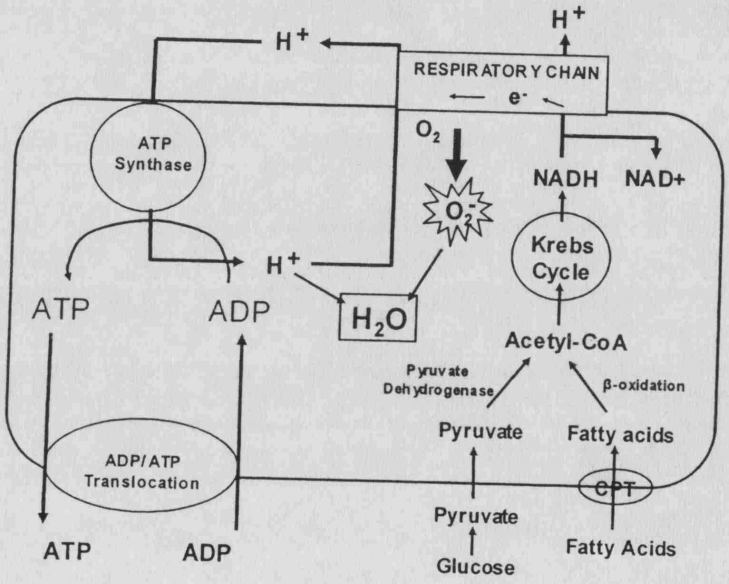
AGEs: Advanced glycosylation end-products, $O_2^{\cdot-}$: Superoxide radical, $NOO^{\cdot-}$: Peroxynitrite radical

As shown in figure 1.3, hyperglycaemia may result in the glucose-mediated non–enzymatic glycosylation of proteins (the *Maillard* reaction). The result of this is to form a *Schiff* base, whereby a covalent bond forms between the amine group of a protein and the aldehyde group of glucose. Further rearrangement and oxidation results in the formation of advanced glycosylation end products (AGEs). These not only increase ROS production but may also initiate a cascade of events, which have deleterious effects on the vascular system. Alternatively, glucose may undergo auto-oxidation to form a highly reactive enendiol radical. This not only increases AGE formation but may also catalyse the conversion of molecular oxygen to $O_2^{\cdot-}$ (and hence increase ROS). There is also an important direct mitochondrial mechanism by which glucose may increase ROS, which will be discussed later (Evans *et al.* 2002).

Obesity is a typical feature of impaired glucose tolerance and T2DM. As mentioned above, obesity may also contribute further to increase oxidative stress and subsequent insulin resistance in subjects with T2DM. In obese subjects, caloric intake 'usually' exceeds energy expenditure. In adipose and skeletal muscle, excess energy substrate (typically glucose and free fatty acids) enters the citric acid cycle, resulting in the generation of excess mitochondrial NADH and subsequently ROS, as shown in figure 1.4. To protect against the harmful effects of ROS, cells may reduce the formation of ROS and/or enhance ROS removal. Reducing ROS generation may be accomplished by preventing the accumulation of NADH resulting from increased nutrients. This may be accomplished by inhibiting insulin-stimulated nutrient uptake, thus preventing the influx

of high-energy substrates (glucose, pyruvate, fatty acids) into the mitochondria (Ceriello *et al.* 2004).

Figure 1.4: Glucose and fatty acid dependent generation of ROS in the mitochondria



It remains unclear whether free fatty acid (FFA) or glucose is the primary fuel source in over-nourished muscle and adipose tissue. In either case, an influx of substrate into the citric acid cycle generates increased mitochondrial acetyl-coA and NADH (Maddux *et al.* 2001). Acetyl coA, derived from glucose via pyruvate, or by the β-oxidation of FFA, combines with oxaloacetate to form citrate, which enters into the citric acid cycle and is converted to isocitrate, and subsequently, NAD^+-dependent isocitrate dehydrogenase generates NADH. When excessive NADH cannot be dissipitated by oxidative

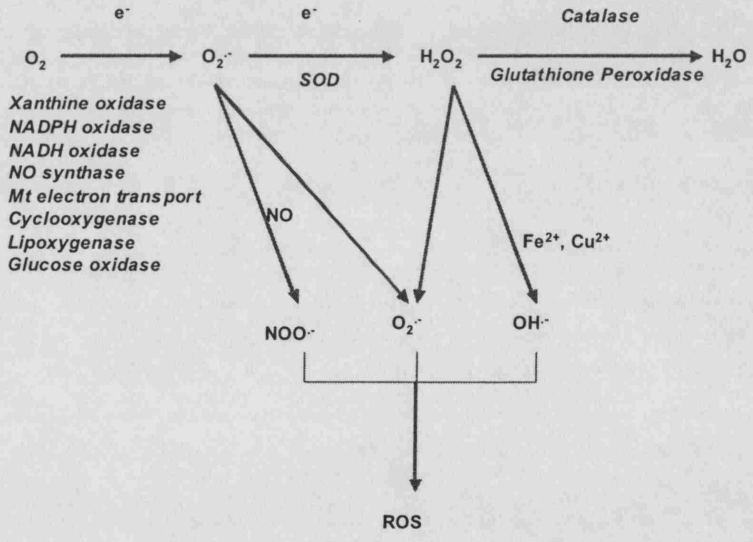
phosphorylation the mitochondrial proton gradient increases, and single electrons are transferred to molecular oxygen, resulting in the formation of superoxide (Maechler *et al.* 1999). Within the cell, an increase in FFA in turn leads to reduced GLUT4 translocation to the plasma membrane, resulting in resistance to insulin stimulated glucose uptake in muscle and adipose tissue (Rudich *et al.* 1998; Tretter *et al.* 2000; Talior *et al.* 2003). In this setting, insulin resistance may be considered a compensatory mechanism that protects the cell against further insulin-stimulated glucose and fatty acid uptake and therefore oxidative damage. This is supported by both *in vitro* and *in vivo* animal models where antioxidants have been shown to improve insulin sensitivity (Paolisso *et al.* 1996).

Pancreatic β-cells and endothelial cells may be particularly affected by overfeeding, as these cells are not dependent on insulin for glucose uptake, and therefore if 'overfed', they cannot down-regulate the influx of nutrients by insulin resistance, and must allow intracellular concentrations to increase further. Many studies have suggested that β-cell dysfunction results from prolonged exposure to high glucose, elevated FFA or both (Evans *et al.* 2003). β-cells are particularly sensitive to ROS because they are low in antioxidants such as glutathione peroxidase, catalase and SOD (Tiedge *et al.* 1997; Robertson *et al.* 2003). Therefore increased oxidative stress may not only result from hyperglycaemia associated with diabetes, but may also have an important causal role in β-cell failure and the development of insulin resistance and T2DM.

1.3.2 Glucose independent sources of oxidative stress

There are in addition, glucose-independent sources of oxidative stress (Harrison *et al.* 2003). These are summarized in figure 1.5. Enzymes such as NADPH oxidase and xanthine oxidase catalyse the conversion of molecular oxygen to $O_2^{\cdot-}$. Under the influence of mitochondrial SOD, this is converted to hydrogen peroxide. Cytoplasmic catalase or glutathione peroxidase then detoxifies this further to form water. Therefore, if there is increased NADPH activity (or any of the enzymes generating $O_2^{\cdot-}$ as a by-productor) or reduced SOD or glutathione activity, ROS generation will be increased.

Figure 1.5: Glucose independent sources of ROS



The influence of antioxidant enzymes (e.g. glutathione enzymes) in determining plasma levels of oxidative stress will be discussed in later chapters.

1.3.3 Oxidative stress and coronary heart disease

Several studies have demonstrated that plasma markers of oxidative stress are elevated in CHD or in the presence of its classical risk factors (Cai *et al.* 2000; Chisolm *et al.* 2000; Harrison *et al.* 2003). As summarized in figure 1.2, *in vitro*, numerous adverse effects on the vascular system are associated with increased oxidative stress. The oxidation of vulnerable cell membrane unsaturated lipids (Evans *et al.* 2002) may modulate diverse signal transduction pathways (Suzuki *et al.* 1997; Harrison *et al.* 2003) leading to numerous adverse effects implicated in the pathogenesis of atherosclerosis. These include increased expression of cell adhesion molecules, induction of pro-inflammatory pathways, activation of matrix metalloproteinase, vascular smooth muscle cell proliferation and death, endothelial dysfunction and lipid peroxidation (LDL-oxidation). *In vivo*, animal studies have shown increased oxidative stress during experimental hypoxia and during cardiac ischaemia (Ceconi *et al.* 2003). In humans, increased oxidative stress has also been demonstrated during coronary by-pass grafting (Ceconi *et al.* 2003), post-myocardial infarction and in congestive cardiac failure (Ceconi *et al.* 2003).

There is therefore, considerable evidence to support the role of oxidative stress in the pathogenesis of CHD at the molecular level; however no prospective studies have been reported showing increased CHD risk in relation to basal plasma markers of oxidative stress. Therefore, whether increased oxidative stress is a 'cause' or 'effect' of CHD remains to be established *in vivo*.

1.3.4 Oxidation of LDL and coronary heart disease

Apart from the global effects associated with increased oxidative stress described above, more specific effects also occur. LDL is an important target of oxidation, and oxidative modification of LDL is a key step in the pathogenesis of atherosclerosis (Witztum *et al.* 2001). The original interest in oxidised-LDL (Ox-LDL) stemmed from two basic observations. The first was that Ox-LDL was cytotoxic to endothelial and smooth muscle cells (Hessler *et al.* 1983). The second was that uptake of native LDL by macrophages occurred at sufficiently low rate to prevent foam cell formation, but uptake of Ox-LDL was unregulated and led to macrophage foam cell formation and subsequent atherosclerosis (Heinecke *et al.* 1984; Steinbrecher *et al.* 1984). Elevated Ox-LDL is independently associated with increased atherosclerotic burden and increased CHD risk (Toshima *et al.* 2000; Ehara *et al.* 2001; Weinbrenner *et al.* 2003). It is now clear that Ox-LDL, with its many oxidatively-modified lipids and degradation products, contributes to the pathophysiology of both the initiation and progression of atherosclerosis (summarised in table 1.2).

Subjects with diabetes (in particular T2DM) typically have elevated plasma triglycerides (Tg) and low HDL. In addition, whilst plasma LDL is not typically elevated, they do have a preponderance of small dense (sd)-LDL. A number of studies have demonstrated that a preponderance of sd-LDL is associated with increased CHD risk (Austin *et al.* 1988; Campos *et al.* 1992; Stampfer *et al.* 1996; Lamarche *et al.* 2001). There are a number of possible reasons why this may be the case. The residence time of sd-LDL is prolonged in the plasma as a result of lower binding affinity to the LDL receptor, and sd-LDL has a

greater affinity to bind to intimal proteoglycans (Anber *et al.* 1996). Because of its size, sd-LDL penetrates more easily into the sub-endothelial space of the arterial wall where oxidative modification takes place (Bjornheden *et al.* 1996). The increased susceptibility of sd-LDL to oxidation was recently demonstrated *in vivo*, where an inverse association has been observed between sd-LDL and Ox-LDL (Scheffer *et al.* 2003).

Therefore, as well as the general harmful effects on the vasculature associated with oxidative stress, more specific effects may be seen at the lipoprotein levels, which are dependent on the properties of these molecules. This thesis will explore these associations further and consider the possible anti-oxidant effect of a major gene involved in lipoprotein metabolism.

Table 1.2: Consequences of elevated Ox-LDL

Increased foam cell formation
Increased monocytes & T-cells chemotaxis
Increased vascular smooth muscle and macrophage production
Altered gene expression (MCP-1, IL-1, ICAMs)
The induction of proinflammatory genes
(PPARγ, Haemoxygenase, SAA, Ceruloplasmin)
Increased Immunogenicity (elicits autoantibody formation & activated T-cells)
Increased LDL susceptibility to aggregation
Enhanced procoagulant pathways
Altered arterial vasomotor properties

1.3.5 Measuring oxidative stress in plasma

By definition, ROS or free radicals are highly reactive and are thus difficult to measure in any biological sample, especially in easily accessible specimens such as serum or plasma. Table 1.3 summarises some of the widely used methods to measure plasma oxidative stress. Many authorities consider plasma or urine F_2-isoprostanes to be the 'gold' standard measure of *in vivo* oxidative damage, however (as discussed below) measurement may be technically difficult. An alternative measure, which is often used is to record the plasma Total Antioxidant Status (TAOS/TAS).

a. Plasma total antioxidant status

Mammals have evolved complex antioxidant strategies to minimise the harmful effects of oxygen in its partially reduced form. Antioxidants within cells, cell membranes and extracellular fluids may be up-regulated and mobilised to neutralise excessive and inappropriate ROS formation. Within the strategy to maintain redox balance against oxidant conditions (e.g. smoking, chronic inflammation), blood has a central role, since it transports and redistributes antioxidants to every part of the body. Plasma components may scavenge long-lived ROS, such as superoxide and hydrogen peroxide, thus preventing reactions with catalytic ions to produce more harmful species (Lynch *et al.* 1978; Halliwell B 1989; Mao *et al.* 1992). Furthermore, plasma components can also reduce oxidised ascorbic acid back to ascorbate (Okamura 1979; Wagner *et al.* 1987; Iheanacho *et al.* 1993; May *et al.* 1995).

Table 1.3: Methods used to measure oxidative stress in plasma

Method	Principle	Comment
Plasma TAOS/TAS	Measures the inhibition of an *in vitro* oxidative process by plasma.	Technically easy and provides an overall measure of antioxidant status. Methods include TAOS/TAS/TRAP.
F_2-isoprostanes	Product of free radical-mediated oxidation of phospholipids containing arachidonic acid	Very specific and the 'gold' standard for assessing lipid peroxidation ex-vivo. However, technically complex requiring mass spectroscopy. Commercially available ELISA kit not validated.
Lipid peroxides	Chemiluminescence, iodometry and ferrous oxidation in xylol orange	Poor specificity. Confounding by proteins and haemoglobin. Commercially available kit not validated.
Thiobarbituric acid-reactive substances	Malonaldehyde, a product of lipid peroxidation is coupled to thiobarbituric acid and the resulting chromogram is measured by flourescence.	Widely used and technically simple. Subject to confounding by compounds of non-peroxidation origin. The specificity may be improved by HPLC. Also affected by Fe content of buffer and reagents.
Conjugated dienes	Early product of lipid peroxidation. Detected at 230nm.	Subjects to confounding by dienes of dietary origin. Some purines, proteins and haem absorb at similar wavelengths. Variation between subjects and laboratories.

Plasma TAOS is therefore, the net effect of many different compounds and systemic interactions. Co-operation among different antioxidants provides greater protection against ROS attack than that provided by any compound alone. A typical example of synergism between antioxidants is glutathione regenerating ascorbate (Packer *et al.* 1979) and subsequently ascorbate regenerating α-tocopherol (Stocker *et al.* 1986). Therefore, plasma TAOS, may give more biologically relevant information than that obtained from measuring plasma concentrations of individual antioxidants (e.g. α-tocopherol, ascorbate).

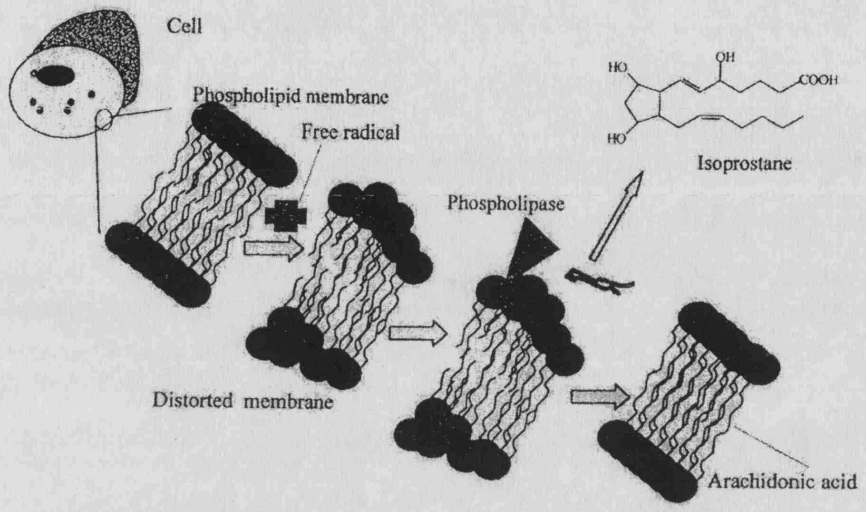
Measuring plasma TAOS has several advantages compared to other measures of oxidative damage in the plasma. Methods employed for measuring serum or plasma concentrations of individual antioxidants, or specific ROS are time-consuming, labour intensive, costly and require complicated techniques (e.g. gas chromatography-mass spectroscopy: GC-MS). For a large number of samples, plasma TAOS is a practical and inexpensive method of assay. Furthermore, the measure of individual antioxidant molecules separately is not practical and much of their antioxidant effects are additive. Plasma TAOS has been widely used clinically to assess plasma antioxidant depletion and oxidative stress, and has been shown to correlate well with other indices (Miller *et al.* 1993; Rice-Evans *et al.* 1994; McLemore *et al.* 1998). Previous studies have suggested that much of the plasma antioxidant capacity may be related to plasma levels of proteins and uric acid (Erel 2004). Unfortunately, these measures were not available in this study and hence could not be adjusted for in the analysis. However, as described in this thesis, correlation with other biochemical intermediate phenotypes will be explored.

b. Plasma F_2-isoprostane

Plasma F_2-isoprostanes are a class of prostanoid-like compounds derived from arachidonic acid, by free radical attack on cell membrane phospholipids (Morrow *et al.* 1990) or circulating LDL (Lynch *et al.* 1994; Morrow *et al.* 1997). After their formation, isoprostanes are released from the membrane phospholipids in response to cellular activation, presumably through a phospholipase-mediated mechanism (figure 1.6), circulate in the plasma, and are excreted in the urine. They may circulate as the free form, or esterified in phospholipids. The factors that regulate release and inter-conversion between the free and esterified form are poorly understood. Given the ubliquitous distribution of the precursor arachidonic acid, isoprostane synthesis can occur in virtually all of the cellular layers of the atherosclerotic lesion (Mezzetti *et al.* 2000).

The measurement of plasma F_2-isoprostane is considered the best measure of plasma oxidative stress. The main advantage of the technique is the high degree of specificity and sensitivity provided by GC-MS. However, measurement requires a relatively large volume of plasma (0.5-1.0ml) and is a multi-step complex process (described in chapter 2) with the potential for errors at many points. Furthermore, measurement must be performed in an experienced specialist centre with GC-MS. The measure of F_2-isoprostane concentration in 10 samples takes between 48-72 hours.

Figure 1.6: Synthesis and cleavage of isoprostane in the cell membrane



Free radical attack of arachidonic acid forms an isoprostane esterified to membrane phospholipids and this perturbs structure. Phospholipase cleavage restores membrane integrity and releases the free isoprostane.

c. Other specific measures of oxidative damage

As described in section 1.3, oxidative stress has numerous adverse effects on the vascular system. Within these, LDL is an important target of oxidation with ox-LDL having a major pro-atherosclerotic role. As well as measuring the indices described in table 1.3, ox-LDL may also be specifically measured. This would be a useful measure as unlike the above markers of overall lipid or protein damage, this would give a specific measure of oxidative damage to the LDL particle. Using both of these measures would therefore provide knowledge of the general oxidative stress of an individual and a focused measure of LDL-oxidation. This thesis will explore the relationship between plasma TAOS and LDL-oxidation.

1.4 Common gene variants and their role in investigating the pathogenesis of CHD

1.4.1 Methods employed to study complex disease

The genetics of multifactorial disorders such as CHD and diabetes mellitus is complex, as susceptibility does not follow simple Mendelian monogenic inheritance and does not exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus (Lander *et al.* 1994). Three methods have been proposed to study complex disorders. These are:- association studies, linkage analysis and allele-sharing studies.

a. Association studies

Association studies are powered to detect a small effect of variation in one gene, but such effects can only be confirmed with statistical certainty in large samples drawn from the population as a whole. Association studies performed on samples of the population, investigate whether genetic variation, within or close to a gene of interest, is associated with inter-individual differences in the intermediate phenotype (biochemical or clinical), or with increased risk of disease. This may occur either because the genetic variation is causal (for example a sequence change leading to an amino acid substitution) or because it is in linkage disequilibrium (LD) with another sequence change which is causal, and lies elsewhere in the gene. Two different samples are typically studied in association studies. Prospective cohorts are the best for measuring risk associated with a specific gene variant, as they measure risk over time. Case-control studies are those where the allele frequency of a specific gene variant is compared in groups of unrelated subjects with and without disease. Case-control studies, are easier and since they do not require long-term follow-up, results can be made available quickly. Case-control studies also

allow a more precise characterisation of outcome, as well as greater statistical power to detect association than would be feasible with a prospective design, by ensuring all cases are scanned properly for a particular phenotype. In this respect, case-control studies are useful to study the association between gene variants and various intermediate phenotypic measures. The main disadvantage of these studies is that errors in the measurements of exposures, (e.g. age) can differ systematically between cases and controls, and therefore can give misleading results. Several association studies with respect to many different gene variants have failed to show consistent results with replication (Colhoun *et al.* 2003). One possible explanation for this is that often, these studies focus on different samples of subjects (e.g. diabetes/no diabetes, males/females, different cultural origin). Furthermore, many studies do not stratify by risk factors or look for evidence of a gene-environment interaction (e.g. with smoking). Clearly, key metabolic genes will respond differently to environmental exposure and altered homeostasis. There are many examples where this approach has been successfully employed to clarify underlying disease aetiology (Zito *et al.* 1997; Humphries *et al.* 1998; Gardemann *et al.* 1999; Humphries *et al.* 2001).

b. Linkage analysis

With linkage analysis, the whole genome is scanned in a number of families with a history of the disease, to identify chromosomal regions linked to disease and related factors. It involves constructing a disease model to explain the inheritance pattern of the disease, by comparing the observed segregation of gene markers and trait in affected pedigrees. Linkage is the method of choice for studying simple Mendelian traits in

monogenic disorders because the allowable models are few and easily tested. However, application to complex, multifactorial disorders are problematic, as the genetic analysis may be confounded by incomplete penetrance, phenocopy, genetic heterogeneity, polygeneic inheritance, absence of large multi-generated pedigrees and mis-inheritence (Lander *et al.* 1994). Linkage is well powered to detect major gene effect but not to detect genes of modest effect.

c. Allele-sharing

The third method for studying complex diseases is the allele-sharing approach. This involves studying affected relatives (or siblings) in a pedigree to see how often they inherit identical copies of the region from a common ancestor (Kurtz *et al.* 1993) and to obtain statistical evidence that the inheritance pattern of the chromosomal region is not consistent with random Mendelian segregation. This method typically applies to a single generation of disease sufferers. It is independent of the pattern of disease inheritance and therefore is frequently used in the analysis of a complex disorder with a late onset, such as CHD risk.

1.4.2 Common gene variants and their biological importance

The structure of a gene is not constant across populations, and variations frequently appear resulting from a permanent change in the DNA base sequence. Many of the variations are the result of single base-pair substitutions. If the sequence change alters the function of the gene (usually having a detrimental effect) they are usually described as mutations.

Common gene variants (or polymorphisms), occur with a population frequency of at least 1%. Whilst many variants are 'silent', others may affect either the structure of the protein transcribed (for instance, the structure of a receptor protein which may influence agonist binding) or the quantity of protein transcription. Sequence changes that have this effect are described as 'functional gene variants' and may have important biological consequences. Functional gene variants may result in the substitution of one base pair for another, resulting in the change of a codon. This may lead either to a missense mutation, in which one amino acid replaces another amino acid in a protein altering the protein structure, or to a nonsense mutation in which a termination codon appears in the middle of a gene. When a nonsense codon appears there is no transfer RNA molecule to recognise the codon, therefore protein synthesis terminates and a truncated protein is produced. Variation within RNA-splicing sites may cause mis-splicing in the intron or exon, and this is likely to result in a protein lacking part or all of an exon (or having additional amino acids or in an in-frame 'stop' codon), again altering the structure of the gene product. In addition to changes in the structure of a gene-product, variations may impact upon the *quantity* of protein produced. This occurs if there are changes in the rate of gene transcription or messenger RNA stability. Variations in RNA processing and translation will affect the stability of messenger RNA, which in turn may affect the amount of gene product. Variations arising in gene regulatory sequences, such as the promoter region, may alter the rate of gene transcription. The function of the promoter region is to set the location and direction of transcription on a DNA template. RNA polymerase must be recruited to the promoter site, a task mediated by a number of

proteins called transcription factors. Transcription factors bind to sequences within the promoter, or may bind to one another to instruct the RNA polymerase whether or not to transcribe the particular gene. For these reasons, variants present with the gene promoter region are of particular interest.

There is one circumstance in particular where caution is required before ascribing a difference in biological activity to any one variant site. This occurs when two polymorphic sites are in complete (or very strong) allelic association (linkage disequilibrium-LD). In such a situation one allele of the polymorphism of interest almost always occurs in the presence of a specific allele of the second linked variant. In this circumstance, the gene variant under investigation merely acts as a marker for the presence of the second variant. Thus, the 'effect' of variation in the candidate gene is in fact a reflection of the function of the adjacent mutation. LD is a population-based concept and is detected in a sample of unrelated subjects, as the prevalence together of two alleles of gene variants, more frequently than expected by chance alone. It is usually measured as delta (D) or D', and values range from -1 (complete negative association) to +1 (complete positive association). Values of 0, represent little or no association. D' differs from D, in that the difference in allele frequency between the two alleles under study is considered in the calculation.

1.5 Common gene variants which may influence oxidative stress

Many common gene variants linked with oxidative stress have been studied in relation to CHD risk. The list is long and includes those implicated in the generation of cellular ROS and those with anti-oxidant properties. However, association with intermediate biochemical risk factors and interaction with pro-oxidant environments (e.g. cigarette smoking) has not frequently been reported.

The focus of this thesis was to examine the association between plasma markers of oxidative stress and LDL-oxidation with variants from three specific candidate genes:- glutathione-s-transferase (a cellular antioxidant protein), apolipoprotein E (a plasma lipoprotein) and the mitochondrial uncoupling protein 2 (responsible for uncoupling the electrochemical proton gradient generated across the mitochondrial membrane by the electron transport chain). Each of these candidate genes code for proteins which may have anti-oxidant effects, in three different surroundings (cytoplasm, plasma and mitochondria). No previous studies have been reported studying possible associations between variants in these genes and plasma markers of oxidative stress and LDL-oxidation in subjects with diabetes.

1.5.1 Variation in the apolipoprotein E (*APOE*) gene

Mature apolipoprotein E (apoE) is a 299 amino acid glycoprotein (Rall *et al.* 1982) synthesised in the liver and intestine, and is found in association with trigyceride-rich lipoproteins (Talmud *et al.* 2002). It is the ligand for removal of these particles from the plasma and thus, in determining the metabolic fate of these lipoproteins. Of the candidate

genes involved in determining plasma lipid levels and CHD risk, *APOE* is the most comprehensively studied (Stephens *et al.* 2003).

The human *APOE* gene is located on chromosome 19, consisting of 3.7kb and four exons. Genetic variation in exon 4 results in the three common gene variants ε2, ε3, and ε4 which have strong and consistent influences on plasma lipids (Davignon *et al.* 1988) and CHD risk (Wilson *et al.* 1996). Each isoform differs by one amino acid (Davignon *et al.* 1988) resulting in the presence of an Arg (CGC) or Cys (TGC) at amino acid positions 112 and 158, resulting in Cys112/Arg158 in apoE3, Arg112/Arg158 in apoE4 and Cys112/Cys158 in apoE2 (Richard *et al.* 1995). Plasma apoE levels differ by genotype such that ε4ε4 subjects have the lowest levels and ε2ε2 the highest (Smit *et al.* 1988). With respect to receptor binding activity, apoE3 and apoE4 bind with equal affinity, while apoE2 is defective, with between 1-2% of the binding activity of the other isoforms (Weisgraber *et al.* 1982). ε3 is the most common isoform with a frequency of 0.77 in Caucasian populations, while ε4 and ε2 are seen at frequencies of 0.15 and 0.08 respectively (Stephens *et al.* 2003).

Association with lipids and CHD risk

Carriers of the ε2 allele, who represent about 12% of the population, have cholesterol levels approximately 10% lower than ε3 homozygotes, whilst ε4 carriers who represent approximately 25% of the population, have cholesterol levels approximately 5% higher than ε3 homozygotes. Compared to men homozygous for the ε3 allele, those carrying ε2 show protection from both CHD and stroke (Kessler *et al.* 1997), whereas those with the

ε4 allele have a higher risk (Gerdes *et al.* 2000). With respect to CHD, two meta-analyses have demonstrated increased risk in ε4 allele carriers (Wilson *et al.* 1996; Song *et al.* 2004). The latter showed that ε4 carriers had a 42% higher CHD risk compared to ε3ε3 and ε2+ subjects. However, the picture appears more complex than this, with risk associated with the ε4 allele being seen mainly, or to a large extent in smokers, and a modest or no increase in risk associated in ε4 non-smokers. In the UK based NPHSII study, compared to never smokers, smoking was observed to increase risk significantly (NPHSII OR: ε4+ smokers 3.17, ε3ε3 smokers 1.38, ε2+ smokers 1.18, ε4+ ex-smoker 0.84, ε3ε3 ex-smokers 1.74, ε2+ ex-smokers 0.48; all compared to never smokers). Importantly, the risk associated with the *APOE* genotype in NPHSII was independent of classical risk factors including lipid levels (Humphries *et al.* 2001).

Interestingly, even though ε2 is not generally associated with increased risk in published studies, ε2 is associated with elevated Tg compared to the other isoforms. Homozygosity for ε2 predisposes to the development of type III hyperlipoproteinaemia due to delayed metabolic clearance of apoE containing lipoproteins. The result is that there is an accumulation of chylomicron and VLDL remnants in the plasma (Weisgraber *et al.* 1982).

Association with biochemical markers of oxidative stress

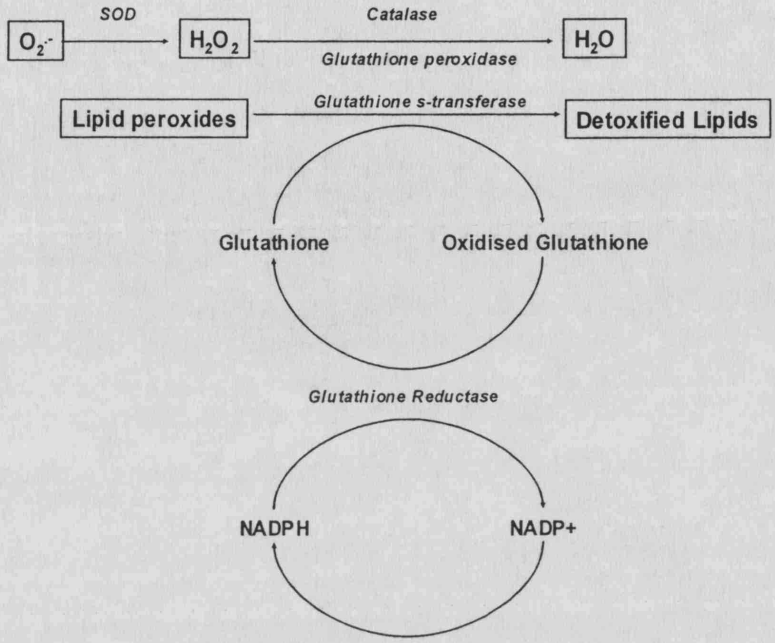
The different isoforms of apoE possess different antioxidant activity. Studies have been observed showing differential effects on susceptibility to oxidation in plasma, as well as specifically in relation to LDL. *In vitro*, the antioxidant efficacy of apoE in relation to

hydrogen peroxide cytotoxicity was E2>E3>E4 (Miyata *et al.* 1996; Jolivalt *et al.* 2000). Furthermore, serum lipid peroxides are higher in ε4+ subjects (Smith *et al.* 1998), and in subjects with Alzheimer's disease, products of lipid peroxidation in plasma and urine are lower in ε2+ compared to ε4+ subjects (Fernandes *et al.* 1999). The antioxidant effect associated with the ε2 allele may be related to the fact that E2 has two free –SH groups, E3 one –SH group and E4 none. Moreover, plasma lipoproteins from apoE-deficient mice are more prone to *in vitro* oxidation than lipoproteins from wild-type mice (Hayek *et al.* 1994). This effect may also be reversed with supplementation with vitamin E (Palinski *et al.* 1994).

1.5.2 Variation in the glutathione s-transferase gene

Glutathione s-transferases (GSTs) play a major role in cellular antioxidant defense mechanisms, by catalysing the reduction of potentially harmful peroxides (Berliner *et al.* 1995). GSTs are a family of detoxification enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (Mannervik *et al.* 1988; Ketterer *et al.* 1992). These enzymes catalyse the interaction of glutathione with a wide variety of endogenous and exogenous electrophilic compounds including potential atherogenic substances, such as lipid peroxides, to form thioesters (figure 1.7), a reaction that may be the first step in the detoxification process (Rebbeck 1997). During this process, reduced glutathione is oxidised, which is subsequently recycled back to reduced glutathione by the action of NADPH-dependent glutathione reductase.

Figure 1.7: The role of glutathione s-transferase



As shown in figure 1.7, the anti-oxidant role of glutathione peroxidase is also dependent on the oxidation of glutathione. Therefore, these two enzymes compete for reduced glutathione availability to exert their anti-oxidant role. Thus, if the anti-oxidant activity of GST is increased, it might be that glutathione peroxidase would be less efficient in detoxification (alternatively the reverse may apply).

Human GSTs are divided into two distinct super families;- microsomal and cytosolic. Microsomal GSTs play a key role in the endogenous metabolism of leukotrienes and prostaglandins. Cytosolic GSTs are divided into six classes as shown in table 1.4. *GST* expression is induced by compounds known to result in chemical stress and carcinogenesis including phenobarbitol and other aromatic compounds. GSTs may also

form protein:protein interactions with members of the mitogen activated pathway (MAP) kinase pathway thereby serving a regulatory role in the balance between cell survival and apoptosis.

Table 1.4: Cytosolic GST

Class	Gene	Chromosome
Alpha (a, α)	*GSTA1-2*	6
Mu (M, μ)	*GSTM1-5*	1
Omega (O, ω)	*GSTO1*	10
Pi (P, π)	*GSTP1*	11
Theta (T, θ)	*GSTT1-2*	22
Zeta (Z, ζ)	*GSTZ1*	14

GST genes are highly polymorphic. Most work has previously focused on the *GSTM1* and *GSTT1* deletion variants. *GSTM1* (chromosome 1p13.3) and *GSTT1* (chromosome 22q11.2) encode the cytosolic enzymes GST-μ and GST-θ respectively (Pemble *et al.* 1994). Approximately 50% of the Caucasian population are homozygous for the deletion of *GSTM1* (Rebbeck 1997) and 20% homozygous for the null variant gene for *GSTT1*. In both variants the homozygosity for the deletion allele, results in deletion of the entire gene (Pemble *et al.* 1994). Polymorphic deletion variants in the *GSTM1* and *GSTT1* genes therefore produce either a functional protein (non-deletion alleles or heterozygous deletion, *GSTM1-1* and *GSTT1-1*) or result in the complete absence of the protein

(homozygous deletion alleles, *GSTM1-0* and *GSTT1-0*) (Pemble *et al.* 1994). Considerable interest has focused on the association of these gene variants in relation to tobacco related cancers (Rebbeck 1997), since *GSTM1* and *GSTT1* enzymes detoxify not only products of oxidative stress but also carcinogenic compounds such as polycyclic aromatic hydrocarbons, a major constituent of tobacco smoke (Rebbeck 1997). Several epidemiological studies have suggested that *GSTM1-0* and *GSTT1-0* status are associated with increased risk of smoking related cancers including lung, bladder, ovarian and colorectal cancers (Bell *et al.* 1993; Chenevix-Trench *et al.* 1995; McWilliams *et al.* 1995; Brockmoller *et al.* 1996; Coughlin *et al.* 2002).

Association with CHD risk

In contrast to the extensive investigation of the *GSTM1* and *GSTT1* gene variants in relation to malignancy, their role in CVD risk and the pathophysiology of atherosclerosis, particularly in relation to exposure to cigarette smoke, remains unclear (Li *et al.* 2000; Wilson *et al.* 2000; de Waart *et al.* 2001; Li *et al.* 2001; Wang *et al.* 2002; Masetti *et al.* 2003; Olshan *et al.* 2003). Recent studies have suggested that variation in the *GSTM1* or *GSTT1* genes may alter the susceptibility to atherosclerosis (Li *et al.* 2000; Wilson *et al.* 2000; de Waart *et al.* 2001; Li *et al.* 2001; Wang *et al.* 2002; Masetti *et al.* 2003; Olshan *et al.* 2003). Many of the previous association studies looking at these gene variants have focused on the interaction with cigarette smoking, therefore in the setting of a high ROS environment. Previously, studies in smokers have shown that subjects with the *GSTM1-0* compared to *GSTM1-1* variant had a higher risk for CHD, but not of lower extremity arterial disease (Li *et al.* 2000; Li *et al.* 2001). In another study focusing on smokers and

using carotid intimal thickness (IMT) as an outcome, *GSTM1-0* compared to *GSTM1-1* had increased carotid atherosclerosis over a 2-year period (de Waart *et al.* 2001). Contradictory to these findings, two further studies focusing on a sample of UK Caucasian smokers and secondly a UK sample of subjects with a South Asian origin, showed a *reduced* risk of acute myocardial infarction in *GSTM1-0* subjects (Wilson *et al.* 2000; Wilson *et al.* 2003). The literature is also unclear with respect to role of the *GSTT1* variant in relation to CVD risk. The "Atherosclerosis Risk In Communities" (ARIC) study, showed *GSTT1-1* smokers to be at an increased risk of CHD, lower extremity arterial disease and carotid artery atherosclerosis (Li *et al.* 2000; Li *et al.* 2001; Olshan *et al.* 2003). However, two other published studies showed an association between the GSTT1-0 variant and CVD risk (Masetti *et al.* 2003; Park *et al.* 2003).

Association with biochemical markers of oxidative stress

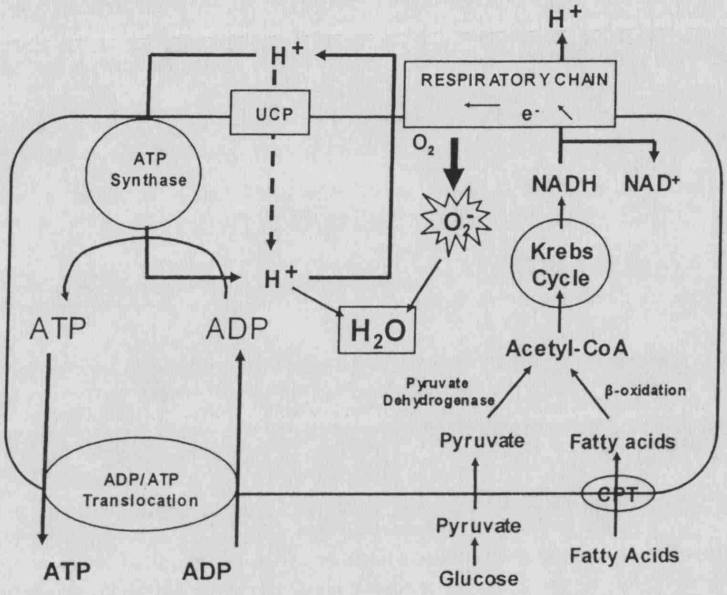
Surprisingly, despite the interest between the *GSTT1* and *GSTM1* variants and CVD risk, within the literature, there is only one report looking at the association between these genotypes and a plasma marker of inflammation and oxidative stress (Rahman *et al.* 2004). This study was performed in 90 subjects with severe acute pancreatitis (AP) and 253 healthy controls. The *GSTT-1* was more prevalent in those with AP compared to the controls (odds ratio: 6.6 [95%CI: 2.3-18.7]) and was associated with increased plasma CRP. Although the levels of reduced glutathione were higher in those with severe AP, no *GST* genotype effect on the levels was observed.

1.5.3 Variation in the mitochondrial uncoupling protein 2 gene

As described in section 1.3.1, the mitochondrial electron transport chain (ETC) is associated with substantial, innate and mandatory ROS generation as a by-product of aerobic metabolism (Shigenaga *et al.* 1994; Turrens 1997). Furthermore, the ETC may be the predominant source of ROS in non-phagocytic cells (Chance *et al.* 1979). Uncoupling proteins (UCPs 1-3) (Bouillaud *et al.* 2001) dissipate the inner mitochondrial membrane proton electrochemical gradient that drives ATP synthesis (Garlid *et al.* 2001), figure 1.8. UCP1 expression is restricted to brown adipose tissue (BAT) (Lin *et al.* 1980), whilst UCP3 is predominantly expressed in skeletal muscle (Boss *et al.* 1997). UCP2 is ubiquitous and is likely to be the ancestral UCP, and shares 59% and 73% sequence homology with UCP1 and UCP3 respectively (Fleury *et al.* 1997; Gimeno *et al.* 1997; Duval *et al.* 2002). The ubiquity and electrochemical actions of UCP2, make it a plausible negative regulator of ROS production (Negre-Salvayre *et al.* 1997; Casteilla *et al.* 2001). Uncoupling leads to a higher flow rate though the electron chain and a reduced half life of the radical intermediates (figure 1.8). This leads to a reduction in the formation of ROS, at the cost of wasting energy as heat. Therefore, it may be that UCP2 has a role in protecting the cell from increased oxidative stress. Interestingly, UCP2 activity itself is induced by by-products of lipid peroxidation (Echtay *et al.* 2002) and intramitochondrial superoxide (Echtay *et al.* 2002), which may serve to protect against further ROS generation. As described in section 1.3.1, excess nutrient intake leads to increased ROS production by the ETC, and interestingly UCP2 is over-expressed in animal models and humans under these conditions. The widespread UCP2 tissue

distribution is to some extent, due to expression in immune cells which are generators of large amounts of ROS especially under conditions of stress.

Figure 1.8: The role of UCP2



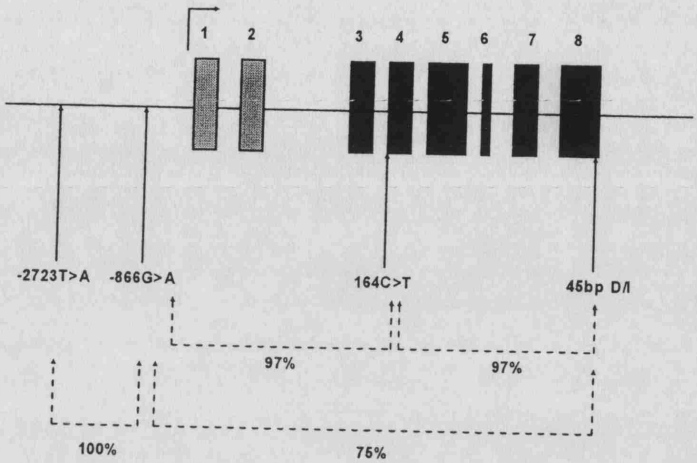
The human *UCP2* gene is located on chromosome 11q13, and consists of 8 exons, of which exons 1 and 2 are non-coding (Pecqueur *et al.* 2001). The transcription site is preceeded by a region that contains a strong *cis*-acting positive regulatory element (-141 to -65) which may underlie the ubiquitous expression of *UCP2*. Several consensus sequences exist for transcription control elements, such as C/EBP-β (important for IL-6 gene expression), CREB-1 (cAMP response binding protein 1), 2 PPARγ responsive elements, 2 TREs (thyroid hormone response elements), and NFκ-B. Promoter construct

work has further delineated regions containing further positive (-1398 to -884) and negative (-3271 to -1398, -1388 to -884, -884 to -141) control elements.

Four common gene variants have been described in the *UCP2* gene. As shown in figure 1.9, a high degree of LD exists across this gene. The -866G>A promoter variant is in 100% LD with another -2723T>A promoter variant. The -866G>A variant is also at the junction between a negative and positive cis-acting DNA regions, and within a region that contains binding sites for hypoxia, aromatic hydrocarbons and inflammatory cytokines (Esterbauer *et al.* 2001). In exon 8, a 3'UTR 45 base pair deletion/insertion (D/I) variant exist, and is in moderate LD with the -866G>A variant (75%) (Esterbauer *et al.* 2001; Wang *et al.* 2004). In exon 4, a +164C>T variant results in an amino acid change of alanine to valine (Ala55Val). This is in strong LD with the -866G>A variant (97%) and with the D/I variant (97%). The -866G>A variant has been reported to explain 71% of the variation in mRNA transcript ratio of the exon 8 D/I variant (Esterbauer *et al.* 2001).

Previous studies have demonstrated the -866G>A variant to be functional. In adipose tissue, the G allele has been associated with reduced mRNA expression *in vivo* and *in vitro* (Esterbauer *et al.* 2001). Furthermore, studies in rodent pancreatic β-cells have shown the A allele to be associated with increased mRNA expression (Krempler *et al.* 2002).

Figure 1.9: Linkage disequilibrium across the *UCP2* gene



Previous associations with the UCP2 gene variants

Considerable interest has developed in the role that the *UCP2* -866G>A promoter variant may play in determining obesity and insulin sensitivity (Esterbauer *et al.* 2001; O'Rahilly 2001; Krempler *et al.* 2002; Sesti *et al.* 2003; Wang *et al.* 2004). These studies showed an association between the -866G allele with higher BMI and also relative protection from T2DM. Furthermore, the -866A allele was associated with β-cell dysfunction as determined by lower plasma insulin (Krempler *et al.* 2002). O'Rahilly has previously described this association as "UCP2 being an adiposity angel and diabetes devil" (O'Rahilly 2001). The mechanism for this can be understood in terms of mitochondrial physiology. As described above, in adipose, the A allele compared to the G allele, is associated with higher UCP2 expression (Esterbauer *et al.* 2001). Therefore, in relation to obesity, the G allele would be associated with less uncoupling of the proton gradient across the mitochondrial membrane. This would hypothetically results in increased ATP

synthesis and storage. Conversely, with the A allele, increased UCP2 expression would increase uncoupling of the proton gradient, leading to reduced ATP synthesis and storage and increased energy loss as heat. With respect to pancreatic β-cell dysfunction in rat cells, insulin secretion is an active process dependent on ATP (Krempler *et al.* 2002). Therefore, increased UCP2 expression in these cells would result in increased uncoupling of the proton gradient and reduced ATP synthesis and subsequent insulin secretion. Previous studies looking at the D/I variant in relation to obesity have provided conflicting results (Dalgaard *et al.* 2001; Esterbauer *et al.* 2001; Marti *et al.* 2004). In a large prospective study of 544 obese Danish men and 872 healthy controls no association was observed respect to BMI and changes in body weight (Dalgaard *et al.* 1999).

For these reasons it is understandable why much of the previous work relating to variation in the *UCP2* gene has been related to energy expenditure, obesity and insulin secretion. However if this gene has such a 'major' effect on the mitochondrial proton gradient and hence in allowing the neutralisation of superoxide, a valid hypothesis would be that this may variant alter cellular ROS production and hence play an important antioxidant role and protect against CHD.

Evidence for the role of UCP2 in modulating oxidative stress

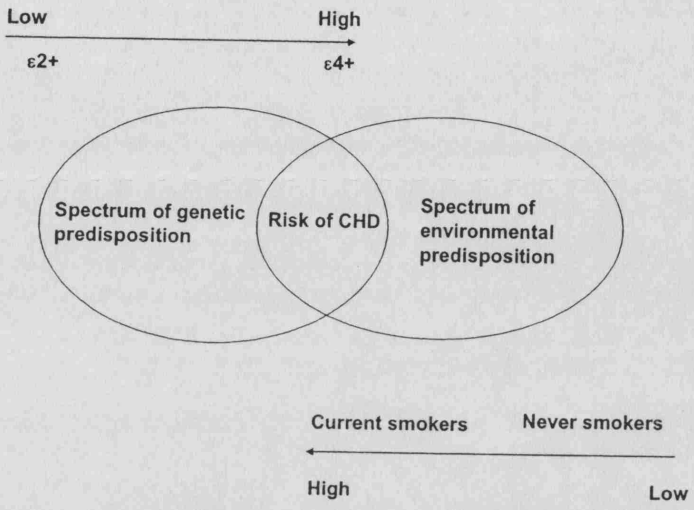
The UCP2 knockout mouse is able to generate more ROS during infection with *T. gonadii,* and clear an infection normally fatal in the wild type mice (Arsenijevic *et al.* 2000). The knockout mouse has higher levels of ROS and also had a greater burden of arterial disease. Further support for an antioxidant role of UCP2 comes from cell and

animal transgenic studies. Decreased UCP2 expression (through endothelial anti-sense strategies (Duval *et al.* 2002) or macrophage gene-deletion (Arsenijevic *et al.* 2000)) increases ROS generation and bone marrow transplant from UCP2 knockout donor mice increases markers of oxidative stress and lesion size in atherosclerotic-prone mice (Blanc *et al.* 2003).

1.6 Gene-environment interaction

Almost all chronic diseases, such as diabetes and CHD, result from gene-environment interactions (Sing *et al.* 2003). This effect is illustrated in figure 1.10. In a population there is a range of genetic risk profiles with each individual occupying a position along the genetic risk spectrum (e.g. ε4 allele). Similarly, individuals adopt a different position on the environmental spectrum of risk by the lifestyle choices they make (e.g. smoking, diet-induced obesity). The importance of gene-environment interaction is that, only when an individual with a high-risk genetic profile enters into a high-risk environment, will the effect be so great that premature CHD will develop. In this way, a gene variant may be compared to the analogy of a 'loaded gun' and the environment to a 'firing trigger'. The result is *'Genes load the gun, but the environment pulls the trigger' (Elliot Joslin1921).* There are several examples where gene-environment interaction has been studied both in relation to CHD risk and biochemical intermediate phenotypes (Stephens *et al.* 2003). An example may be observed in individuals who are carriers for the ε4 allele, as described in section 1.5.1. In the NPHSII study, male smokers with this genotype had a hazard ratio of 3.17 for future CHD.

Figure 1.10: Gene:environment interaction



1.7 Aims of thesis

General hypothesis

Diabetes and CHD are closely linked, and both are associated with increased oxidative stress. The association between oxidative stress and other biochemical intermediate risk factors remains unclear. Exploring these associations, the effects of candidate anti-oxidant genes and gene-environment interaction will clarify this, improving our understanding of the pathophysiology of CHD and diabetes. Studying these associations in a high-risk group of subjects with diabetes (a 'stressor state') may reveal important associations not seen in normal ('unstressed') subjects. Thus, the aims of this study were:-

1. To recruit a large cross-sectional sample of patients with diabetes and record all relevant clinical and biochemical data. Blood for DNA extraction, plasma and serum for further analysis of intermediate traits will also be collected.

2. To study phenotypic measures of oxidative stress and LDL-oxidation in relation to CHD status and associated risk factors.

3. To study the association of variants in the *APOE*, *GST* and *UCP2* genes in relation to the biochemical intermediate phenotypes described in aim 2. Gene-environment interaction in relation to cigarette smoking and CHD risk will also be examined.

4. Expression studies will be performed in relation to *UCP2*, as no previous studies have explored the association of variants in this gene with CHD risk and oxidative stress in humans. Further studies will be performed on monocytes from subjects selected by genotype to study cellular oxidative burden and gene expression.

CHAPTER TWO

METHODS

CONTENTS OF CHAPTER TWO

2.1 Reagents and commonly used stock solutions

Reagents

Plasma Total antioxidant status: 2,2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid (ABTS), Horseradish Peroxidase (HRP) and Hydrogen Peroxide (H_2O_2). All supplied by Sigma (Poole, UK).

Plasma F_2-isoprostane: D_4-8-iso-$PGF_{2\alpha}$, and 5ng of D_4-$PGF2_\alpha$ standards supplied by Cayman Chemical (MI, USA). tC18 (-Si-C_{18}-H_{37}: tc18 Sep-Pak) columns (Waters, MA, USA). N,N-di-isopropylethylamine Pentaflourobenzylbromide, dimethylformamide (DMF) and bis-siylyltrimethylfluoroacetamide (BSFTA) and TLC plates (Whatman 60A, Linear K6D, 5x20cm, 250μm thick) were supplied by Sigma-Aldrich (Poole, UK).

Plasma CRP: Anti-CRP antibody, CRP Standards and conjugated anti-CRP antibody was supplied by DAKO (Glostrup, Denmark). All other reagents were supplied by Sigma (Poole, UK).

DNA Extraction: All reagents were supplied by Sigma (Poole, UK).

Polymerase Chain Reaction: PCR primers and *Taq* polymerase were supplied by Gibco-BLR Ltd (Paisley UK). Restriction enzyme (Mlu-I) was supplied by New England Biolabs Inc (UK). dNTPs were supplied by Pharmacia Biosystems Ltd (Milton Keynes, UK).

Gels: 37.5:1 acrylamide:N,N'-methylenebenebisacrylamide was supplied by Protogel, National Diagnostics (Hull, UK). TEMED (NNN',N'-tertramethylethylethylenediamine and ammonium persulphate (APS) were supplied by BDH (Leicestershire, UK).

Cell culture: RPMI-1640 medium was supplied by Gibco (Paisley, UK). Ficoll-plaque was supplied by Amersham chemicals (Amersham, UK). DCF-DA was supplied by Molecular Probes (Eugene, USA). All other reagents were supplied by Sigma (Poole, UK). Menadione was supplied by Sigma-Aldrich (Poole, UK). Diphenyleneiodonium chloride (DPI) was supplied by Calbiochem (Darmstadt, Germany).

Commonly used stock solutions

CRP Assay:

Coating buffer: 10.6g Na_2CO_3 (0.1M), 8.4g $NaHCO_3$ (0.1M) dissolved in 1 litre of H_2O and adjusted to pH 9.6.

10x Wash Buffer (PBST (Phosphate Buffered Saline and Tween)): 160g NaCl, 3g Na_2HPO_4 (anh) or 57.3g $Na_2HPO_4.12H_2O$, 4g KCl , 4g KH_2PO_4 (anhydrous) dissolved in 2 litres of H_2O (for 10x PBS), to which 0.05% Tween 20 was added.

Blocking Buffer: 1% BSA (Bovine Serum Albumin) in PBS (0.1g per 10ml).

Diluting Buffer: 0.2% BSA in PBST (0.1g per 50ml).

Substrate: 0.9g Na acetate in 10ml H_2O (10x solution). This was diluted, 1ml in 9ml of H_2O and 1 tablet of TMB (3,3', 5,5' tetramethyl-benzene dihydrochloride) added.

Coating antibody: 6µl of anti-CRP antibody was added to 6ml coating buffer per plate to be coated (1:1000 dilution).

CRP Standards: 10µl of CRP standards was diluted in 327.5µl of diluting buffer. 10µl of this was added to 990µl of diluting buffer for the first standard (48µl/l). Serial dilutions were then made (1:2), by aliquoting 500µl of standard into 500µl aliquots of diluting buffer to produce the standard series: Standard 1: 48µg/l; 2: 24µg/l; 3: 12µg/l; 4: 6µg/l; 5: 3µg/l; 6: 1.5µg/l.

Conjugate: 3µl of HRP (horseradish peroxidase)-conjugated anti-CRP antibody to 6ml of diluting buffer (a 1:2000 dilution) per plate.

Wash Buffer: For 500mls wash buffer, 50mls of 10xPBS to added to 450mls of H_2O. 250µl Tween 20 was then added.

DNA Extraction:

1M $MgCl_2$: 20.33g $MgCl_2$ dissolved in 100ml in dH_2O.

1M Tris pH 7.5: 12.11g Tris, made up to 100ml in dH_2O, Correct pH to 7.5 and autoclaved.

Sucrose lysis mix: 109.54g sucrose, 5ml 1M $MgCl_2$, 10ml 1M Tris pH7.5, 10ml Triton-X-100, made up in 1000ml in dH_2O and stored at 4°C.

0.5M Na_2EDTA: 37.22g EDTA, made up to 200ml in dH_2O. Adjust with NaOH to pH 8.0

10%SDS: 10g Sodium dodecyl sulphate, made up in 100ml in dH_2O.

Nuclear lysis mix: 1ml 1M Tris-HCl pH8.2, 2.34gNaCl, 0.4ml 0.5M Na_2EDTA pH 8.0, 10ml 10%SDS, made up to 90ml in dH_2O.

5M Sodium perchlorate: 70.24g sodium perchlorate, made up to 100ml in dH_2O.

TE buffer pH 7.6: 1.21g Tris, 0.37g EDTA, made up to 1000ml in dH_2O.

MADGE gels: For each gel a solution was made up containing:-

5mls of 10xTBE , 12.2mls 30% acrylamide-bisacrylamide (in a ratio of 19:1), 32.5mls of distilled dH_2O, 150µl N'-tetramethylethylenediamine (TEMED), 150µl 25% ammonium per sulphate

TBE (10x Tris-Boric acid-Ethylenediaminetetraacetic acid contained:- 0.9M Tris, 0.9M Ortho-boric acid, 0.2M ethylenediaminetetraacetic acid

Krebs buffer: For 1l: NaCl (118mM) 6.896g; KCl (4.8mM) 0.358g; KH_2PO_4 (1.2mM) 0.163g; $MgSO_4.7H_2O$ (1.2mM) 0.296g; $CaCl_2$ (1.0mM) 0.111g; HEPES (25mM) 5.958g; 990ml of dH_2O.

This solution was autoclaved and 1.009g of glucose in dH_2O added to give a final concentration of 5.6mM.

2.2 Methods

2.2.1 Study samples

a. UCL Diabetes and Cardiovascular Disease Study

The University College London Diabetes And Cardiovascular disease Study (UDACS) is a cross-sectional sample of subjects designed to study the association between common variants in inflammatory/metabolic genes and biochemical risk factors implicated in CHD in patients with diabetes.

(i) Recruitment

All subjects were recruited by me, between December 2001 and January 2003 from the diabetes clinic at University College London Hospitals NHS Trust (UCLH). Clinical information was gathered from the computerised clinic database, which was first established in 1983. The database contains demographic and clinical information on patients attending the diabetes clinic. All patients had diabetes according to WHO criteria (Alberti *et al.* 1998; Alberti *et al.* 1998). From the database, patients were categorised by the presence/absence of clinically manifest CHD. The presence of CHD was recorded if any patient had positive coronary angiography/angioplasty, coronary artery bypass, cardiac thallium scan, exercise tolerance test, myocardial infarction or symptomatic/treated angina. Any individual who was asymptomatic or had negative investigations was categorised as 'no CHD'. Table 2.1 summarises the characteristics of the UDACS sample. Analysis in this thesis will focus on Caucasian subjects only, as these individuals made up the majority of the sample (approximately 80%). Furthermore, the frequency of gene variants may differ between different ethnic groups and subjects

from certain racial origins (e.g. South India) with diabetes are at an increased risk of CHD. (Of note, within the sample, the next most common ethnic group were those from the Indian continent, and comprised 11% of the sample).

Ethical approval was obtained from UCL/UCLH Ethics Committee and the project was registered with the Department of Research and Development at UCLH. All subjects completed a self-assessment questionnaire and gave written consent before being recruited in the study. All subjects were provided with an information leaflet and their family doctor notified of their involvement in the study.

Table 2.1: The University College London Diabetes And Cardiovascular Disease Study (UDACS)

Cohort of 1011 subjects with diabetes	
Age (years)	61.8 (13.2)
Duration of diabetes (years)	11 (5-19)
Type of diabetes	
I	17.8% (n=180)
II	79.1% (n=800)
Pancreatic (pancreatectomy/chronic pancreatitis)	3.1% (n=31)
CHD	20.1% (n=198)
Caucasian	77% (n=780)
Males	62% (n=631)

(ii) Baseline measures

Demographic data including gender, smoking history, family history, medical treatment (diabetes and other), age, date of diagnosis, type of diabetes, date of recruitment, and ethnic origin were recorded. Clinic measurements of blood pressure (supine and lying), weight and height were measured on all subjects. Routine clinic biochemistry was also recorded including HbA_1c, random glucose, total cholesterol, LDL, HDL, random triglycerides, thyroid function, albumin:creatinine ratio, and proteinuria by dipstick. All subjects were free from acute illnesses at the time of recruitment.

(iii) Blood sample collection and storage

Fifteen milliliters of blood was obtained from each subject, at the time of venesection for their routine clinic investigations. This included 5mls of serum, 5mls EDTA and 5mls of citrated plasma. All samples were immediately placed on ice and centrifuged (Sorvall centrifuge at 1800g for ten minutes) within one hour of collection. Serum and plasma samples were divided into three aliquots and immediately frozen at -80°C. The buffy coat from the EDTA/citrate samples was frozen and stored at -20°C for DNA isolation. Of note, EDTA plasma was collected three months into recruitment. This was therefore available on approximately two-thirds of the study. This was used to measure oxidised LDL and LDL particle size.

b. The Second Northwick Park Heart Study Cohort

Subjects were those of the Second Northwick Park Heart Study (NPHSII), detailed elsewhere (Miller *et al.* 1995). In brief, 3012 unrelated healthy Caucasian middle-aged

male subjects (mean age 56.1±3.5 years) recruited from nine UK general practices were prospectively followed for a median 10.2 years (interquartile range 8.1 to 11.4 years). Baseline characteristics were ascertained by means of a questionnaire at entry into the study. Exclusion criteria at baseline were a history of MI, cerebrovascular disease, life-threatening malignancy or regular medication with aspirin or anticoagulants. At entry, a 5ml EDTA blood sample was obtained, from which genomic leukocyte DNA was extracted. Time to first CHD event (defined as sudden cardiac death, symptomatic/silent MI (the appearance of a new major Q wave on the follow up ECG, using Minnesota codes 1_1,$1_{2.1}$ to $1_{2.7}$, $1_{2.8}$ plus 5_1 or 5_2), or coronary revascularisation) was recorded, yielding only one event/subject. To date, 204 events have occurred in the 2775 subjects with DNA available for analysis: 148 (72.5%) acute MI, 38 (18.6%) coronary surgery and 18 (8.8%) silent MI. Obesity and systolic hypertension were defined as body mass index $\geq 30 kg.m^{-2}$ and systolic blood pressure $\geq$160mmHg.

Genotype distribution will be compared between diabetic UDACS subjects and the non-diabetic NPHSII subjects. For this analysis, the diabetic subjects from NPHSII were excluded (n=64).

c. Healthy male volunteers for cell culture studies

Ethical approval was obtained for this study from the UCL/UCLH ethics committee and all subjects gave informed consent. Ten healthy age-matched male volunteers, were selected by homozygosity for the -866G>A gene variant. All subjects were non-smokers,

non-diabetic and were free from any clinically apparent infection and inflammation at the time of venous sampling.

Twenty milliliters of venous blood was collected from each subject into vacutainers containing EDTA. All samples were immediately centrifuged at 1800g for ten minutes and monocyte extraction was performed immediately.

2.2.2 Biochemical assays

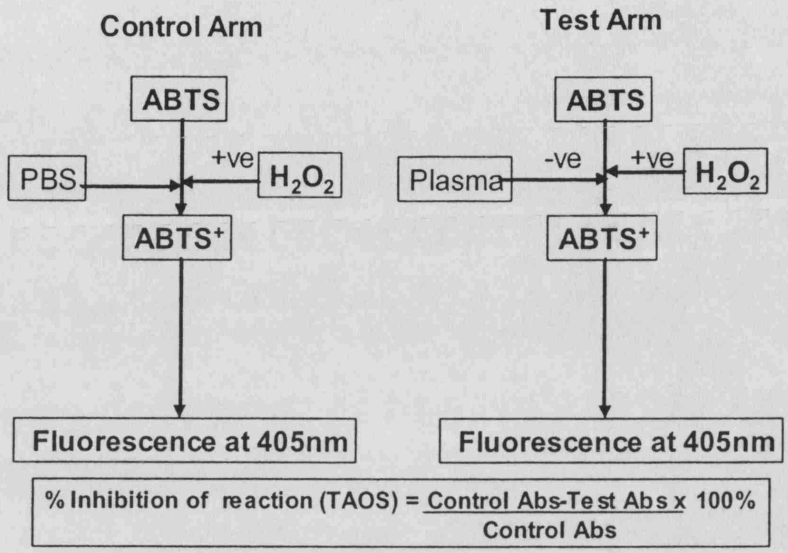
These measures were performed on plasma samples collected in UDACS.

a. Plasma Total antioxidant status

Various methods have been developed to measure plasma Total AntiOxidant Status (TAOS) (Erel 2004). In general, these methods involve the generation of a free radical, and the antioxidant activity of the test sample against this free radical is measured. In this study, plasma TAOS was measured by a photometric microassay previously described by Sampson *et al* (Sampson *et al.* 2002). The TAOS of plasma was determined by its capacity to inhibit the peroxidase-mediated formation of the 2,2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid ($ABTS^{+}$) radical. In the assay, the relative inhibition of $ABTS^{+}$ formation in the presence of plasma is proportional to the antioxidant capacity of the sample. Therefore, there are two arms to the assay, a control arm and test arm, as shown in figure 2.1. In the control arm phosphate buffered saline (PBS) is used instead of plasma. The assay is performed in a 96 well ELISA plate using 2.5μl of plasma. Plasma TAOS is therefore inversely proportional to the degree of oxidative stress in the sample.

Plasma rather than serum was used for the assay, because serum is obtained after clotting blood at room temperature and during this process the aggregation of platelets may release ROS (Leo *et al.* 1997; Ghiselli *et al.* 2000).

Figure 2.1: Measurement of plasma TAOS



(i) Method

Plasma (2.5µl) was placed in duplicate into 90-wells of a 96-well Nunc Immuno Maxisorp 96 well-plate. 2.5µl of PBS was added to 2 wells, an internal control sample to 2 wells and 2 wells were also left empty (as blank controls). The following solutions were subsequently added to each well:-

-20µl of ABTS (20mmol/l).

-20µl of HRP (30mU/ml).

-40µl PBS (pH 7.4).

The reaction was then initiated by the addition of 20µl of H_2O_2 (final concentration 0.1 mmol/l). The increase in absorbance at 405nm over ten minutes was monitored using a Labsystems Multiscan Ex microplate reader. At the end of the ten minutes the absorbance due to the accumulation of $ABTS^+$ in the test sample was read, along with the control

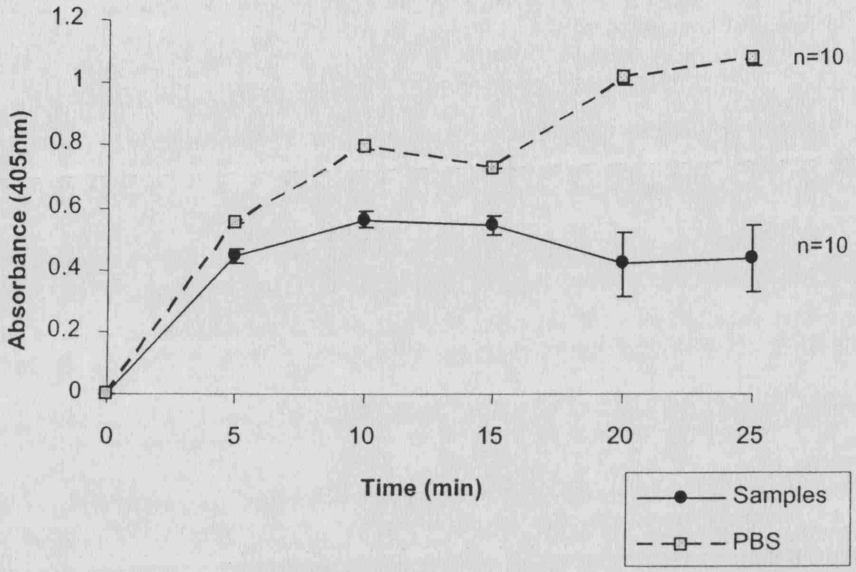
(containing 2.5µl of PBS instead of plasma). The difference in absorbance (control absorbance minus test absorbance) divided by the control absorbance (expressed as a percentage) was used to represent the percentage inhibition of the reaction.

The inter-assay coefficient of variation (CV) was 10.5%, and the intra-assay CV was 4.3%. These figures were derived from repeating the measurement in a single sample 30 times for the intra-assay CV and 15 times for the inter-assays CV.

(ii) Time course of ABTS+ accumulation

Figure 2.2, shows the absorption at 405nm of ABTS+ accumulation with time. As seen, the peak fluorescence in the plasma samples was at 10 minutes, whilst in the PBS samples, ABTS+ accumulation continued beyond this point. Therefore the absorption in all samples was read at ten minutes after the start of incubation.

Figure 2.2: Time course of ABTS+ accumulation *in vitro*



b. Plasma Oxidised LDL

Plasma Ox-LDL was measured using a commercially available Enzyme Linked Immuno Sorbant Assay (ELISA) from Mercodia (Uppsala, Sweden) (Holvoet *et al.* 1998; Scheffer *et al.* 2003). This is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants in the Ox-LDL molecule (mAB-4E6). During incubation, Ox-LDL in the sample reacts with anti-oxidised LDL antibodies bound to each microtitre well. After washing to remove non-reactive plasma components, a peroxidase conjugated anti-human apoB antibody recognises the Ox-LDL bound to the solid phase. After a second incubation and a second wash that removes the unbound enzyme labeled

antibody, the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped with sulphuric acid to give a colorimetric endpoint that is read spectrophotometrically at 450nm. The manufacturer's intra- and inter-assay CV were 4.0% and 4.2% respectively. From my analysis, the inter-assay CV was 14%, and the intra-assay CV was 10%. These figures were derived from repeating the measurement in a single sample 20 times.

c. Plasma F_2-isoprostanes

Measurement of isoprostane was performed by Gas Chromatography Mass Spectroscopy (GC-MS) after extraction, purification and derivitisation (Morrow *et al.* 1994). I performed this work in the laboratories of Professor Kevin Moore in the Centre for Hepatology at the Royal Free campus of UCL. The method involves multiple steps as described below.

(i) Extraction of esterified isoprostanes

A Folch extraction was performed to remove the lipid fraction. Firstly twice the volume of ice cold Folch (chloroform: methanol 2:1 containing BHT 5mg/100ml) was added to 0.5ml plasma with 2.5ml pH 3.0 H_2O. This solution was then vortexed, allowed to stand for thirty minutes at room temperature and then centrifuged for ten minutes at 500g at 4°C. This revealed separation of three phases; organic, precipitate and aqueous. The organic lower layer was removed using a glass pipette without disturbing the precipitate above. This was then dried under nitrogen at 37°C. Resuspension was then performed in 1.9ml methanol (containing BHT 5mg/100ml) and 100µl ethyl acetate. Two nanograms

of D_4-8-iso-$PGF_{2\alpha}$ and 5ng of D_4-$PGF2_\alpha$ were added to this mixture as internal standards and the phospholipids were hydrolysed by adding 2.0ml of 15% potassium hydroxide for sixty minutes at 37°C. The pH was then corrected to 3.0, with 200µl aliquots of 1M HCl. The samples were then made up to a final volume of 15ml with pH 3.0 water. To measure total F_2-isoprostane the Folch extraction was omitted from the above method.

(ii) Extraction and purification of isoprostanes

To purify the sample sufficiently for GC-MS analysis, a series of solid phase chromatography steps were performed. Solid phase cartridges were used to remove water soluble and uncharged organic components, taking advantage of the lipid nature of the molecule combined with the polar –OH and –COOH groups. Thin layer chromatography further separated the compounds according to their chromatographic behaviour, which again depends on charge.

(iii) Solid phase chromatography

Initial extraction of the prostaglandin-like compound was performed using tC18 columns. These samples were prepared by washing with 6ml methanol, followed by 6ml of pH 3.0 water. The sample was then loaded onto the column using a pastette and washed with 6ml of pH 3.0 water to remove water soluble compounds. At this pH, isoprostanes remain relatively uncharged and are bound to the solid phase. The column was then washed with 6ml of heptane to elute completely hydrophobic lipids. Isoprostane was then eluted into polypropylene tubes with 6ml of heptane, ethyl acetate and methanol (40:50:10), and dried under nitrogen.

(iv Preparation of pentaflourbenzyl ester

The pentafourobenzyl ester was prepared by the addition of 20µl 10% DIPEA (N,N-di-isopropylethylamine in acetonitile (AcN) and 40µl 10% PFBR (pentaflourobenzylbromide) in AcN to each sample in the fume hood. This was left at room temperature for thirty minutes and dried under nitrogen.

(v) Thin layer chromatography

The samples then underwent an additional purification with thin layer chromatography (TLC). TLC plates (Whatman 60A, Linear K6D, 5x20cm, 250µm thick) were pre-run in 100ml methanol for sixty minutes and subsequently dried. A TLC tank was prepared with 100ml of chloroform and ethanol (93ml: 7ml) and allowed to equilibrate for sixty minutes. Samples were resuspended in 40µl of methanol and chloroform (1:2), loaded onto the plates and run along with a separate plate loaded with 5ng of the methyl ester of $PGF_{2\alpha}$, which was run as a standard. The solvent front was run to 13cm above the application zone (taking approximately thirty minutes). The PFB derivatives of $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ ran to 3.6cm and 2.9cm respectively, with the methyl ester of $PGF_{2\alpha}$ running to approximately 3.4cm. The plates were then dried and the position of the standard visualised with 2% phosphomolybolic acid. The sample plates were then scraped 1.5cm above and 1.2cm below the solvent front of the standard, and the PFBR esters extracted in 1ml of ethyl acetate and methanol (1:1). The samples were then vortexed for two minutes and centrifuged for two minutes. The supernatant was then removed and dried under nitrogen.

(vi) Preparation of trimethylsilyl derivative

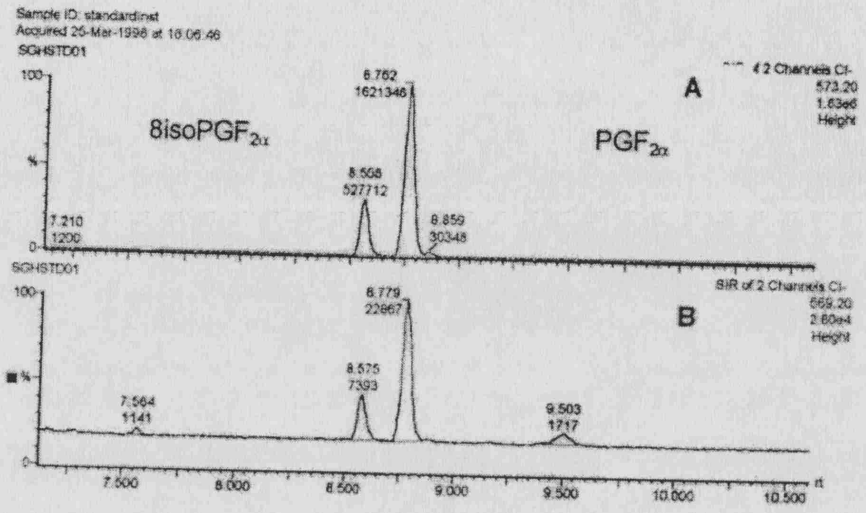
Final esterification was achieved by adding 10µl of anhydrous DMF (dimethylformamide) and 20µl BSTFA (bis-siylyltrimethylfluoroacetamide) for thirty minutes. Samples were dried under nitrogen and subsequently resuspended in 20µl of undecane, transferred to a conical autosampler vial using a Hamilton syringe and sealed.

(vii) Gas Chromatography-Mass Spectroscopy

Four microlitres of each sample was then delivered to the GC-MS apparatus (Fissons GC 8000 series coupled to a Fissons Trio 1000 MS). After injection the isomers were separated by gas chromatography with the 8-iso-$PGF_{2\alpha}$ appearing first.

Negative ion chemical ionization with ammonia was used to determine the mass spectrum. Selective ion monitoring at 569m/z and 573m/z was performed, as these were the masses of the predominant fragments of the undeuterated and deuterated forms of the isomers respectively. The concentration of F_2-isoprostane in the sample was then calculated by dividing the measure from the unknown sample by the standard and multiplying by concentration of the standard. Plasma F_2-isoprostane results were expressed as pg/ml. The inter assay CV is 7%.

Figure 2.3: GC-MS trace of $PGF_{2\alpha}$, 8-iso$PGF_{2\alpha}$ and deuterated derivatives



$PGF_{2\alpha}$ retention time=8.779mins and 8-iso$PGF_{2\alpha}$=8.575mins at m/z 569.20 (B). Similarly for deuterated derivatives at 573.20 (A)

d. Plasma CRP

To limit costs, plasma CRP was measured by an in-house ELISA. This has previously been validated in other studies (Steptoe *et al.* 2001; Paisley *et al.* 2003) and the method was made available from Dr V. Mohamed-Ali (based in the Centre for Clinical Pharmacology) at UCL.

Methods

Fifty microlitres of working coating solution was added to each well of a 92-well Nunc Immuno Maxisorp 96 well-plate. Four wells were left empty as negative controls. The

plate was incubated overnight at 4°C in a closed humid environment. The following morning, the plates were washed twice (using a Denley Well-wash 4 microplate washer) with 250µl per well wash buffer, drying between each wash. One hundred microlitres of blocking buffer was placed in each well and each plate was covered and incubated at 37°C for one hour. Each plate was washed twice. Standards and samples (diluted 1:100- 5µl plasma: 495µl diluting buffer), were added to the plate at 50µl per well. This was covered and incubated at 37°C for two hours. After three washes, 50µl of conjugate was added per well. The plate was then covered and incubated at 37°C for one hour. After a further three washes, 50µl of substrate was added per well. The plate was then incubated in the dark (at room temperature) for exactly ten minutes. The reaction was stopped with 25µl of 2M H_2SO_4 per well and the absorbance was read at 450nm using a Labsystems Multiscan Ex plate reader.

The inter-assay CV was 11.0%, and the intra-assay CV was 10.0%. These figures were derived from repeating the measurement in a single sample 30 times for the intra-assay CV and 15 times for the inter-assays CV.

e. Measurement of LDL particle diameter

This was kindly performed by Dr Muriel Caslake in the laboratories of the department of Vascular Biochemistry of the University of Glasgow. LDL at *d* 1.019-1.063g/ml was isolated from EDTA plasma. Electrophoresis was performed as previously described (Friedlander *et al.* 2000). 2-16% polyacrylamide gels (Alamo gels, San Antonio, Texas), were used to determine LDL peak particle diameter (LDL-PPD) and LDL mean particle

diameter (LDL-MPD) (Belo *et al.* 2002). LDL-PPD was reported as the size of the major LDL fraction. LDL-MPD was calculated to give the mean diameter across the entire LDL profile as previously described (Belo *et al.* 2002).

2.2.3 DNA preparation

a. DNA extraction From Blood by the "Salting Out Method"

DNA was extracted from 5mls of potassium-EDTA or citrated anti-coagulated peripheral blood using a salting out method (Miller *et al.* 1988). Before starting the extraction process (performed in batches of 24 or 36 samples), all samples were carefully logged and entered into a database. The DNA extraction process involves several steps, cellular and nuclear lysis, deproteinisation, extraction and precipitation, as detailed below.

(i) Cell and nuclear lysis

Blood samples were thawed and transferred to a labeled 30ml polypropylene tube. Cold (4°C) sucrose lysis buffer (15ml) was added to each tube and mixed by hand inversion. Tubes were centrifuged at 4°C for ten minutes in batches of 12 at 1800g (Sorvall RC5 centrifuge using rotor SA-600). The supernatant was carefully discarded without disturbing the pellet and 2ml of sucrose lysis buffer was added and the pellets re-suspended using a disposable pastette for each sample. A further 18ml of lysis buffer was added before invert-mixing once more. Samples were then centrifuged again for a further ten minutes. Following centrifugation, the supernatant was discarded, and 2ml of nuclear lysis buffer was added and the pellet re-suspended with a pastette.

(ii) Deproteinisation

Using a 10ml pipette, 1ml of 5M sodium perchlorate was added and the sample inverted. Samples were then left on a shaker for fifteen minutes.

(iii) DNA extraction

Two millilitres of cold chloroform (-20°C) was added to each tube, using a glass 25ml pipette and mixed by hand. Samples were then centrifuged at 1800g for three minutes at room temperature. Following addition of cold chloroform, the DNA from each sample had been partitioned into the upper aqueous phase within each tube. This upper phase was then carefully transferred into a fresh, labeled 30ml polypropylene tube, without disturbing the organic phase.

(iv) Precipitation and washing

Ten millilitres of cold 100% ethanol (stored at -20°C) was poured slowly down the side of each tube to precipitate the DNA. These were then sealed and gently mixed. DNA from each tube was then gently "spooled" using a sterile pastette. Samples were washed in 70% ethanol before being transferred into a sterile labeled microtube containing 1ml Tris-EDTA (TE) buffer. At this final stage, extreme care was taken to ensure that the sample number on the tube corresponded with the code number of the sample as recorded on the microtube label.

(v) Handling DNA samples that fail to spool

Occasionally, no DNA was seen to precipitate after the addition of 100% ethanol. These samples were placed in the freezer overnight at -20°C. They were then centrifuged at 1500g for fifteen minutes, before decanting the supernatant to leave a small DNA pellet. Tubes were allowed to air dry before adding 0.5ml of TE buffer. At this stage samples

were left overnight at 37°C before re-suspending and transferring the contents into labeled microtubes.

(vi) *Dissolving the DNA*

Sealed microtubes were placed in an incubator at 37°C overnight, before being placed in a cold cabinet (at 4°C) for storage. Following DNA extraction, samples were left for a period of at least four weeks to allow the DNA to completely dissolve. At this stage a series of "stock" and "working" 96-well arrays were generated.

(vii) Generating Array Sheets

In order to facilitate high-throughput genotyping technique - microtitre array diagonal gel electrophoresis (MADGE) (Day *et al.* 1994) detailed below; it was firstly essential to generate a clear and accurate array sheet listing the samples by code number within an 8 x 12 grid, as shown in figure 2.4.

Figure 2.4: An Example of a Standard Array "Grid"

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	
F	6	14	22	30	38	46	54	62	70	78	86	
G	7	15	23	31	39	47	55	63	71	79	87	
H	8	16	24	32	40	48	56	64	72	80	88	

b. Manual Standardisation of DNA arrays

This was performed to standardise all DNA samples to 15ng/µl. The steps are described below.

(i) Diluting the samples 1 in 10 for the plate reader

Wearing gloves and using filtered tips, 10µl of each sample was pipetted out into the corresponding well of a 96 well Costar UV plate. Wells 12E, 12F, 12G and 12H were kept empty as blanks (100µl dH_2O). Using a manual Eppendorf 300, 8 channel pipette, 90µl of dH_2O was added to each well and mixed by re-suspending the sample. The plate was then centrifuged (Sigma 4-15) at 200g for thirty seconds, before measurement in the plate reader.

(ii) Measuring the absorption at 260nm and 280nm

This was performed using the Tecan GENios plate reader utilising the Magellan 3 software package. After gently wiping the bottom of the plate with a tissue, the plate was placed into the reader. The 260/280nm filter slide was inserted into the excitation port. The corresponding absorption at 260 and 280nm was then recorded for each well in the plate and exported to an excel file.

(iii) Calculation of the volumes for manual array standardisation

The volume of DNA required (added to 750µl of dH_2O) to achieve a concentration of 15ng/µl was calculated using an Excel programme. If any of the DNA volumes were above 200µl, then the volume was halved and 375µl of dH_2O added to these wells. If any of the DNA concentrations were near 15ng/µl, the DNA was transferred neat.

(iv) Creating Stock and Working Arrays

Stock arrays were created in labeled 96-well Beckman's array. These were stored at -20°C. To create working arrays, 100µl of each sample was removed from the stock array, and transferred to another labeled 96-well array, before storing at 4°C.

2.2.4 Polymerase chain reaction & Restriction Digest

The technique of PCR relies on the fact that double stranded DNA is denatured into single strands by heat, and will anneal with primers, and with the addition of DNA polymerase and nucleotide bases, support the synthesis of double strand on cooling. The first step is therefore a short period of high temperature to denature the DNA. This is followed by cooling in the presence of primers that are complementary to the DNA either side of the sequence to be studied. These primers anneal, and a DNA polymerase adds nucleotides base by base, thus replicating the DNA. The polymerase used is derived from the bacterium *Thermus aquaticus* (*Taq*) and is heat stable. Therefore, it does not need to be replenished after each cycle of heating and cooling. This technique allows very small initial amounts of DNA to be increased in quantity until there is sufficient DNA for genotyping to be performed.

a. Sample Preparation for Polymerase Chain Reaction

Following extraction and dispensing into working 96-well arrays, DNA samples were prepared for PCR by centrifuging the DNA working-array at 200g for one minute. This was to ensure that all the DNA dilutions were at the bottom of their respective wells, thus reducing the possibility of cross-well contamination when the array lid was removed. Two and a half microlitres of each sample (45ng/μl of DNA) was then removed from each array and transferred into a standard 96-well PCR plate (Omniplate, Hybaid) using a Finnipipette multichannel dispenser (Life Sciences, Basingstoke, Hants, UK). Positive and negative controls were utilised to ensure accuracy. Extreme care was taken to ensure that samples were placed in the identical orientation as in the original arrays. Loaded

Omniplates were then centrifuged at 200g for thirty seconds to ensure that the DNA was at the bottom of each well, and then dried on a Thermal Cycler block (MJ Tetrad DNA Engine Thermocycler) at 80°C for ten minutes.

A bulk-mix of reagents was made up for each PCR, allowing adequate volume for the planned number reactions, with an additional 10% added to ensure that the mix would not run short. PCR primers and *Taq* polymerase were kept on ice and added last. PCRs were performed in a total volume of 20µl. Each reaction contained 1x concentration of polmix (50mM KCl, 10mM Tris-HCl (pH 8.3), 0.2mM dATP, dGTP, dTTP and dCTP), $MgCl_2$, 8pmol of each primer and 0.4U of *Taq* polymerase. Magnesium concentration varied with each PCR.

The PCR mix was added to each well of the PCR plate using an automatic Biohit repeating dispenser (Alpha Laboratories, UK). Each sample was overlaid with 20µl of mineral oil to prevent evaporation. The microtitre plate was then sealed with a clear sticky plastic lid and carefully labeled. Plates were centrifuged at 200g for thirty seconds to ensure good mixing of the reaction components in each well. PCR amplification was performed on an MJ Tetrad DNA Engine Thermocycler, using cycle conditions specific to each PCR. The primer sequences, PCR conditions and reagents are shown in tables 2.2 to 2.4 respectively.

b. Restriction digestion

Restriction enzymes are derived from bacteria, and cleave double stranded DNA at a particular sequence. The enzyme translocates along the DNA until a particular recognition site is reached, where the DNA is cut. The restriction enzyme is sensitive even to a single base change in the recognition sequence, and thus can be used to detect point mutations and single base polymorphisms. A single base change can either eliminate or create a cutting site for a particular enzyme.

A bulk mix of restriction enzyme digest mix was made up in a 1ml Eppendorf tube on each occasion, containing sufficient enzyme to digest the PCR products in each well of the PCR plate. 5μl of digestion mix was then added to 10μl of each reaction product using a repeater pipette as for the PCR mix. Each omniplate was then centrifuged at 200g for thirty seconds to ensure that the PCR product and restriction enzyme mixed well. The PCR/digestion mix was then incubated overnight at 37°C. The specific details for each PCR and digestion mix are detailed in table 2.4.

Table 2.2: Primers for the gene variants under study

Primer	Oligonucleotide Sequence 5'-3' Forward/Reverse	Fragment size (bp)
UCP2 -866G>C	5'-CACGCTGCTTCTGCCAGGAC-3'	360
	5'-AGGCGTCAGGAGATGGACCG-3'	
UCP2 45bp ID	5'-CAGTGAGGGAAGTGGGAGG-3'	412 (D)/ 457 (I)
	5'-GGGGCAGGACGAAGATTC-3'	
APOE	5'GAACAACTGACCCCGGTGGCGG-3'	295
	5'-GGATGGCGCTGAGGCCGCGCTC-3'	
GSTM1	5'-GAACTCCCTGAAAAGCTAAAGC-3'	215 (GSTM1-1)
	5'-GTTGGGCTCAAATATACGGTGG-3'	
GSTT1	5'-GCC CTGGCTAGTTGCTGAAG-3'	125 (GSTT1-1)
	5'-GCATCTGATTTGGGGACCACA-3'	
β-globin	5'- CAACTTCATCCACGTTCACC-3'	268
	5'-GAAGAGCCAAGGACAGGTAC-3'	

Table 2.3: PCR conditions for the gene variants under study

Gene variant	Step 1	Step 2	Step 3	Step 4	Number of cycles (Steps 2-4)	Step 5	Step 6	Step 7	Number of cycles (Steps 5-7)	Termination step
UCP2	95°C	95°C	65°C	72°C	30	-	-	-	-	72°C
-866G>C	4 min	45 sec	30 sec	1 min						5 min
UCP2 45bp ID	95°C	95°C	65°C	72°C	30	-	-	-	-	72°C
	4 min	45 sec	30 sec	1 min						5 min
GST-T1/M1	95°C	95°C	61°C	72°C	30	-	-	-	-	72°C
β-globin	5 min	1 min	1 min	1 min						5 min
APOE	95°C	95°C	-	72°C	4	95°C	55°C	72°C	39	-
	5 min	1 min		3 min		1 min	1 min	1 min		

Table 2.4: PCR and Digest components for gene variants under study

Gene variant	Buffer	$MgCl_2$ mmol	BSA	Restriction enzyme	Restriction enzyme buffer
UCP2 -866G>A	Polmix	2.5	+	*Mlu-1* 2U	NEB buffer R^+
UCP2 45bp ID	Polmix	2.5	+	-	-
GST-T1/M1	Polmix	1.5	-	-	-
β-globin					
APOE	Polmix	1.5	-	-	-

2.2.6 Microtitre Array Diagonal Gel Electrophoresis (MADGE)

The DNA fragments produced by restriction enzyme digest were separated using electrophoresis on a non-denaturing polyacrylamide gel, using Microtitre Array Diagonal Gel Electrophoresis (MADGE). Utilisation of this technique makes it possible to electrophorese the entire 96 wells of a standard PCR plate on a single gel, by allowing the samples to run diagonally. Use of MADGE was an essential part of the project as it allowed high throughput screening of the PCR product, and allowed the 96 well DNA array format to be retained throughout the screening process. Standard 7.5% MADGE gels were made up in batches for economy of time.

MADGE consists of an open arrangement of 8x12 wells each 2mm deep. The wells are arranged at an angle of 71.2° to the short axis of the array, but perpendicular to the long-axis of the Perspex formers used. Thus the maximum track length is 26.5mm allowing sufficient travel for genotype resolution.

The components required to make a singe 7.5% MADGE are listed on page 72. Before making the mix, glass plates of appropriate size (160 x 100 x 2mm) were rigorously cleaned and hand dried. Once dry, 5 drops of γ-methacryloxypropyltrimethoxysilane ("sticky" silane) were spread across the plates and left to air-dry. Silane was used to ensure that the MADGE gel would adhere to the glass plates. The MADGE mix was then made up in bulk in a conical flask.

Polymerisation was initiated by the addition of APS, which was added last (as the combined solution begins to set within thirty seconds of mixing) before the solution was mixed and quickly poured into the three-dimensional former. A glass plate was then gently placed over the mould (silanised side facing downwards) taking care not to trap any air bubbles. This was then left for fifteen minutes to set, using a small weight to ensure that the glass did not slip whilst the gel was setting. Excess gel was trimmed from the edges of the MADGE former before the glass plate and attached gel were then prized away from the plastic former. MADGE gels were stored in a plastic Stuart box containing neat TBE (1x concentration).

Gel Staining and Loading

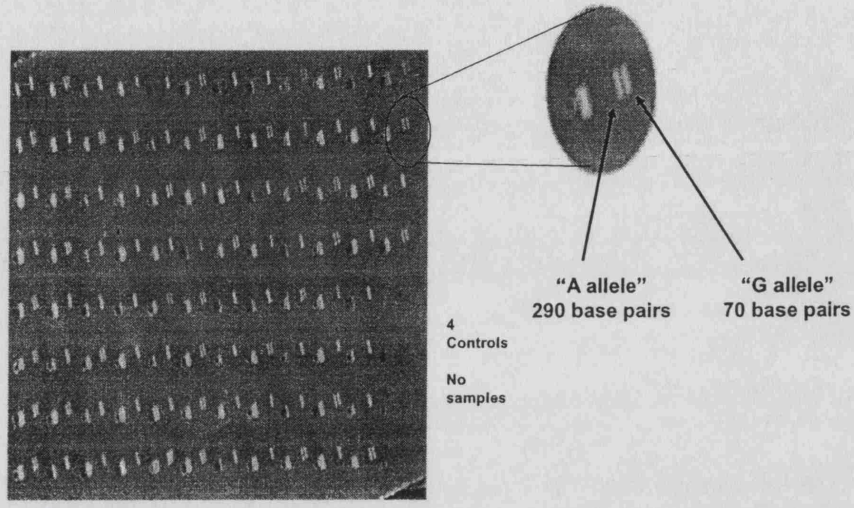
Prior to loading a gel with digested PCR product, each gel was stained with Ethidium Bromide (EtBr). This was achieved by placing them individually in a Stuart box, shielded from direct light, containing 100ml of 1x TBE and 10µl EtBr for 20 minutes.

Whilst the gels were being stained, the PCR-digest product was prepared for loading onto the MADGE gel. 2µl of formamide dye (98% formamide, 10mmol/l EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue) was added to each well of a new, round-bottomed, loading tray, followed by 5µl of each digested sample, using a multi-channel pipette to pick up the samples from under the oil in the omniplates. The digested samples were gently mixed, by aspirating the formamide dye–digest mixture up and down several times into the pipette, before dispensing them on the digest plate.

After placing the stained MADGE gel into an electrophoresis tank containing 1000ml of 1xTBE, a multi-channel pipette was then used to transfer 5µl of this digest/dye mixture to the wells of the gel. At all times the samples were kept in the same layout as on the PCR tray, allowing each sample to be easily identified without being re-labeled. The gel was electrophoresed at 150 volts for forty minutes.

Following electrophoresis the gel was viewed and photographed under ultraviolet light using the UVP Gel Documentation System. Care was once more taken to ensure the correct orientation of the MADGE under UV. This was ensured by always placing the well corresponding to grid-reference "A1" in the bottom left-hand corner of the UV-viewing box. The image thus produced was used for genotyping. An example of a typical MADGE gel is shown in figure 2.5.

Figure 2.5: MADGE gel for the *UCP2* -866G>A gene variant



MADGE gel of digest in 92 samples with 4 blank controls

2.2.7 Genotyping quality control

All genotyping was performed in a double blind fashion using both positive and negative controls. The results were rechecked by two individuals at the time of MADGE imaging and during data entry into the computer database. Any apparent genotype differences were resolved by repeat PCR. Overall there was excellent reproducibility with >95% consistency between samples (as will be described later).

2.2.8 Genotyping for specific gene variants

a. *UCP2* variants

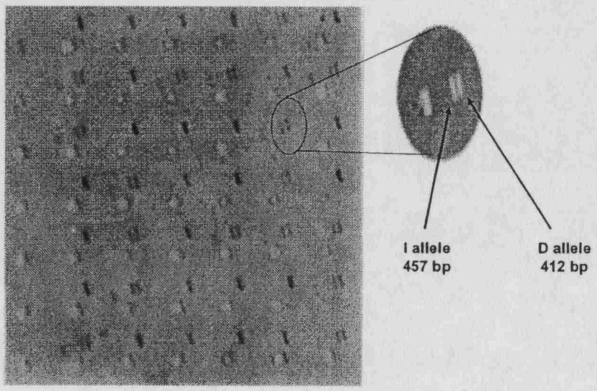
UCP2 -866G>A

Primers and PCR conditions for the *UCP2* -866G>A variant were a kind gift from Harold Esterbauer (Department of Medicine, Landeskliniken, Austria). The PCR conditions and restriction enzyme digestion conditions are summarised in tables 2.2-2.4. PCR resulted in a 360bp fragment, which following digestion with *Mlu*-I, resulted in 290bp fragment (A allele) and a 70bp fragment (G allele), as shown in figure 2.5.

UCP2 45bp Deletion/Insertion

Primer sequences were obtained from previously published studies (Dalgaard *et al.* 2001; Marti *et al.* 2004). PCR conditions were optimised to those shown in tables 2.1-2.3. The PCR resulted in a 412bp product (Deletion-D) or a 457bp product (Insertion-I) as shown in figure 2.6.

Figure 2.6: MADGE gel for the *UCP2* D/I gene variant



b. *APOE* variants

(i) APOE genotyping by heteroduplex analysis

The 2 common variants within the human *APOE* gene resulting in the 3 allelic isoforms ε2, ε3 and ε4 were determined using a heteroduplex generator (Bolla *et al.* 1999). Briefly, the method involves PCR amplification of the region containing the two polymorphic sites at amino acid positions 112 and 158, followed by hybridisation of this PCR product to a universal heteroduplex generator (UHG). The UHG is used to induce heteroduplex formation, which is visualized on a 10% non-denaturing polyacrylamide mini-gel using ethidium bromide. The UHG is a synthetic DNA molecule, which is an amplifiable copy of the target sequence, containing strategic sequence modifications in close proximity to the point of mutation. When a UHG is mixed, denatured and reannealed with the amplican of interest, DNA heteroduplexes form, which are subject to far greater electrophoretic retardation than homoduplexes and in-trans heteroduplexes.

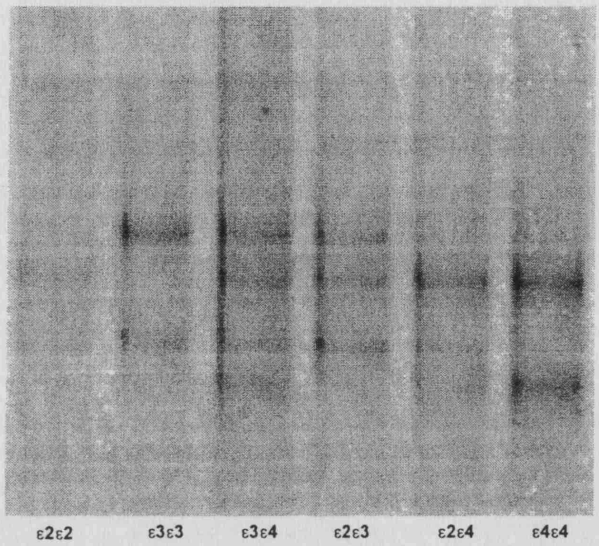
The method involves 2 separate PCR reactions. Firstly, the genomic DNA sample undergoes PCR with the primers shown in tables 2.2-2.4 (Bolla *et al.* 1999). UHG (available in our laboratory) underwent PCR under the identical conditions. Amplification of the genomic DNA results in a PCR product of 295bp, whilst the amplification of the UHG results in a product of 303bp. For the generation of heteroduplexes, 10μl of amplified UHG PCR product and 15μl amplified genomic DNA were mixed together in a clean microtitre plate and denatured on a PCR block at 94°C for ten minutes. The samples were allowed to cool slowly for fifteen minutes to allow renaturation and formation of the heteroduplexes.

Visualisation of heteroduplexes

For visualisation of the heteroduplexes, vertical 10% mini gel electrophoresis was performed (figure 2.7). Two sets of glass plates (20cm x 18.5 cm; Cambridge Electrophoresis, Cambridge, UK) were used to pour polyacrylamide gels. For each pair of gels, 100mls of polyacrylamide was made using:- 33.4ml 30% acrylamide (0.8% w/v acrylamide; 37.5:1 acrylamide:N,N'-methylenebenebisacrylamide), 20ml 10xTBE pH8.3 and 46.6ml dH_2O. To polymerise each gel (50ml), 100µl TEMED and 300µl 10% APS were added and the mix was poured onto a set of 2 plates. A comb with 24 teeth (each 5mm wide) was inserted along the edge of the gel, and the gel allowed to set for thirty minutes. Once set, the combs were removed and both sets of plates clamped onto each side of the vertical gel electrophoresis apparatus (Cambridge Electrophoresis, Cambridge, UK).

25µl of the product following the second PCR was loaded with 4µl of loading dye into the mini gel. This was then electrophoresed at 130V for twenty hours at 19°C in 1xTBE. Once electrophoresis was complete, the gels were removed and stained for twenty minutes with 1xTBE containing 0.1% ethidium bromide and visualized with a UV transilluminator.

Figure 2.7: Heteroduplex patterns of the *APOE* gene variants



(ii) APOE sequencing

This was performed by Mert Sozen a visiting PhD student from Hacettepe University, Ankara, Turkey under my supervision. PCR products were purified using the GFX ™ PCR DNA and Gel Band Purification kit (Amersham Biosciences). Subsequently, automated sequence analysis was performed with the ABI Prism 377 DNA Sequencer (PE Applied Biosystems) using the Big Dye Terminator v 3.1 Cycle Sequencing kit.

(iii) ApoE phenotype

Further phenotypic characterisation for *APOE* gene variants were performed by isoelectric focusing and the measurement of plasma apolipoprotein E. These measurements were kindly performed by Dr Muriel Caslake in the laboratories of the department of Vascular Biochemistry in the University of Glasgow.

Isoelectric focusing

Apolipoprotein E phenotyping was performed on 10μl of serum from the three subjects with the newly identified mutations by isoelectric focusing (IEF) followed by Western blotting using specific monoclonal antibodies to apoE (Dako, Ely, Cambridgeshire) (Havekes *et al.* 1987).

Plasma apolipoprotein E measurement

Plasma apoE concentrations were analysed using a commercially available immunoturbidimetric kit from Wako Chemicals (*Osaka, Japan*).

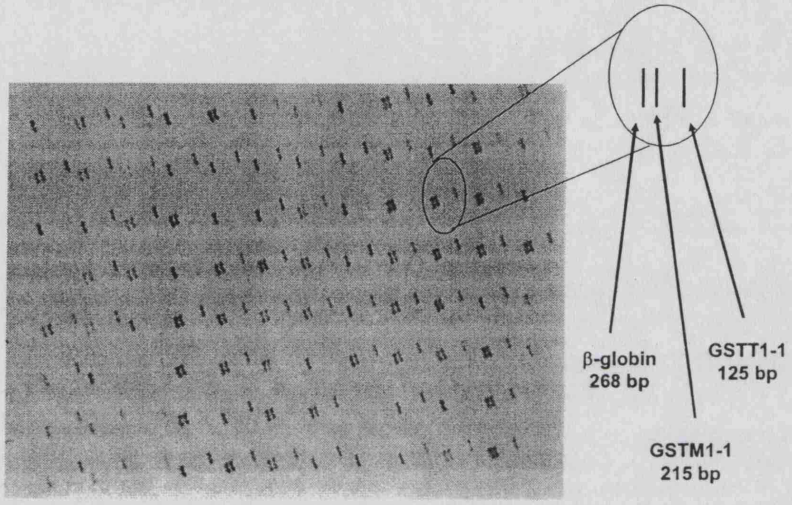
c. *GST* Genotyping

Genotypes for *GSTM1* and *GSTT1* were determined using PCR with published primers (Li *et al.* 2000) including β-globin as a positive control for amplification. The PCR protocol is summarized in tables 2.2-2.4. Three possible PCR products can be observed. *GSTM1*- and *GSTT1*- specific products of 215 and 125 bp respectively, and a 268 bp β-globin product, as shown in figure 2.8. Absence of *GSTM1*- or *GSTT1*- specific PCR product is the result of DNA amplification with *GSTM1* or *GSTT1* gene deleted on both alleles. Gene-specific PCR product indicates at least one functional allele. Heterozygous (+/-) status cannot be differentiated from homozygous (+/+) status.

In the NPHSII study, genotyping for the *GST* variants was performed by Mr Jamie Acorn. Within UCADS, this genotyping was performed by Professor Tony Hayek

(visiting from The Bruce Rappaport Faculty of Medicine, Haifa, Israel), under my supervision.

Figure 2.8: MADGE gel for the *GST* gene variants



2.2.9 Cell culture studies

Cell culture was performed in the tissue culture suite of the Rayne building at UCL, which is a dedicated, protected environment with air filtration and two door locks before entry. Gloves, cell culture dedicated laboratory coats and overshoes were worn at all times. Sterility was maintained by handling all open culture media, culture-ware and liquids within a class II microbiological safety cabinet with unidirectional laminar flow (Envair (UK) Ltd). Surfaces were cleaned with 1% virkon and 70% ethanol solutions. Materials for use within the laminar flow hoods were pre-sprayed with 70% ethanol. Cell contaminants, such as media used for growth, were removed to 1% Virkon-containing vessel and stored for at least twenty-four hours before disposal. Galaxy R CO_2 incubators (Wolf Laboratories) humidified and set to 5%CO_2, 37°C were used for cell incubation. This work was performed on monocytes collected from the subjects described in section 2.2.1.c.

a. Monocyte preparation for culture

Monocyte extraction was performed using a previously described technique (Wan *et al.* 1993). After centrifugation of EDTA collected venous blood (1800g for ten minutes), 100µl of plasma from each subject was aliquoted into wells of a sterile 96 well Nunc cell culture plate (Roskilde, Denmark). Care was taken to carefully label the wells for individual subjects. The plasma was left coating the wells for forty-five minutes at 37°C. Preincubating the plates with plasma in this way increases the success of monocyte adhesion. The wells were then washed twice with 100µl of PBS.

The remaining blood was transferred to a 30ml sterile centrifuge tube with 6mls RPMI-1640 medium (supplemented with, 2mM glutamine, 10% heat inactivated bovine serum, penicillin at 100units/ml and streptomycin at 100μg/ml), pre-warmed by incubation in a water bath at 37°C. The samples were mixed gently by inversion. Four millilitres of Ficoll-plaque (warmed to room temperature) was dispensed to a fresh 30ml tube. The blood-RPMI mix was then gently layered onto the surface of the Ficoll-plaque. The samples were then centrifuged at 200g for twenty minutes at room temperature.

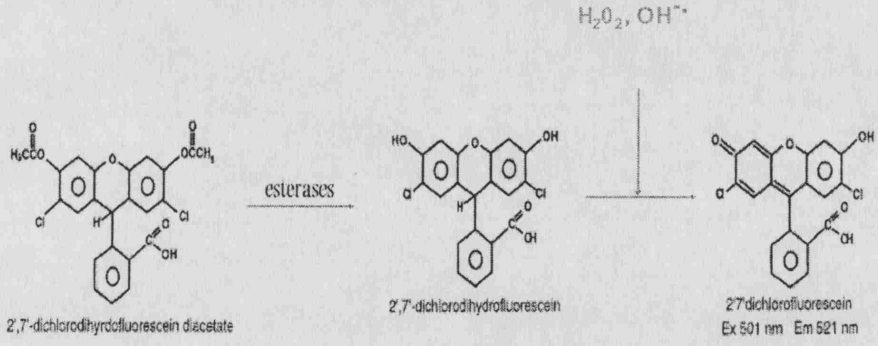
After centrifugation, the monocyte layer is visible between the Ficoll-Plaque and RPMI-plasma layer. The monocytes were then recovered with a 100μl pipette and transferred to a 15ml tube. An equal volume of RPMI was added, and the sample mixed gently and centrifuged at 200g for five minutes. Following this, the monocytes were recovered from the formed pellet. The supernatant was aspirated, carefully ensuring that the pellet was left intact. A repeat wash was then performed.

The pellet was then resuspended in RPMI and the number of monocytes counted. Using this technique the recovery of monocytes is around $5x10^6$ from 4mls of blood. Within three hours, 50-70% of monocytes should adhere, however all were left overnight to ensure a higher percentage adhered. Monocytes were plated at $1x10^6$ cells/well in a sterile 96 well Nunc cell culture plate (Roskilde, Denmark).

b. Measurement of cellular ROS

Cellular ROS was measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein (H_2-DCF-DA). This passively diffuses into cells, where intracellular esterases cleave off the diacetate group and thereby trap the H_2-DCF within the cytoplasm. In the presence of cellular ROS, H_2-DCF is rapidly oxidised to highly fluorescent DCF (figure 2.9). Flourescence was read at 485nm excitation and 530nm emission wavelengths using a Tecan GENios plate reader utilising the Magellan 3 software package. This method has successfully been used to measure cellular ROS formation in a variety of different cell types and shown good reproducibility with other measures of cellular ROS in cell culture (Wan *et al.* 1993; Kim *et al.* 2003; Voelkel *et al.* 2003; Iuvone *et al.* 2004).

Figure 2.9: Measurement of cellular ROS with DCF-DA



c. Cell culture and treatment

All cell culture was performed in a 5%CO_2 environment. Following a twelve hour culture, the cells were inspected (to look for any evidence of fungal infection and to assess adherence) and the medium was changed. Four hours later, the medium was removed from each well, and the cells were washed with Krebs buffer. The cells were then incubated with 100µl Krebs buffer containing DCF-DA, at a final concentration of 5µM (Iuvone *et al.* 2004) for one hour. After this the Krebs- DCF-DA was removed and the cells incubated for sixty minutes with the selected cellular pro-oxidants listed in table 2.5.

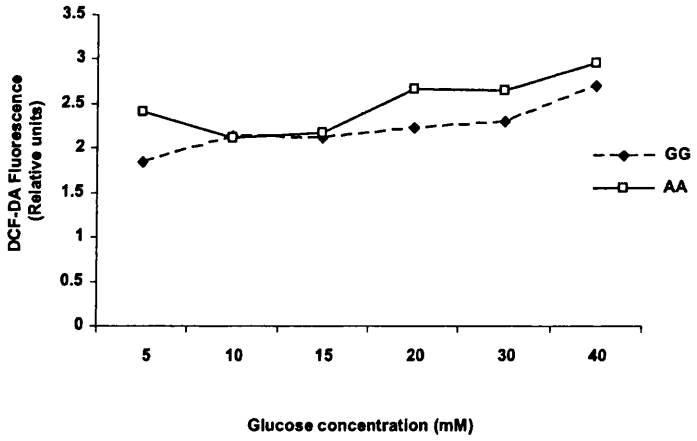
Table 2.5: *In vitro* pro-oxidant 'stressors'

'Stressor'	Mechanism	Final concentration	Reference
Menadione	A mitochondrial ETC electron donor	1µM	(Luczak *et al.* 2004)
LPS	Bacterial lipopolysaccharide	1µg/ml	(Kizaki *et al.* 2002)
High glucose	Unclear	20mM	(Quagliaro *et al.* 2003)
High glucose+Insulin	Unclear	20mMGlucose + 1µmol insulin	(De Mattia *et al.* 1999)
Angiotensin II	A direct stimulator of NADPH dependent ROS production	1.10^{-7} M	(Desideri *et al.* 2003)

Dose response curve for DCF-DA fluorescence in relation to Glucose concentration

Previous studies assessing cellular response to glucose in culture have used varying concentrations of glucose in the culture medium. Therefore, initially, a dose-response experiment was performed to study the dosages of glucose which may alter cellular ROS production in 1 AA and 1 GG subject. This experiment was performed in triplicate in a range of glucose concentrations ranging from 5 to 40mM. Subsequent studies utilised 20mM glucose. *In vivo*, this would relate to the plasma concentration found in a poorly controlled subject with diabetes. Furthermore, as shown in figure 2.10, apart from the basal 5mM concentration (present in the basal Krebs buffer), the greatest difference between the AA and GG subject was at this point.

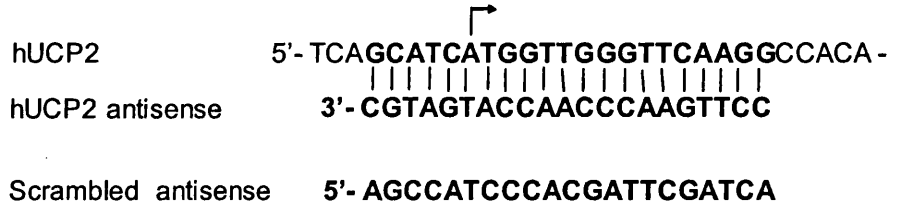
Figure 2.10: Dose response curve for DCF-DA fluorescence with glucose concentration



d. Antisense studies

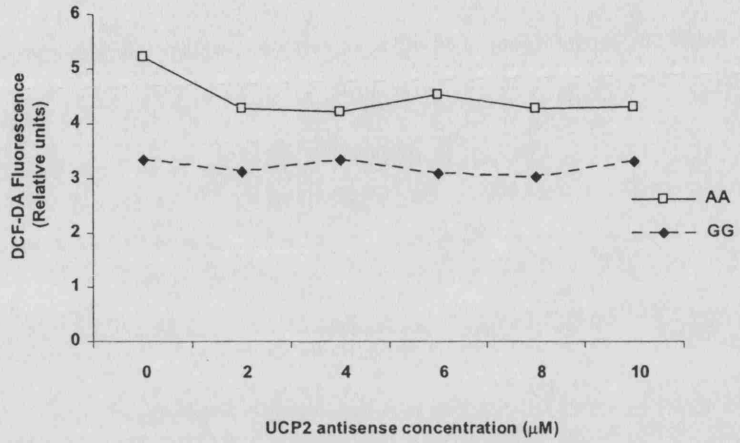
An antisense strategy was employed to selectively downregulate UCP2 protein expression in monocytes, using a similar approach to that previously described in murine endothelial cells (Duval *et al.* 2002). Complementary oligonucleotide sequences (21mer) were designed to straddle the published translation start sites as shown in figure 2.11.

Figure 2.11: *UCP2* antisense oligonucleotide



Previous studies have used *UCP2* antisense 1-10μM (Duval *et al.* 2002). Initially, a dose response curve was performed to establish whether differing concentration of *UCP2* antisense influenced DCF-DA fluorescence in 1 AA and 1 GG subject (figure 2.12). As shown no difference was observed in fluorescence by *UCP2* antisense concentration in either the GG or AA subject. In the following studies 10μM, as in the original studies by Duval.

Figure 2.12: Dose response curve for DCF-DA fluorescence with antisense concentration



e. Reducing extra-mitochondrial ROS production with a NADPH oxidase inhibitor

Studies were also performed using the NADPH oxidase inhibitor Diphenyleneiodonium chloride (DPI). Experiments were performed in the presence and absence of DPI (final concentration 10μM) in the presence of 20mM glucose, insulin+glucose and menadione. This incubation was performed for sixty minutes as described above.

2.2.10 mRNA expression and the *UCP2* -866G>A variant

a. Subjects

This was performed on the same subjects described in section 2.2.1.c above. *UCP2* expression by genotype was determined in monocytes extracted without further culture, and in those cultured in the presence of a pro-oxidant stimulus.

b. Monocyte preparation for culture

Monocytes were isolated from 30mls of EDTA blood as described in section 2.2.9. Cell culture was then performed in a sterile 6 well Nunc cell culture plate (Roskilde, Denmark) in a 5%CO_2 environment. Following a twelve hour culture in RPMI, the cells were inspected, and the medium was changed. Four hours later, the medium was removed from each well, and the cells were washed with Krebs reagent. The cells were then incubated for ten hours with 2000µl Krebs (5mM glucose), or Krebs with 20mM glucose+insulin, or Krebs with menadione (in the concentrations shown in table 2.5). After this period the supernatants, were removed and mRNA isolation performed as described below.

c. mRNA isolation

All materials were handled with gloves, pipettes autoclaved prior to use for RNA work, and bench-space cleaned meticulously with 70% ethanol. Isolation was performed using the RNeasy kit (Qiagen, Crawley, UK) spin protocol. This entails the use of a high-salt buffer system which allows up to 100µg of RNA longer than 200bp to bind to a silica-gel based membrane with a RNeasy min column. Initially, 350µl of lysis buffer (RLT buffer)

together with β-mercaptoethanol (β-ME) were added to stabilize the sample. Following this, 350μl of 70% ethanol was added and the suspension vortexed. The sample was then added to a spin column and centrifuged at 100g for fifteen seconds. The flow-through was discarded and 350μl of buffer RW1 added to the column, and the sample centrifuged at 100g for fifteen seconds. 80μl DNase I solution was added directly to the silica gel membrane of the spin column for fifteen seconds followed by a second RW1 wash. The sample was then washed twice with 500μl of buffer RPE. Finally, the RNA was eluted with 50μl of RNase free water added directly to the silica membrane with subsequent centrifugation for one minute at 100g into a fresh eppendorf. The RNA was stored at -80°C.

d. cDNA synthesis

The concentration of mRNA was determined by measuring the absorption at 260nm in a spectrophotometer. RNA (1.5μg) was added to 2μl random primers ($pd[N]_6$) and the reaction volume made up to 12μl with RNase free water. The sample was heated to 70°C for five minutes and then chilled on ice for one minute. 4μl of 1st strand buffer (Invitrogen), 2μl 0.1M DTT and 1μl 10mM dNTP mix was mixed gently, followed by incubation at 42°C for two minutes. After this stage, 1μl Superscript II reverse transcriptase (Invitrogen) was added, the sample mixed and incubated at 42°C for one hour. The incubation temperature was increased to 55°C for five minutes and subsequent addition of 1μl RNaseH (Invitrogen) and further incubation for ten minutes at 55°C. The resulting DNA samples were stored at -20°C.

e. Design of primers

Forward and reverse primers and probes were ordered and supplied on demand (*UCP2*: HS00163349_M1 [FAM-TACAAAGCCGGATTCCGGCAGAGTT-NFQ]; *β-Actin*: HS99999903_M1 [FAM-TCGCCTTTGCCGATCCGCCGCCGCCCGT-NFQ]; ABI systems). The probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) and at the 3' end with a Taqman probe and a non-fluorescent quencher. Thermal cycling was performed as follows:- 50°C for two minutes, 95°C for ten minutes, followed by 40 cycles of 95°C for fifteen seconds and 60°C for one minute.

f. Real time PCR

The reverse transcribed cDNA was used as the template for 'real time' PCR. Relative quantification of *UCP2* mRNA was performed with Taqman, after amplification and detection with the ABI Prism 7900 Sequence Detection System. This was performed using β-actin as an endogenous control, to normalise for differences in total amounts of RNA or DNA that may be present in the samples. The Cycle threshold (Ct), at which a significant increase in PCR cycle threshold was detected, was automatically set at the beginning of the logarithmic phase of each PCR amplification, in each well on the plate. The Ct value was defined as the fractional PCR cycle number at which the fluorescence emitted from a particular well rose above the threshold. The difference in Ct values for the endogenous reference β-actin, and *UCP2* was used as a basis for the relative expression analysis. Comparisons were then made between changes in expression by *UCP2* genotype (GG v AA) in a basal state (Krebs solution) and following stimulation (insulin+glucose or menadione). The GG cells in basal Krebs solution were set as the

reference point. Each sample was run in triplicate and the mean Ct value used for analysis. The lower the Ct value, the higher the amount of mRNA in the sample tested. The difference for each sample relative to *β-actin* was calculated (following the manufacturers instructions) as follows:-

$$\Delta Ct = (Ct\ UCP2) - (Ct\ \beta\text{-}Actin)$$

A comparative Ct (ΔΔCt) was obtained for the stimulated sample relative to the reference sample using the formula:-

$$\Delta\Delta Ct = (\Delta Ct\ Stimulated) - (\Delta Ct\ Reference)$$

The amount of mRNA, normalized to an endogenous reference and relative to a calibrator is given by:-

$$2^{-\Delta\Delta Ct} \text{ or } 2^{-(\Delta Ct\ Stimulated)-(\Delta Ct\ Reference)}$$

Each sample was run in triplicate and processed in 386 well plates. Each PCR mixture was 5μl in volume, consisting of 2.5μl Taqman Universal PCR master mix, 0.25μl of (20x) assay on demand probes (containing relevant primers), 2.25μl of RNase free water and 1μl of DNA.

The expression work described in section 2.2.10 was performed with help from Ka-Wah Li (MSc student, under my supervision)

2.2.11 Statistical analysis

Analysis was performed using SPSS (version 10.1, SPSS Inc., Chicago). For gene association analysis, data are reported for those individuals amongst whom high-throughput genotyping was successful. Results are presented as mean ± standard deviation and for data that was not normally distributed, as the geometric mean ± approximate standard deviation or median (interquartile range), as indicated in the results. The geometric mean and approximate standard deviation is shown for plasma CRP, Ox-LDL, Ox-LDL:LDL and F_2-isoprostane.

Deviations from Hardy-Weinberg equilibrium were considered using chi-squared tests. Hardy-Weinberg equilibrium gives the expected genotype distribution based on the observed frequency of the rare allele (q) and common allele (p) as $p^2+2pq+q^2$, where p^2 is the predicted frequency for homozygosity of the common allele, q^2 is the predicted frequency for homozygosity of the rare allele and $2pq$, the heterozygotes. These frequencies are expected provided the sample is drawn from a population with random mating and no strong selection. Deviations from the expected frequencies may suggest selection bias or technical problems with the method of genotyping.

Allele frequencies are shown with the 95% confidence interval. Log-transformations were conducted for data which were not normally distributed (except for duration of diabetes, were a square root transformation was required). Analysis of variance (ANOVA) was used to assess the association between genotypes and baseline characteristics on normally distributed data, or after appropriate transformation (log or

square root). In UDACS, the relationships between baseline parameters and plasma TAOS were tested by Pearson correlation co-efficient. ANCOVA was performed to test the association between genotype and plasma TAOS, Ox-LDL and Ox-LDL:LDL, after adjustment for potential confounders, using multiple regression analysis to obtain a residual. The interaction between genotype and smoking status in determining plasma TAOS, Ox-LDL and Ox-LDL was performed using linear regression where the combined effects were compared to the individual effects of these variables (likelihood ratio test). Chi-squared tests were used to compare differences in categorical variables by genotype and smoking status. In all cases a P value of less than 0.05 was considered statistically significant.

In NPHSII, analysis was performed by either Emma Hawe or Jackie Cooper (statisticians based in the department) using the 'Intercooled STATA' package (version 7.0, STATA Corporation, Texas). Survival analysis with respect to the *UCP2* genotype was carried out using Cox proportional hazards model, 'failure' being the first CHD event. Results are presented as hazard ratios (HR) with their corresponding 95% confidence interval (CI), and the CHD event rate per 1000 patient years calculated from survival analysis. To allow for differences in baseline data according to age and practice, age was included as a covariate in the model and data stratified by practice (using the strata option in STATA). The assumption of proportional hazards was checked by testing for a non-zero slope in a generalized linear regression of the scaled Schoenfeld residuals on time (using the stphtest command in STATA). The relative excess risk due to interaction (RERI) was used as a measure of deviation from additive effects. This was calculated as in the

following example:- If we consider a genotype (11 v 12/22) by smoking interaction, the RERI would be calculated as OR for 12/22 smokers (v 11 non-smokers) - OR for 12/22 non-smokers (v 11 non-smokers) - OR for (11 smokers (v 11 non-smokers) + 1. A value of zero (or above) represents no deviation from additive effects, and 95% CI were calculated using bootstrapping (Assmann *et al.* 1996).

No adjustment was made for multiplicity of testing. Whilst making such an adjustment reduces the type I error, it leads to increases in the type II error, and fewer errors of interpretation occur when no adjustment is made (Rothman 1990).

In the analysis of measures within UDACS, no differentiation was made between type 1 and type 2 diabetes mellitus, in the analysis.

All graphs show the arithmetic/geometric mean and standard error (calculated from the approximate standard deviation of the geometric mean for non normally distributed data).

CHAPTER THREE

PLASMA MARKERS OF OXIDATIVE STRESS AND LDL-OXIDATION

CONTENTS OF CHAPTER THREE

3.1 Plasma TAOS as a measure of oxidative stress

3.1.1 Introduction

As described in chapter 1, plasma markers of oxidative stress may be technically difficult to measure. Plasma TAOS provided a simple estimate of the net anti-oxidant capacity of 'overall' plasma. Few reports are published looking at the association of plasma TAOS with biochemical risk factors associated with CHD. Furthermore, there are no prospective studies looking at plasma markers of oxidative stress with future CHD risk.

3.1.2 Aims

The purpose of the work described in this section, was firstly to confirm that plasma TAOS is a useful surrogate marker for the measure of plasma oxidative stress and secondly to study the association between plasma TAOS and 'traditional' risk factors for CHD.

3.1.3 Validation with plasma F_2-isoprostanes

3.1.3a Methods

Plasma esterified F_2-isoprostane was measured successfully in 18 of 20 subjects selected from UDACS. These subjects were Caucasian males. The baseline characteristics of these subjects are shown in table 3.1. Male subjects and those with T2DM were selected to avoid any influence of gender and type of diabetes might have on F_2-isoprostane concentration. Furthermore, since CHD is a pro-oxidant state, equal numbers of those with and without CHD were selected. As shown in table 3.1, the selected subjects were comparable to males with T2DM from the whole sample, with respect to many variables.

Stored plasma was used to measure plasma TAOS and F_2-isoprostane, as described in chapter 2.

Table 3.1: Baseline characteristics of subjects with F_2-isoprostane measurements

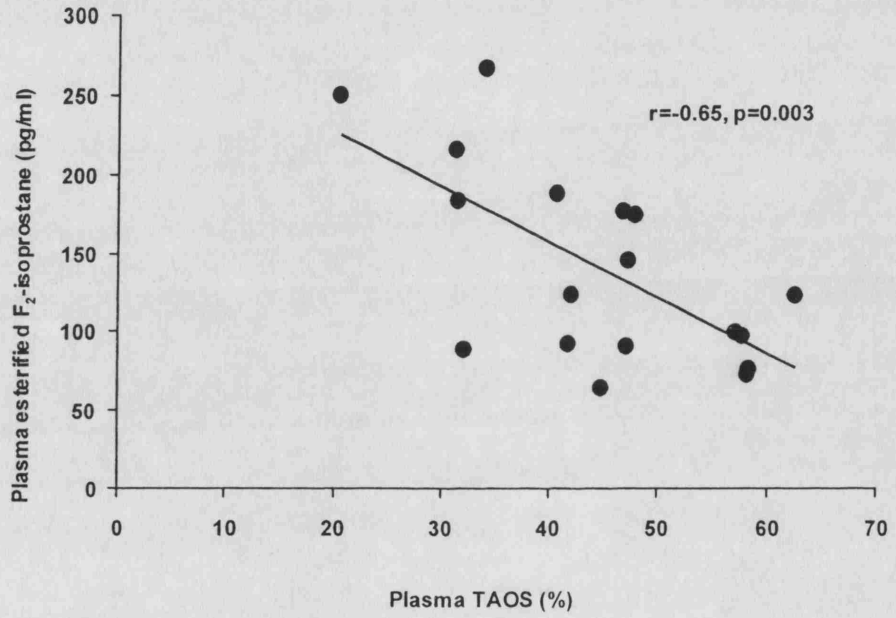
Measure	Subjects (n=18)	UDACS-Others (n=439)	p
Age (years)	64.8 (5.4)	61.1 (13.9)	0.25
BMI (Kg/m^2)*	31.1 (8.0)	28.3 (5.1)	0.05
Duration DM (years)$^+$	7.5 (6.0-17.0)	11.6 (5-20)	0.32
HbA1c (%)*	7.6 (1.5)	7.7 (1.6)	0.86
Glucose (mmol/l)*	9.8 (3.8)	9.5 (4.8)	0.83
Cholesterol (mmol/l)	5.0 (0.9)	4.9 (1.1)	0.76
LDL (mmol/l)	2.7 (0.8)	2.7 (0.9)	0.89
HDL (mmol/l)*	1.2 (0.3)	1.3 (0.4)	0.58
Tg (mmol/l)*	2.2 (1.2)	1.7 (1.1)	0.07
CRP (mg/l)*	2.11 (1.38)	1.43 (1.24)	0.06
TAOS (%)	44.6 (11.6)	42.6 (13.5)	0.53
F_2isoprostane (pg/ml)	140.01 (62.66)	-	-
Systolic BP (mmHg)*	138 (15)	137 (20)	0.69
Diastolic BP (mmHg)*	78 (12)	80 (12)	0.47
% CHD	50	21.5	0.005
% Smokers	17	18	0.86
% on insulin	39	44	0.64
% on Aspirin	44	48	0.80
% on Statin	44	25	0.06
% on ACEI	44	48	0.79

*Log transformed, $^+$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^+$duration of diabetes

3.1.3b Results

As illustrated in figure 3.1, there was a significant correlation between plasma TAOS and F_2-isoprostane (Pearson correlation: r=-0.65, p=0.003).

Figure 3.1: Correlation between plasma TAOS and F_2-isoprostanes



3.1.4 Association between plasma TAOS, risk factors & medication

3.1.4a Methods

This was explored in UDACS. Since OS may differ between gender (Ide *et al.* 2002), males and females were analysed separately. The purpose was to explore the association of plasma TAOS with a range of intermediate risk factors for CHD recorded in UDACS and to study the effect of ACEI , aspirin and statin therapy on plasma TAOS.

3.1.4.b Results

With respect to gender, no significant difference was observed between males and females (43.1±13.2% v 41.7±13.1% respectively, p=0.14). Plasma TAOS was significantly correlated with the biochemical parameters shown in table 3.2. The same significant correlates were identified in both males and females. Plasma HDL and LDL-PPD had positive correlations, whilst random glucose and random Tg had negative correlations. Stepwise backward logistic regression showed glucose and Tg to be strongest independent predictors of plasma TAOS (glucose p=0.004, Tg p=0.005). In the further analysis, when mentioned adjustment will be made to plasma TAOS for glucose and Tg.

Figure 3.2, shows the association between quartiles of plasma TAOS with glucose and Tg. The data is shown for all subjects and those without CHD. There was evidence of a linear association between both glucose and Tg with plasma TAOS.

Table 3.2: Significant correlations between plasma TAOS and biochemical intermediate risk factors in diabetes

Males

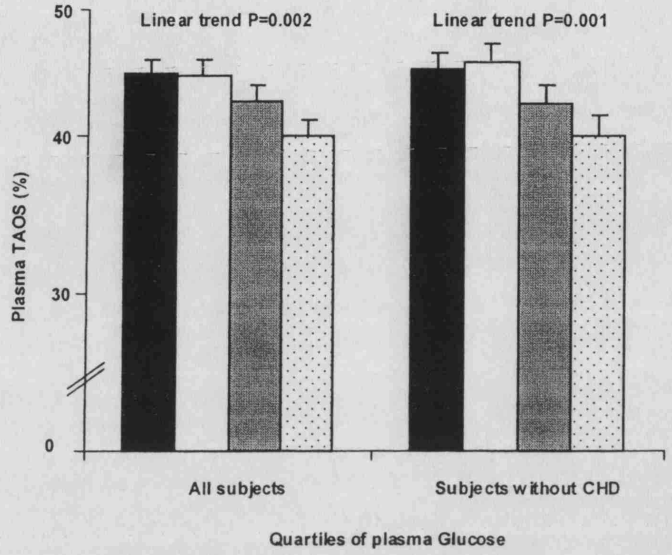
	Glucose	HDL	Tg	LDL-PPD
TAOS	r=-0.10	r=0.10	r=-0.10	r=0.14
	p=0.04	p=0.03	p=0.04	p=0.02
	n=457	n=457	n=457	n=319
Glucose		r=-0.10	r=0.24	r=-0.13
		P=0.03	p<0.001	p=0.02
		N=474	n=474	n=315
HDL			r=-0.52	r=0.51
			p<0.001	p<0.001
			n=474	n=315
Tg				r=-0.50
				p<0.001
				n=315

Females

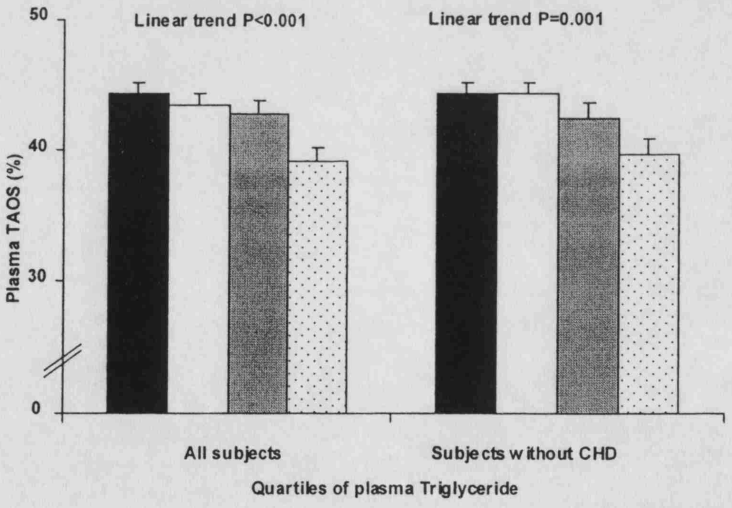
	Glucose	HDL	Tg	LDL-PPD
TAOS	r=-0.17	r=0.12	r=-0.19	r=0.17
	p=0.003	p=0.03	p=0.001	p=0.01
	n=304	n=302	N=303	n=204
Glucose		r=-0.10	r=0.20	r=-0.18
		p=0.10	p<0.001	p=0.01
		n=309	n=310	n=206
HDL			r=-0.52	r=0.33
			p<0.001	p<0.001
			n=309	n=206
Tg				r=-0.42
				p<0.001
				n=206

Figure 3.2: Association of plasma TAOS with glucose and triglycerides

Plasma glucose



Plasma triglycerides



The association between TAOS and smoking, urinary protein excretion, hypertension and type of diabetes was also explored. Since CHD may be an independent pro-oxidant, this analysis focused on those subjects without CHD. In males, there was a non-significant trend for plasma TAOS to be lower in current-smokers compared to never and ex-smokers (p=0.08, table 3.3). Of note when never-smokers were compared to ex-smokers and current-smokers there was a non-significant linear trend was observed (44.4 ±12.0 v 43.5 ±15.5 v 41.1 ±15.3, p=0.08). No significant associations were observed between urine protein excretion, hypertension or type of diabetes and plasma TAOS.

Table 3.3: Association of plasma TAOS with risk factors

Risk factor	Males		P	Females		p
Non smoker v smoker	44.0 ±12.7 (n=270)	41.1 ±15.0 (n=76)	0.08*	41.6 ±13.3 (n=216)	42.1 ±12.5 (n=36)	0.84
Normo- v microalbum/prot	43.7 ±12.4 (n=195)	43.0 ±14.3 (n=156)	0.62	42.3 ±12.8 (n=152)	40.5 ±13.6 (n=98)	0.27
Hypertension/Rx	43.1 ±13.1 (n=153)	43.5 ±13.4 (n=199)	0.82	41.6 ±13.2 (n=111)	41.8 ±13.1 (n=143)	0.91
Type 1 v type 2	44.4 ±12.0 (n=90)	43.5 ±12.9 (n=248)	0.55	40.0 ±13.5 (n=57)	42.2 ±13.2 (n=189)	0.28

*p=0.04 after adjusting plasma TAOS
Hypertension is defined as systolic BP>135mmHg and/or diastolic BP>85mmHg
Ex-smokers are those who have stopped for up to 12 months

No significant effects were observed by ACEIs, statins or aspirin on plasma TAOS. Nevertheless, there were some interesting trends. Males taking aspirin had elevated TAOS (not statistically significant), whilst in females the mean TAOS was lower in those taking aspirin. Conversely, with statin therapy, the reverse trends was observed with females being treated with statins having the highest plasma TAOS of all groups.

Table 3.4: Association of plasma TAOS with different therapies

Treatment	Males		P	Females		P
No ACEI v ACEI	43.9 ±13.0	42.6 ±13.6	0.36	42.1 ±12.8	41.1 ±13.5	0.55
	(n=199)	(n=153)		(n=150)	(n=105)	
No Aspirin v Aspirin	42.4 ±12.8	44.6 ±13.8	0.13	42.2 ±12.7	40.7 ±13.8	0.39
	(n=208)	(n=144)		(n=165)	(n=89)	
No statin v statin	43.8 ±12.7	41.1 ±16.1	0.17	40.9 ±13.1	44.4 ±12.8	0.09
	(n=299)	(n=51)		(n=195)	(n=59)	

No change was observed after adjusting plasma TAOS for correlates

3.1.5. Association with CHD in subjects with diabetes

3.1.5a Methods

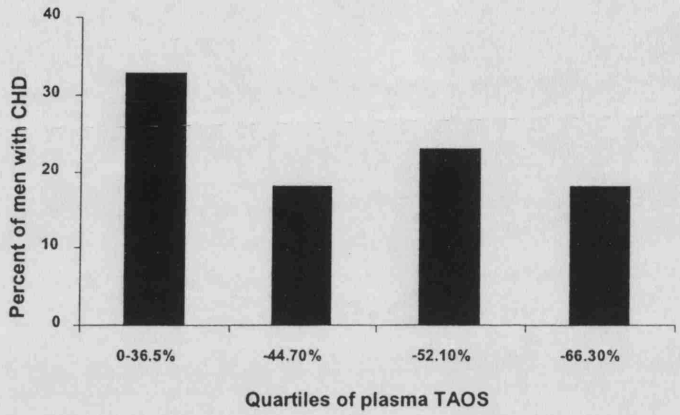
The association between CHD status and plasma TAOS was investigated in the subjects from UDACS.

3.1.5b Results

In males, there was a significant association between plasma TAOS and CHD status (No CHD v CHD: 43.4±13.2% v 40.3±13.8% respectively, p=0.04). After adjustment of plasma TAOS (for plasma glucose and Tg), this association was no longer significant (p=0.06). Figure 3.3 shows the proportion of men with CHD grouped by quartiles of TAOS. There were a significantly higher percentage of men with CHD in the lowest quartile compared to the other quartiles, suggesting that there was a threshold at which lowers plasma TAOS was associated with CHD. Furthermore, there was a highly significant result when men in the lower quartile was compared to other quartiles combined (% of men with CHD: Lower quartile v other quartiles 32.7% v 19.7%, p=0.004). In the women, no association was seen between plasma TAOS and CHD status (No CHD v CHD: 41.7±13.1% v 43.9±11.4% respectively, p=0.26). No difference was observed after adjustment and no effect was observed by dividing the group by quartiles of plasma TAOS.

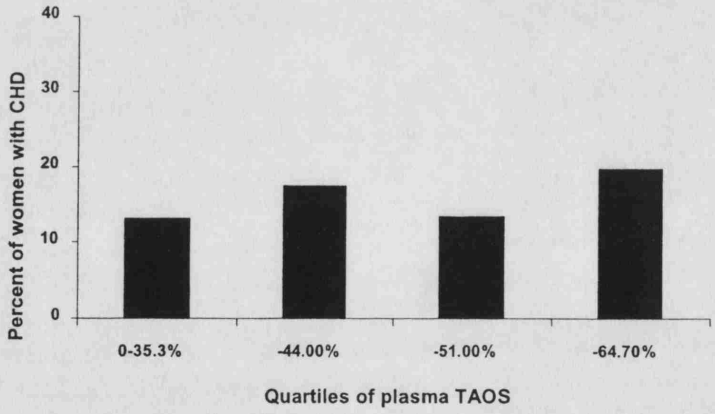
Figure 3.3: Association of plasma TAOS with CHD

Men



ANOVA between groups, χ^2=9.93, p=0.03, Linear trend p=0.03

Women



ANOVA between groups, χ^2=1.75, p=0.63

3.1.6. Plasma TAOS and prospective CHD risk

3.1.6.a Methods

Plasma TAOS was measured in 310 plasma samples from the NPHSII sample. Analysis was performed on baseline samples (stored at -80°C) collected at the time of recruitment ten years earlier. The baseline characteristics of these subjects grouped by CHD status, are shown in table 3.5.

Table 3.5: Baseline differences in subjects by CHD status in NPHSII

Trait	No CHD (n=211)	CHD (n=99)	P
Age (years)	56.3 (3.6)	56.4 (3.58)	0.83
Systolic blood pressure (mmHg)*	138 (19)	143 (21)	0.03
Diastolic blood pressure (mmHg)	85 (12)	86 (11)	0.37
Body mass index (kg/m^2)*	26.8 (3.4)	26.8 (3.4)	0.82
Current smoking % (n)	25.6% (54)	46.5% (46)	<0.0001
Cholesterol (mmol/l)	5.8 (1.00)	6.1 (1.00)	0.02
Triglyceride (mmol/l)*	1.9 (1.0)	2.1 (1.1)	0.17
CRP (mg/l)*	1.15 (1.28)	2.05 (2.26)	<0.0001
Fibrinogen (g/l)*	2.73 (0.55)	2.93 (0.56)	0.003
Plasma TAOS (%)	37.1 (7.9)	35.1 (8.0)	0.04

Mean (SD) shown. Geometric mean and approximate SD shown for systolic blood pressure, body mass index and triglyceride, CRP. *Log transformed.

3.1.6.b Results

In NPHSII, plasma TAOS was negatively correlated with total cholesterol and Tg (r=-0.23, p=0.02 and r=-0.20, p=0.06 respectively). No glucose levels were available on the subjects. There was a borderline significant with fibrinogen (r=0.18, p=0.07).

Of the 310 samples analysed 99 subjects went onto develop a CHD event over the following ten years. Plasma TAOS was significantly lower in those males who subsequently developed CHD (table 3.5). A 1 standard deviation (SD) decrease in TAOS of 7.9% was associated with a modest increase in CHD risk (1.30 95%CI: 1.02-1.64, p=0.04). This association remained significant after adjustment for classical risk factors associated with increased risk in the NPHSII (age, systolic BP, BMI, Tg, fibrinogen, smoking and diabetes). After adjustment for these risk factors, the OR for CHD associated with a 1 SD decrease in plasma TAOS was 1.30 (95%CI 1.08-1.45), p=0.01, suggesting that a lower plasma TAOS had an independent effect on ten-year CHD risk. Table 3.6, shows the OR for CHD by quartiles of plasma TAOS (the upper quartile [quartile 4] being the reference point, OR=1.00). The pattern is similar to that observed for the prevalence of CHD in males from UDACS (section 3.1.5). Again a threshold effect may be observed, with the OR for CHD being significantly higher in the lowest quartile of plasma TAOS.

Table 3.6: CHD risk by baseline plasma TAOS in NPHSII

Quartiles	No CHD	CHD	OR (95% CI)	OR (95% CI)[†]	OR (95% CI)[‡]
1 (<31%)	43 (20.4)	35 (35.4)	1.91 (0.99-3.70)	2.22 (1.08-4.56)	1.81 (0.84-3.90)
2 (31-37.3%)	57 (27.0)	20 (20.2)	0.82 (0.41-1.67)	0.89 (0.41-1.91)	0.94 (0.42-2.09)
3 (37.4 - 42.1%)	57 (27.0)	21 (21.2)	0.86 (0.43-1.74)	0.88 (0.41-1.89)	0.95 (0.43-2.11)
4 (>42.1%)	54 (25.6)	23 (23.2)	1.00	1.00	1.00
P value			P=0.04	P=0.03	P=0.03

[†]adjusted for age, Systolic BP, BMI, cholesterol, Tg, fibrinogen, smoking and diabetes.
[‡] adjusted for age, Systolic BP, BMI, cholesterol, Tg, fibrinogen, smoking, diabetes and CRP.

3.2 Plasma Oxidised-LDL as a marker of oxidation

3.2.1 Introduction

As described in chapter 1, oxidative stress has numerous adverse effects on the vascular system. LDL is an important target of oxidation with oxidised LDL (Ox-LDL) having a major pro-atherosclerotic role. The measurement of plasma Ox-LDL gives a specific measure of oxidation. Unlike the generic measurement of plasma TAOS or F_2-isoprostane, this measure is specific to LDL (a key atherogenic risk factor). In the body as a whole, the contribution of Ox-LDL to whole plasma oxidative status is not clear, as LDL is only a small fraction of the entire plasma. Ox-LDL concentrations are also strongly correlated with plasma LDL levels, and the latter is thus is a key factor in determining absolute plasma Ox-LDL concentration. To overcome this, researchers have calculated the proportion of Ox-LDL per LDL, using the formula below (Scheffer *et al.* 2003). This results in a ratio of Ox-LDL to LDL-C, expressed as Ox-LDL U/mmol LDL. In this study, I will consider both the absolute Ox-LDL and the OxLDL:LDL ratio as outcome variables.

$$\textbf{Ox-LDL:LDL U/mmol} = \frac{\textbf{Ox-LDL}}{\textbf{LDL}} \quad \frac{\textbf{U/l}}{\textbf{mmol/l}}$$

Ox-LDL and Ox-LDL:LDL therefore provide focused measures of LDL oxidation, complementing that of plasma TAOS, which provides a more general measure of oxidative stress in plasma.

3.2.2 Aims

The purpose of the work described in this section was to study the association between plasma Ox-LDL, Ox-LDL:LDL with TAOS, and risk factors for CHD.

3.2.3 Correlation of Ox-LDL with plasma TAOS and F_2-isoprostanes

3.2.3a Methods

The association between plasma Ox-LDL and plasma TAOS and F_2-isoprostane was examined in the 18 subjects with F_2-isoprostane measurements (section 3.1.3). The association with plasma TAOS was also explored in the whole UDACS sample.

3.2.3b Results

No correlation was observed between Ox-LDL and plasma TAOS or F_2-isoprostane (TAOS $r=0.17$, $p=0.51$; F_2-isoprostane $r=-0.07$, $p=0.80$). Furthermore, no correlation was observed with Ox-LDL:LDL (TAOS $r=-0.02$, $p=0.94$; F_2-isoprostane $r=-0.04$, $p=0.90$). In UDACS, no correlations were observed between plasma TAOS with Ox-LDL ($r=-0.04$, $p=0.34$) or Ox-LDL:LDL ($r=0.04$, $p=0.36$). No correlations were observed in UDACS after excluding subjects with CHD, or those taking statins.

3.2.4 Association between plasma Ox-LDL, risk factors & medication

3.2.4a Methods

These associations were studied in subjects from UDACS. The association of total Ox-LDL and Ox-LDL:LDL ratio was examined in relation to the collected biochemical and clinical data within UDACS. Again analysis focused on those subjects without CHD.

3.2.4b Results

With respect to gender, Ox-LDL was not significantly between gender (46.8±19.6U/l v 44.7±17.0U/l respectively, p=0.26). In both sexes (table 3.7), plasma Ox-LDL had significant positive correlations with LDL and Tg, and a negative correlation with LDL-PPD. Within males, there was a further positive correlation with BMI and a negative correlation with HDL. In females, the correlation between HDL and Ox-LDL was not significant (r=-0.11, p=0.12). LDL particle size is known to be dependent on both plasma LDL and Tg concentration, and, in line with previous observations, the strongest correlation in both sexes was with plasma LDL. When all of the above correlates were included in a statistical model, stepwise elimination revealed that plasma LDL and Tg concentration were the strongest independent predictors of plasma Ox-LDL (LDL p<0.001, Tg p<0.001). When LDL-PPD was added to this model all three variables remained statistically significant (LDL-C p<0.001, Tg p=0.02, LDL-PPD p=0.04). Because of this, further analysis, adjustment will be made to plasma Ox-LDL for Tg and LDL.

Table 3.7: Significant correlations between plasma absolute Ox-LDL and biochemical intermediate risk factors in diabetes

Males

	LDL	HDL	Tg	BMI	LDL-PPD
Ox-LDL	r=0.32	r=-0.17	r=0.24	r=0.14	r=-0.19
	p<0.001	p=0.003	p<0.001	p=0.01	p=0.001
	n=297	n=309	n=309	n=307	n=310
LDL		r=0.05	r=0.09	r=0.1	r=0.08
		p=0.28	p=0.05	p=0.04	p=0.17
		n=456	n=456	n=452	n=310
HDL			r=-0.52	r=-0.32	r=0.51
			p<0.001	p<0.001	p<0.001
			n=474	n=470	n=315
Tg				r=0.37	r=-0.50
				p<0.001	p<0.001
				n=470	n=315
BMI					r=-0.20
					p<0.001
					n=313

Females

	LDL	HDL	Tg	BMI	LDL-PPD
Ox-LDL	r=0.29	r=-0.11	r=0.22	r=0.03	r=-0.17
	p<0.001	p=0.12	p=0.02	p=0.64	p=0.01
	n=200	n=202	n=202	n=202	n=198
LDL-C		r=-0.03	r=0.07	r=0.07	r=0.01
		p=0.64	p=0.20	p=0.20	p=0.88
		n=307	n=306	n=306	n=204
HDL			r=-0.52	r=-0.31	r=0.33
			p<0.001	p<0.001	p<0.001
			n=309	n=308	n=206
Tg				r=0.29	r=-0.42
				p<0.001	p<0.001
				n=309	n=206
BMI					r=-0.23
					p<0.001
					n=206

Table 3.8: Significant correlations between OX-LDL:LDL and biochemical intermediate risk factors in diabetes

Males

	LDL	HDL	Tg	LDL-PPD
Ox-LDL:LDL	r=-0.50	r=-0.20	r=0.13	r=-0.22
	p<0.001	p<0.001	p=0.02	p<0.001
	n=297	n=297	n=297	n=293
LDL-C		r=0.05	r=0.09	r=0.08
		p=0.28	p=0.05	p=0.17
		n=456	n=456	n=310
HDL			r=-0.52	r=0.51
			p<0.001	p<0.001
			n=474	n=315
Tg				r=-0.50
				p<0.001
				n=315

Females

	LDL	HDL	Tg	LDL-PPD
Ox-LDL:LDL	r=-0.48	r=-0.08	r=0.11	r=-0.13
	p<0.001	p=0.24	p=0.13	p=0.07
	n=200	n=200	n=200	n=194
LDL-C		r=-0.03	r=0.07	r=0.01
		p=0.64	p=0.20	p=0.88
		n=307	n=306	n=204
HDL			r=-0.52	r=0.33
			p<0.001	p<0.001
			n=309	n=206
Tg				r=-0.42
				p<0.001
				n=206

In both sexes, Ox-LDL:LDL, showed a strong negative correlation with LDL (table 3.8). This is expected as LDL is the denominator for the calculation of Ox-LDL:LDL. There also was a negative correlation with LDL-PPD size (borderline in females), suggesting that smaller, more dense LDL is associated with increased Ox-LDL:LDL. In males, Ox-LDL:LDL had a negative correlation with HDL and a positive correlation with plasma Tg. The association with Tg was of borderline significance in females. When all of the above correlates, apart from LDL-C, were included in a statistical model, stepwise regression revealed LDL-PPD to be the strongest independent predictors of plasma Ox-LDL:LDL ($p<0.001$). Because of this, in further analysis, adjustment was made to plasma Ox-LDL:LDL for LDL-PPD, where described to assess independent associations with Ox-LDL:LDL.

The association between absolute plasma Ox-LDL and Ox-LDL:LDL were also examined with respect to smoking, urinary protein excretion, hypertension and type of diabetes was also explored (tables 3.9 and 3.10). Since CHD may be an independent pro-oxidant stress, this analysis focused on those subjects without CHD. In males, plasma Ox-LDL was significantly higher in subjects with evidence of microalbuminuria/proteinuria ($p<0.001$) and those with hypertension ($p=0.05$). After Ox-LDL was adjusted for LDL and Tg, Ox-LDL remained significantly higher in those with evidence of microalbuminuria/proteinuria ($p=0.003$), but not in subjects with hypertension ($p=0.32$). No other significant associations were observed. With respect to Ox-LDL:LDL, plasma levels were again significantly higher in those with

microalbuminuria/proteinuria. After adjusting Ox-LDL:LDL for LDL-PPD, this association remained statistically significant (p=0.04). No other significant associations were observed.

No significant effects were observed with the administration of ACEIs, statins or aspirin on plasma Ox-LDL or Ox-LDL:LDL (tables 3.11 and 3.12). Of interest, after adjusting absolute Ox-LDL, male subjects prescribed statins had a borderline significantly lower Ox-LDL (p=0.08). With respect to Ox-LDL:LDL, no difference was observed by statin therapy after adjustment for LDL-PPD (p=0.63).

Table 3.9: Association of plasma absolute Ox-LDL with risk factors

	Men		P	Women		p
Smoker v Non-smoker	44.1 ±7.3	45.7 ±8.0	0.56	45.9 ±8.7	52.8 ±7.2	0.17
	(n=184)	(n=47)		(n=142)	(n=20)	
Normo- v microalbum/prot	41.1 ±7.3	49.3 ±6.9	<0.001*	47.2 ±8.9	46.3 ±8.2	0.79
	(n=128)	(n=111)		(n=100)	(n=62)	
Hypertension/Rx	42.3 ±6.9	46.6 ± 7.7	0.05	48.2 ±8.8	45.6 ±8.3	0.40
	(n=101)	(n=138)		(N=78)	(N=76)	
Type 1 v type 2	44.2 ±7.3	44.8 ±7.4	0.81	47.8 ±6.7	46.5 ±9.0	0.72
	(n=61)	(n=178)		(N=40)	(N=124)	

*P=0.003 after adjustment of Ox-LDL for LDL and Tg

Table 3.10: Association of plasma Ox-LDL:LDL with risk factors

	Men		P	Women		p
Smoker v Non-smoker	18.6 ±2.9	17.4 ±3.7	0.52	16.7 ± 3.5	16.5 ± 2.5	0.91
	(n=176)	(n=46)		(n=141)	(n=20)	
Normo- v microalbum/prot	15.8 ±2.9	18.2 ±3.2	0.02*	17.3 ± 3.4	16.0 ± 3.3	0.30
	(n=124)	(n=106)		(n=99)	(n=62)	
Hypertension/Rx	16.2 ±3.0	17.4 ±3.2	0.20	16.2 ± 3.3	17.2 ± 3.5	0.40
	(n=98)	(n=132)		(n=77)	(n=86)	
Type 1 v type 2	16.8 ± 2.6	16.9 ± 3.1	0.96	16.5 ± 2.5	16.8 ±3.6	0.86
	(n=60)	(n=230)		(n=40)	(n=123)	

*P=0.04 after adjustment of Ox-LDL:LDL for LDL-PPD

Table 3.11: Association of plasma absolute Ox-LDL with medication

Treatment	Men		P	Women		P
No ACEI v ACEI	43.4 ±7.5	46.4 ±7.3	0.19	48.8 ±8.4	44.0 ±8.6	0.12
	(n=134)	(n=105)		(n=98)	(n=66)	
No Aspirin v Aspirin	44.9 ±7.3	44.3 ±7.7	0.80	46.7 ±7.8	46.9 ±10.2	0.97
	(n=149)	(n=90)		(n=115)	(n=49)	
No statin v statin	45.2 ±7.2	41.7 ±8.1	0.24*	47.1 ±8.0	45.7 ±9.9	0.69
	(n=202)	(n=36)		(n=123)	(n=41)	

*P=0.08 after adjustment of Ox-LDL for LDL and Tg

Table 3.12: Association of plasma Ox-LDL:LDL with medication

Treatment	Men		P	Women		P
No ACEI v ACEI	16.7 ± 3.1	17.1 ± 3.1	0.64	16.8 ± 3.4	16.6 ± 3.4	0.91
	(n=131)	(n=99)		(n=97)	(n=66)	
No Aspirin v Aspirin	17.2 ± 3.0	16.4 ± 3.2	0.43	16.6 ± 3.2	17.0 ± 3.9	0.81
	(n=145)	(n=85)		(n=115)	(n=48)	
No statin v statin	16.9 ± 3.0	16.5 ± 3.5	0.74	16.4 ± 3.0	17.8 ± 4.4	0.30
	(n=197)	(n=32)		(n=122)	(n=41)	

No associations were observed after adjusting Ox-LDL:LDL

Effect of lipid lowering therapy on lipid fractions and Ox-LDL and Ox-LDL:LDL

This was explored in all subjects. The rational for using a statin in the UCL diabetes clinic was to treat hypercholesterolaemia (dictated by guidelines at specific times). Therefore, a higher proportion of subjects with complications should be prescribed statins. The association between statin treatment and its effect on measures of LDL oxidation and lipid parameters was explored in all subjects. The aim was to ascertain whether Ox-LDL and Ox-LDL:LDL were different in relation to statin use, as would be expected for total cholesterol and LDL measures.

Table 3.13, shows the results of statin therapy on lipid fractions. As expected, total cholesterol and LDL were significantly lower in those receiving treatment. No significant difference was observed with respect to Ox-LDL, however as shown, the Ox-LDL:LDL was higher in those receiving statins.

Table 3.13: Statin therapy, lipids and Ox-LDL in all subjects

	No statin (n=574)	Statin (n=210)	P
Cholesterol (mmol/l)	5.2 (1.1)	4.8 (1.1)	<0.001
LDL (mmol/l)	2.9 (0.9)	2.5 (0.9)	<0.001
Ox-LDL (U/l)	45.3 (18.0) (n=367)	45.0 (19.7) (n=143)	0.87
Ox-LDL:LDL (U/mmol)	16.5 (7.1) (n=367)	19.2 (9.9) (n=143)	0.001
LDL-PPD (nm)	26.74 (0.91) (n=378)	26.49 (1.11) (n=142)	0.009

3.2.5 Association with CHD in subjects with diabetes

3.2.5a Methods

The association between CHD status and plasma Ox-LDL and Ox-LDL:LDL was investigated in the subjects from UDACS.

3.2.5b Results

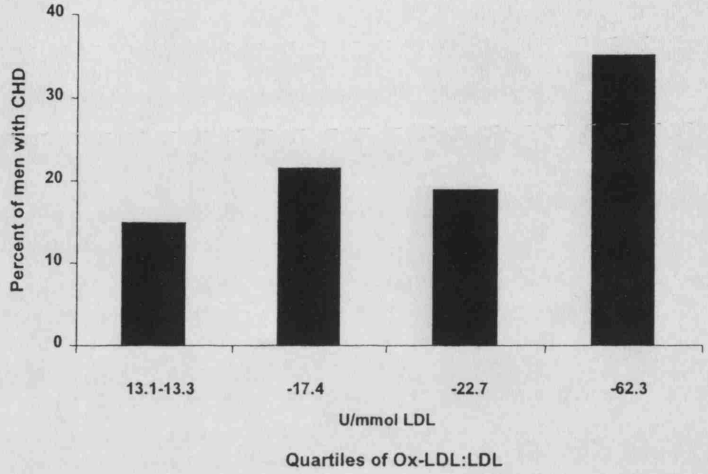
No association was observed between absolute plasma Ox-LDL and CHD status, before or after stratifying by gender (Males, No CHD v CHD: 44.7±6.4U/l v 42.0±9.5U/l, p=0.41; Females, No CHD v CHD: 46.8±8.6U/l v 46.5±6.3U/l, p=0.93). No change was observed after adjustment of the absolute Ox-LDL, for Tg and LDL.

In all subjects, plasma Ox-LDL:LDL was significantly higher in those with CHD (No CHD v CHD: 16.8±7.4U/mmol v 19.0±10.0U/mmol, p=0.02). In males, there was a significant association between plasma Ox-LDL:LDL and CHD status (No CHD v CHD: 16.9±3.1U/mmol v 19.3± 5.0U/mmol respectively, p=0.04). After adjustment for LDL-PPD, this association was no longer significant (p=0.10). Figure 3.4, shows the proportion of males with CHD grouped by quartiles of Ox-LDL:LDL. There were a significantly higher percentage of men with CHD in the highest quartile compared to the lower three quartiles, suggesting that there was a threshold at which Ox-LDL:LDL was associated with CHD. Consistent with this, there was a highly significant result when men in the upper quartile was compared to other quartiles combined (% of men with CHD: Lower quartiles v upper quartile: 18.4% v 35.1%, p=0.003). In the females, no

significant association was seen between plasma Ox-LDL:LDL and CHD (No CHD v CHD: 16.7±3.4U/mmol v 18.4±(2.8U/mmol respectively, p=0.25). No effect was observed by dividing the group by quartiles of plasma TAOS as shown in figure 3.4.

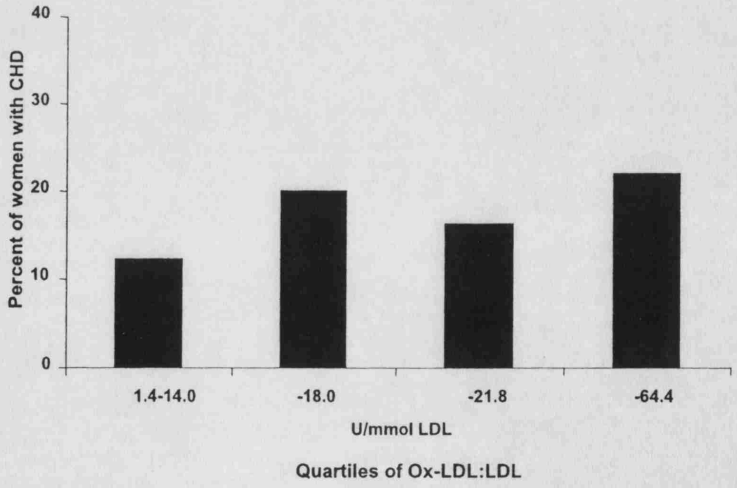
Figure 3.4: Association of Ox-LDL:LDL with CHD

Men



ANOVA between groups, χ^2=9.90, p=0.02, Linear trend p=0.008

Women



ANOVA between groups, χ^2=1.83, p=0.60

3.3 Discussion

In this chapter, plasma TAOS and Ox-LDL have been described as measures of total plasma oxidation and specific LDL-oxidation respectively. Plasma TAOS was correlated with plasma esterified F_2-isoprostanes, supporting its use as a cheaper, practical measure of total plasma oxidative stress. Plasma TAOS was modestly and non-significantly different between males and females, with women having mean levels 4% lower than males. Previously, the converse relationship has been observed between oxidative stress and gender (Ide *et al.* 2002). One possible explanation for this finding might be that the majority of women were post-menopausal, which is associated with increased CHD risk, particularly in females with diabetes. No studies are published reporting plasma measures of oxidative stress in post-menopausal females. Plasma TAOS was correlated with glucose, HDL, Tg and LDL-PPD. This is in line with previous observations suggesting that HDL exhibits anti-oxidant properties (Durrington *et al.* 2001), and increasing plasma glucose is associated with increased oxidative stress (Davi *et al.* 1999). Plasma Tg is negatively associated with HDL (in UDACS, r=-0.52), which may account for the association with HDL. Furthermore, increased Tg levels are also associated with increased Sd-LDL, and this may be responsible for the correlation between LDL-PPD and plasma TAOS. Further analysis showed plasma glucose and Tg were the strongest independent predictors of plasma TAOS. Smoking is well-established as a pro-oxidant (Morrow *et al.* 1995), and in line with this plasma TAOS was non-significantly lower in current smokers. An interesting observation is the linear trend observed from never- to ex to current-smokers, observed in males. No significant effects were observed with respect

to treatment with ACEIs, statins or aspirin. CHD was associated with lower plasma TAOS in males. A 'threshold' was observed whereby approximately 13% more men had CHD in the lower quartile of plasma TAOS compared to the combined upper quartiles. In the females, no such association was observed. The association between plasma markers of oxidative stress and CHD in men has previously been described in cross sectional studies (Weinbrenner *et al.* 2003), but no studies are published looking at this association in females. The association with CHD was further explored in the prospective NPHSII sample of men. Subjects in the lowest quartile had an approximate doubling in prospective CHD risk, even after adjusting for well-established risk factors associated with CHD. Again, in this sample of subjects there was a 'threshold' at which risk was increased. No previous reports have been published reporting the association between baseline measures of plasma oxidative stress and prospective risk.

With respect to plasma Ox-LDL, both the absolute level and the Ox-LDL:LDL levels were considered. The latter provides an estimate of the proportion of oxidatively modified LDL per LDL particle. In line with previous studies, looking at similar measures of total plasma oxidative stress and specific antioxidant levels in relation to Ox-LDL (Kopprasch *et al.* 2002; Weinbrenner *et al.* 2003), no association was observed between the measures of LDL-oxidation with plasma TAOS or F_2-isoprostanes. This may be accounted for by the fact that Ox-LDL does not provide a large contribution to overall plasma oxidation, but is a specific measure of LDL oxidative modification. In line with the results for plasma TAOS, there was a non-significant trend for absolute Ox-LDL to be

higher in females. Within both males and females, Ox-LDL had a significant positive correlation with LDL and Tg, and a negative correlation with LDL-PPD size. This is in line with previous published studies, The LDL-PPD is associated with both LDL and Tg, resulting in a smaller and denser LDL particle which is more readily oxidised. This may explain why both LDL and Tg were the strongest independent predictors of Ox-LDL. With respect to Ox-LDL:LDL the strongest correlation was with LDL-PPD. This has been observed in one previous study (Scheffer *et al.* 2003), supporting the view that increased Sd-LDL is more prone to oxidative modification. Strangely, no significant difference was observed in the Ox-LDL measures with respect to smoking. In the males, both Ox-LDL and Ox-LDL:LDL were significantly higher in subjects with microalbuminuria and proteinuria. This is in line with a previous study in Japanese subjects (Ujihara *et al.* 2002). A plausible explanation for this might be that Ox-LDL is taken up by scavenger receptors on the mesangial cells, resulting in foam cell formation macrophage infiltration and alteration in renal haemodynamics.

An important observation is the effect that statins have on Ox-LDL and Ox-LDL:LDL. The absolute Ox-LDL is largely dependent on the proportion of LDL present in the plasma. Therefore, if subjects are treated with cholesterol-lowering therapies, the LDL, and subsequently the absolute Ox-LDL, will decrease. To date, no studies have been published showing that statins alter the proportion of Ox-LDL per LDL particle. The Ox-LDL:LDL ratio adjusts for the total LDL concentration in the plasma. As observed, in UDACS, those being treated with statins had a significantly higher Ox-LDL:LDL but

lower total cholesterol, LDL and Ox-LDL. This observation is interesting, as those prescribed statins would presumably have had higher cholesterol fractions (including LDL and Ox-LDL) prior to starting therapy. However, it might be that statin therapy does not alter the proportion of Ox-LDL per LDL, despite lowering LDL (and absolute Ox-LDL concentrations). This concept is supported by results from a published study, where similar results were observed, using Ox-LDL:apoB ratio (Tsuzura *et al.* 2004). Confirmation in a prospective sample of subjects before and after the commencement of statin is required.

With respect to CHD, no significant difference was observed in absolute Ox-LDL. As described above, this may be accounted for by the increased statin use in this group. However, Ox-LDL:LDL was significantly higher in males with CHD. No significant difference was observed in females. In males there appeared to be a 'threshold' in which men in the upper quartile had a significantly increased prevalence of CHD (approximately 17% higher in the upper quartile compared to the lower quartiles).

In summary, this chapter shows plasma TAOS to be lower in subjects with CHD and to be strongly associated with plasma glucose and Tg. Increased plasma levels of both glucose and Tg, are typical of subjects with T2DM, and this may in part explain the previous observations showing reduced plasma TAOS and increased markers of ROS generation in subjects with diabetes.

Circulating Ox-LDL:LDL (and not absolute Ox-LDL) is greater in subjects with CHD. LDL and Tg were the strongest predictors of absolute Ox-LDL and LDL-PPD of Ox-LDL:LDL. These measures of LDL-oxidation were not associated with plasma TAOS in the circulation, suggesting that they are independently associated with risk and other biochemical risk factors. As described above, this may be accounted for by the fact that Ox-LDL does not provide a large contribution to overall plasma oxidation, but is a specific measure of LDL-oxidative modification. Both measures are however useful. Ox-LDL as described in table 1.2, chapter 1, is associated with processes involved in plaque formation (e.g. increased foam cell, LDL aggregation, enhanced monocyte chemotaxis). The TAOS of plasma may influence or regulate other processes involved in the pathogenesis of atherosclerosis (e.g. MMP activation, apoptosis, expression of cell adhesion molecules, altered vasomotor activity). Later in this thesis, these biochemical measures of oxidation will be explored in relation to variants in specific genes involved in lipid metabolism, general antioxidant protection and mitochondrial ROS production, to explore the role of these different processes in determining plasma TAOS and LDL oxidation.

CHAPTER FOUR

VARIATION IN THE HUMAN APOLIPOPROTEIN E GENE AND ASSOCIATIONS WITH MARKERS OF OXIDATIVE STRESS

CONTENTS OF CHAPTER FOUR

4.1 Apolipoprotein E (*APOE*) gene variants and markers of oxidative stress

4.1.1 Introduction

As described in chapter 1, variation in the human apolipoprotein E gene (*APOE*) has been associated with increased risk of both CHD and Alzheimer's disease. An important observation was that in the NPHSII cohort, there was a significant interaction with cigarette smoking to increase risk in the ε4 allele carriers (Humphries *et al.* 2001). Apart from its effect on CHD risk, the ε4 allele has also been associated with early onset of Alzheimer's dementia. With respect to biochemical intermediate phenotypes, ε4+ subjects typically have higher plasma LDL and lower plasma Tg concentrations compared to ε2+ subjects. To date, only one study consisting of 62 subjects has explored the association of variation in the *APOE* gene with a marker of oxidative stress (Metso *et al.* 2003). This study showed that ε4 allele carriers had increased autoantibodies to Ox-LDL compared to both ε3ε3 and ε2+ subjects. No studies have explored association between variation in the *APOE* gene with both a plasma marker of oxidative stress and LDL oxidation together, particularly in a pro-oxidant state (such as diabetes or smoking).

4.1.2 Aims

The purpose of the work described in this section, was firstly to study the effects of variation in the *APOE* gene with plasma TAOS and measures of LDL-oxidation. *APOE* gene interaction with smoking will also be studied in relation to the above measures. Analysis in the presence of a pro-oxidant state such as smoking might reveal differences, otherwise not observed in a 'normal' state.

4.1.3 Methods

This work was performed on those samples obtained from UDACS. *APOE* genotyping was performed as described in chapter 2. Analysis focused on the association of genotype with the measures of oxidative stress and LDL-oxidation already described in chapter 3. In the analysis, raw and adjusted values for markers of oxidative stress are shown (as described in chapter 3: TAOS adjusted for Tg and glucose, Ox-LDL for Tg and LDL, Ox-LDL:LDL for LDL-PPD).

4.1.4 Results

4.1.4.a APOE genotype distribution and clinical data

Seven hundred and sixty one (98%) of the 780 Caucasian subjects were successfully genotyped for the *APOE* variants. Sixteen of these subjects were ε2ε4, and in 2 (and a further 1 non-Caucasian) subjects an aberrant heteroduplex pattern was observed resulting from new mutations (described in section 4.2). These were excluded from further analysis leaving 743 subjects. Exclusion of the ε2ε4 genotype is a common analytical strategy (Davignon *et al.* 1988; Gerdes *et al.* 2000; Humphries *et al.* 2001), based on the observation that the ε2 allele is associated with lower cholesterol levels and the ε4 allele with higher levels and CHD risk, and thus carriers of both alleles will have intermediate effects. The genotype distribution was in Hardy Weinberg equilibrium, as shown in table 4.1. The allele frequencies were as follows:- ε2: 0.07, ε3: 0.81; ε4: 0.12. Of the 743 subjects included in the analysis, 95 were ε2+, 495 were ε3ε3 and 153 were ε4+.

The baseline characteristics of the subjects (by CHD status) for whom genotyping was successful are summarised in table 4.2. Of the 743 diabetic Caucasian subjects, CHD was present in 153 individuals (20.5%), whose mean age, BMI, Tg, CRP and Ox-LDL:LDL was significantly higher, and HDL and LDL-PPD lower than those without CHD. Duration of diabetes, plasma TAOS, absolute Ox-LDL and HbA1c were not significantly different in both groups. A significantly higher proportion of subjects with CHD were treated with aspirin, statins and ACE inhibitors (ACEI) which may account for their lower diastolic BP and the observation that LDL was higher in those without CHD. There was no significant difference in *APOE* genotype distribution between those with and without CHD (p=0.91).

Table 4.1: Frequency distribution for *APOE* genotype in subjects

	APOE genotype					
	ε2ε2	ε2ε3	ε2ε4	ε3ε3	ε3ε4	ε4ε4
No. subjects (%)	2 (0.3%)	93 (12.3%)	16 (2.1%)	495 (65.2%)	146 (19.2%)	7 (0.9%)

APOE genotype were in Hardy-Weinberg equilibrium, χ^2=2.34, p=0.23.
The 2 Caucasian subjects with new mutations are excluded

4.1.4.b APOE genotype, lipids & measures of oxidative stress

In line with previous data, ε2 allele carriers had lower plasma total cholesterol, LDL and higher Tg compared to ε3ε3 or ε4 allele carriers (for ε2+ v ε3ε3/ε4+ grouped: total cholesterol p=0.01, LDL p<0.001, Tg p=0.004). Across the genotypes (table 4.3), there was a non-significant linear trend for Ox-LDL to be higher in ε4+ subjects (ε4+>ε3ε3>ε2+). With respect to Ox-LDL:LDL, there was no significant linear difference across the genotype groups, although ε2+ had higher levels compared to ε4+ subjects (p=0.02, after adjustment p=0.03), and compared to ε3ε3/ε4+ subjects grouped (p=0.02, p=0.03 after adjustment). No difference was observed in LDL-PPD and LDL-MPD, or plasma TAOS. Plasma CRP was lower in the ε4+ subjects with a significant linear trend across genotype groups (p=0.01).

Table 4.2: Baseline characteristics of subjects by CHD status

Trait	No CHD (n=590)	CHD (n=153)	P Value
Age (years)	60.8 (14.0)	68.7 (10.3)	<0.001
Duration (years)$^{+}$	11 (5-21)	11 (6-18)	0.79
Systolic blood pressure (mmHg)*	149 (19)	138 (22)	0.94
Diastolic blood pressure (mmHg)*	80 (11)	77 (12)	0.001
Body mass index (kg/m^2)*	28.3 (5.6)	29.5 (5.4)	0.02
HbA_{1c} (%)*	7.8 (1.6)	7.6 (1.5)	0.10
LDL-Cholesterol (mmol/l)	2.9 (0.9)	2.5 (0.90)	<0.001
HDL-Cholesterol (mmol/l)*	1.4 (0.5)	1.2 (0.4)	<0.001
Tg (mmol/l)*	1.6 (0.9)	1.9 (1.0)	0.003
CRP (mg/l)*	1.54 (1.33)	1.84 (1.58)	0.02
TAOS (%)	42.7 (13.3) (n=569)	41.2 (13.3) (n=184)	0.27
Ox-LDL (U/l)*	45.8 (18.3) (n=383)	44.2 (19.3) (n=102)	0.43
Ox-LDL:LDL (U/mmol)*	16.8 (7.4) (n=383)	19.0 (9.8) (n=102)	0.002
LDL-MPD (nm)	26.88 (0.85) (n=393)	26.71 (0.78) (n=103)	0.06
LDL-PPD (nm)	26.71 (0.98) (n=393)	26.53 (0.92) (n=103)	0.09
Sex (F/M)	240/350 (40.6/59.4%)	49/104 (32.2/67.8%)	0.06
Never/Ex/Current smokers	300/185/9105 (50.9/31.3/17.8%)	66/68/19 (43.3/44.7/12.0%)	0.007
APOE (ε2+/ε3ε3/ε4+)	74/395/121 (12.5/66.9/20.5%)	21/100/32 (13.7/65.4/20.9%)	0.91
ACEI (No/Yes)	337/253 (57.1/42.9%)	68/85 (44.4/55.6%)	0.005
Aspirin (No/Yes)	369/221 (62.6/37.4%)	39/114 (25.2/74.8%)	<0.001
Insulin (No/Yes)	315/275 (53.4/46.4%)	98/55 (64.1/35.9%)	0.02
Statin (No/Yes)	483/107 (81.9/18.1%)	60/93 (39.5/60.5%)	<0.001

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

Table 4.3: *APOE* genotype, lipids and measures of oxidative stress in all subjects

Trait	ε2+ (n=95)	ε3ε3 (n=495)	ε4+ (n=153)	P
Total Cholesterol (mmol/l)	4.9 (1.0)	5.2 (1.1)	5.2 (1.1)	0.04
LDL (mmol/l)	2.5 (0.8)	2.9 (0.9)	2.9 (1.0)	0.001
TC:HDL*	3.5 (1.0)	3.9 (1.3)	4.0 (1.4)	0.33
Tg (mmol/l)*	2.0 (1.2)	1.6 (0.9)	1.70 (1.1)	0.01
CRP (mg/l)*	1.73 (1.57)	1.66 (1.40)	1.33 (1.22)	0.02
Adjusted CRP (mg/ml)*	2.02 (1.74)	1.98 (1.61)	1.64 (1.41)	0.04
TAOS (%)	43.0 (14.2) (n=90)	42.4 (13.9) (n=476)	42.3 (11.3) (n=151)	0.913
Adjusted TAOS (%)	43.7 (28.1)	42.3 (27.2)	42.2 (21.9)	0.89
Ox-LDL (U/l)*	44.7 (19.4) (n=62)	45.1 (18.2) (n=326)	47.5 (19.0) (n=97)	0.52
Adjusted Ox-LDL (U/l)	45.2 (19.1)	44.8 (17.1)	47.2 (17.9)	0.54
Ox-LDL:LDL (U/mmol)*	19.1 (8.3) (n=62)	16.7 (6.7) (n=326)	17.5 (7.0) (n=97)	0.10
Adjusted Ox-LDL:LDL	19.1 (9.2)	16.9 (7.5)	17.5 (7.7)	0.08
PPD (nm)	26.61 (1.07) (n=65)	26.71 (0.93) (n=329)	26.58 (0.93) (n=102)	0.44
MPD (nm)	26.80 (0.88) (n=65)	26.87 (0.83) (n=329)	26.79 (0.86) (n=102)	0.61

*Log transformed
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data

4.1.4.c Interaction between APOE genotype and smoking

Since cigarette smoking is both pro-inflammatory and pro-oxidant, I further examined the association between *APOE* genotypes and measures of oxidative stress and LDL-oxidation in subjects by smoking status. Since CHD may itself be pro-inflammatory and is associated with increased plasma markers of oxidative stress and LDL-oxidation (as described in chapter 3), the focus of analysis was on those subjects without manifest CHD. As shown in table 4.4, the associations between genotypes with lipid parameters and measures of oxidative stress were similar to that of all subjects (regardless of CHD status). The association of smoking with plasma markers is shown in table 4.5.

In those without CHD, Ox-LDL was significantly higher in ε4+ subjects (ε4+ v ε2+/ε3ε3 grouped, p=0.02, unchanged after adjustment). As shown in figure 4.1, in ε4+ subjects there was a linear increase in plasma Ox-LDL from never smokers to ex-smokers to current smokers (ANOVA p=0.02, linearity p=0.006, after adjustment this remained unchanged). Ox-LDL in ε4+ current smokers was 25% greater than in ε4+ never smokers (p=0.02). No significant difference was observed in Ox-LDL in ε2+ or ε3ε3 subjects by smoking status. No significant interaction was observed between genotype (ε2+, ε3ε3, ε4+) and smoking status (never, ex, current) in determining Ox-LDL, p=0.06 (unchanged after adjustment). In the interaction model, when the ε4+ genotype was compared to ε2+/ε3ε3 grouped together, this interaction was more apparent (p=0.02, p=0.01 after adjustment).

Table 4.4: *APOE* genotype, lipids and measures of oxidative stress in subjects without CHD

Trait	ε2+ (n=74)	ε3ε3 (n=395)	ε4+ (n=121)	P
Total Cholesterol (mmol/l)	5.0 (1.0)	5.2 (1.0)	5.2 (1.1)	0.18
LDL (mmol/l)	2.6 (0.8)	3.0 (0.9)	3.0 (0.9)	0.007
TC:HDL*	3.5 (1.0)	3.7 (1.3)	3.8 (1.4)	0.29
Tg (mmol/l)*	2.0 (1.2)	1.6 (0.9)	1.60 (1.1)	0.005
CRP (mg/l)*	1.60 (1.38)	1.63 (1.40)	1.24 (1.13)	0.009
Adjusted CRP (mg/ml)*	1.90 (1.56)	2.00 (1.62)	1.55 (1.31)	0.01
TAOS (%)	43.7 (15.1) (n=71)	42.5 (13.6) (n=379)	42.7 (10.9) (n=119)	0.80
Adjusted TAOS (%)	45.2 (29.0)	42.6 (27.0)	42.9 (21.0)	0.74
Ox-LDL (U/l)*	45.7 (18.8) (n=50)	44.5 (18.4) (n=255)	50.5 (16.5) (n=78)	0.05
Adjusted Ox-LDL (U/l)	45.8 (18.5)	44.2 (17.4)	50.1 (15.5)	0.05
Ox-LDL:LDL (U/mmol)*	18.7 (8.7) (n=50)	16.1 (7.1) (n=255)	18.0 (7.0) (n=78)	0.03
Adjusted Ox-LDL:LDL	18.7 (8.8)	16.3 (7.2)	18.0 (7.0)	0.04
PPD (nm)	26.65 (1.09) (n=53)	26.76 (0.93) (n=258)	26.59 (1.05) (n=82)	0.36
MPD (nm)	26.83 (0.88) (n=53)	26.93 (0.84) (n=258)	26.77 (0.88) (n=82)	0.31

*Log transformed
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data

Table 4.5: Plasma Markers of oxidation by smoking status in subjects without CHD

	Never	Ex	Current	P[a]	P[b]	Adj P[a]	Adj P[b]
Ox-LDL (U/l)*	44.6 (19.6)	45.9 (15.7)	48.6 (18.7)	0.09	0.14	0.14	0.18
Ox-LDL:LDL (U/mmol)*	15.8 (7.3)	18.0 (6.9)	17.0 (8.0)	0.45	0.20	0.43	0.20
TAOS (%)	43.2 (12.8)	42.9 (13.2)	40.9 (14.6)	0.21	0.20	0.24	0.25

[a]Comparing current to ex and never smokers
[b]Comparing current to never smokers

With respect to Ox-LDL:LDL, ε2+ and ε4+ subjects had similar levels before stratifying by smoking history. As shown in table 4.4, ε3ε3 subjects had the lowest levels. Figure 4.2 shows the result after stratifying by smoking history. In line with the above results for Ox-LDL, there was a linear association by smoking status in ε4+ subjects (ANOVA p=0.07, linear trend p=0.03, after adjustment p=0.02, 0.02 respectively). No difference was observed in ε2+ and ε3ε3 subjects by smoking status. Of interest in never smokers, ε2+ subjects had a significantly higher Ox-LDL:LDL compared to ε3ε3/ε4+ subjects (p=0.04, after adjustment p=0.03). The former also had higher Tg (ε2+ v ε3ε3/ε4+: 1.9 (1.2) v 1.5 (0.9) mmol/l, p=0.02). There was no evidence for an interaction between genotype and smoking status in determining Ox-LDL:LDL, p=0.23 (p=0.15 after adjustment). When the ε4+ genotype was compared to ε2+/ε3ε3 grouped together, this interaction was of borderline significance (p=0.07, p=0.05 after adjustment).

With respect to plasma TAOS (Figure 4.3), ε2+ smokers had a non-significantly higher plasma TAOS compared to ex-smokers and never smokers (p=0.13). There was no overall significant interaction between genotype (ε2+, ε3ε3, ε4+) and smoking status (never, ex, current) in determining TAOS, p=0.39 (unchanged after adjustment).

However, a significant interaction was observed when the model considered ε2+ v grouped ε3ε3/ε4+ and smoking status (current v never and ex-smokers grouped), p=0.02.

Figure 4.1: Plasma Ox-LDL by *APOE* genotype and smoking status

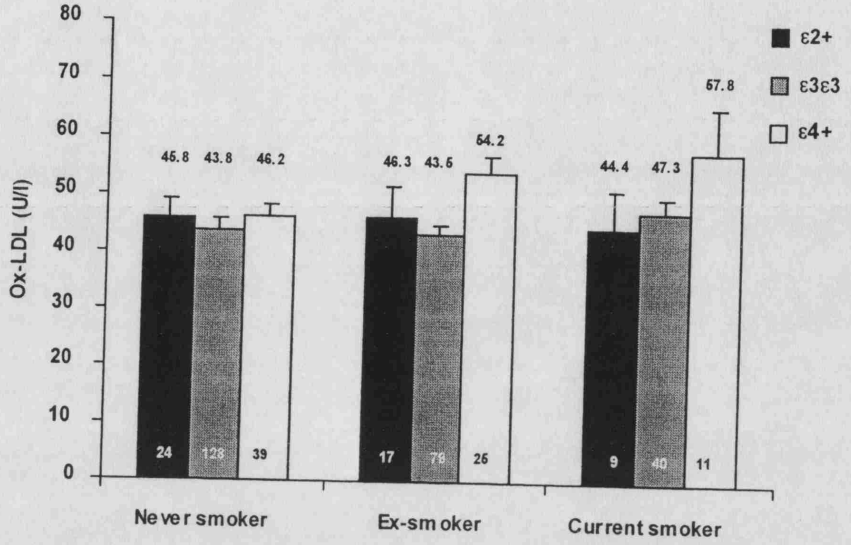


Figure 4.2: Plasma Ox-LDL:LDL by *APOE* genotype and smoking status

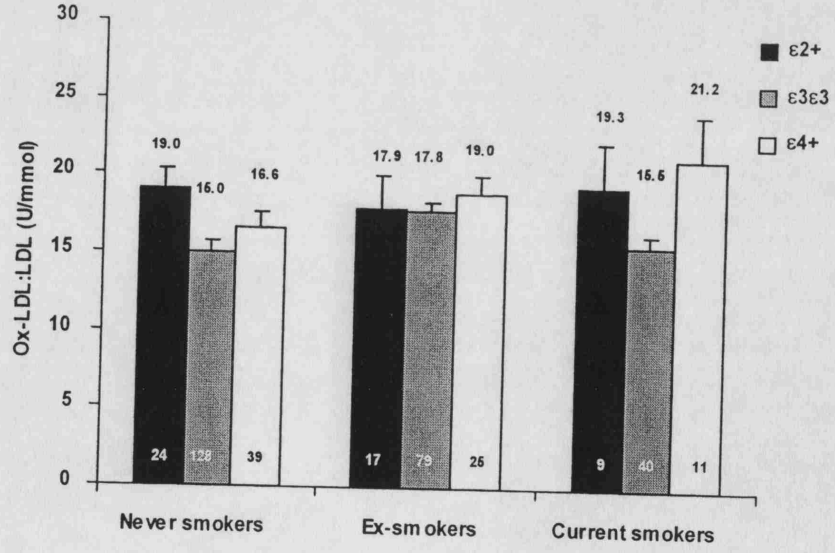
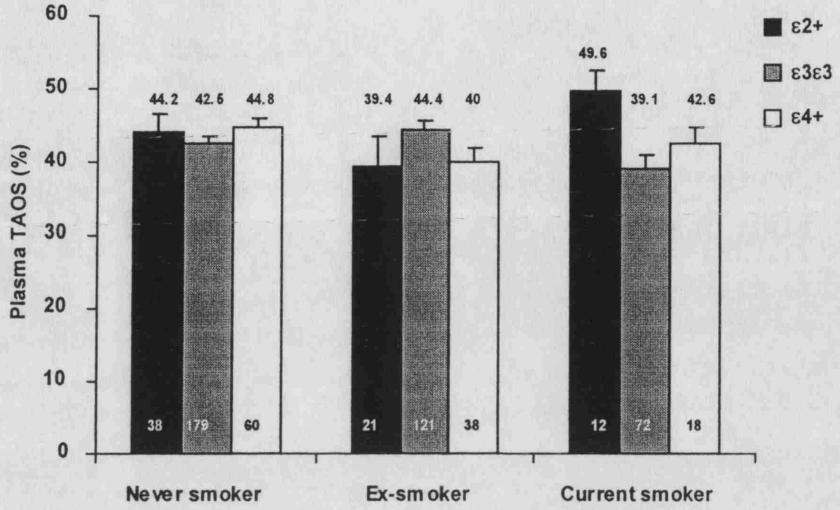


Figure 4.3: Plasma TAOS by *APOE* genotype and smoking status



4.1.4.d Interaction of the APOE genotype, smoking and LDL size

Since sd-LDL is more prone to oxidation, the association between smoking status and *APOE* genotype was also explored by LDL particle size. Since analysis would be performed on a small numbers of subjects, associations were explored in current smokers v ex-/never smokers grouped. Subjects with measurements of LDL-PPD were divided into 2 groups by the median value. Group 1-'small' had a mean LDL-PPD of 25.93nm (range 23.53-26.79) and group 2-'large' a mean LDL-PPD of 27.47nm (range 26.80-29.86). Since LDL-PPD is a more accurate measure of sd-LDL, the analysis focused on LDL-PPD. The results however were similar with respect to LDL-MPD.

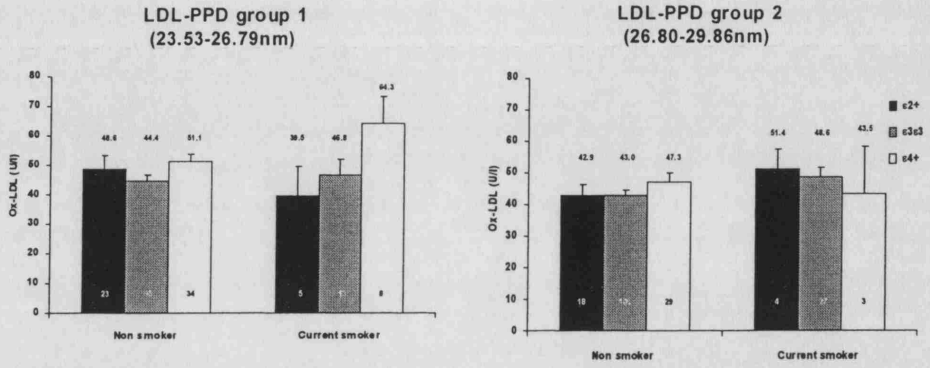
As shown in figure 4.4A, in the 'small' LDL-PPD group, ε4+ current smokers had the highest Ox-LDL levels (in smokers: ε4+ v grouped ε2+/ε3ε3 p=0.04, adjusted p=0.02). In the smokers, there was a significant linear association across genotype (ANOVA

p=0.10, linear trend p=0.04, adjusted ANOVA=0.01, linear trend p=0.01). In the non-smokers, no association was observed across genotype (ANOVA p=0.41) or when ε4+ were compared to ε2+/ε3ε3, (p=0.33). In the 'small' LDL-PPD group there was a significant interaction by genotype (ε2+, ε3ε3, ε4+) and smoking to increase Ox-LDL (p=0.05, adjusted p=0.02). When the ε4+ genotype was compared to ε2+/ε3ε3 grouped, this interaction remained unchanged (p=0.08, p=0.03 after adjustment). In the 'large' LDL-PPD group, no difference was observed in smokers or non-smokers by genotype. No overall interaction was observed across genotype with smoking (p=0.47).

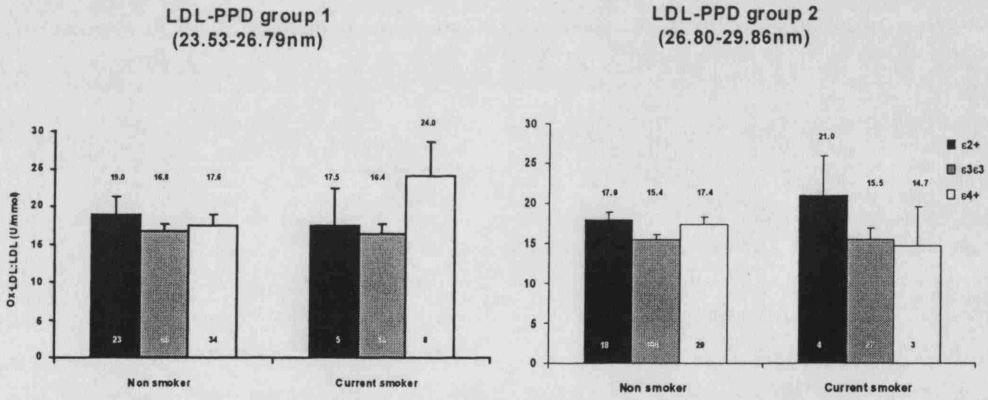
With respect to Ox-LDL:LDL (figure 4.4B), in the 'small' LDL-PPD group, in line with the above observations, ε4+ current smokers had the highest Ox-LDL:LDL ratio (in smokers: ε4+ v grouped ε2+/ε3ε3 p=0.04 for ε2+ v ε3ε3 v ε4+ p=0.09). In the non-smokers, no association was observed across genotype (ANOVA p=0.45) or when E4+ subjects were compared to ε2+/ε3ε3 grouped, p=0.93). In the 'small' LDL-PPD group the interaction between genotype (ε2+, ε3ε3, ε4+) and smoking to increase Ox-LDL:LDL was not significant (p=0.07). When the ε4+ genotype was compared to ε2+/ε3ε3 grouped, this interaction was significant (p=0.03). In the 'large' LDL-PPD group, no difference was observed by genotype in smokers or non-smokers. No interaction was observed across genotype with smoking (p=0.08).

Figure 4.4: Association between *APOE* genotype and smoking by LDL particle size on markers of oxidative stress

A: Ox-LDL



B: Ox-LDL:LDL



4.1.4.e Genotype distribution difference compared to the non-diabetic NPHS sample

Genotype data for the *APOE* gene variants was also available on non-diabetic subjects from the NPHSII study. The genotype distribution between this sample (at relatively low risk for CHD) was compared with the diabetes subjects in UDACS. Of the UDACS (n=743) sample, 12.8% were ε2+, 66.7% were ε3ε3 and 20.5% were ε4+ (table 4.6). In the NPHSII subjects (2229 non diabetic), the distribution was ε2+ 14.3%, ε3ε3 59.8% and ε4+ 25.9%, which was significantly different from that of UDACS (χ^2=11.9, p=0.003). Since the NPHSII sample is all male, we also compared the genotype distribution between NPHSII and men only (n=454) from UDACS, this association remained significant (χ^2=7.3, p=0.03).

Table 4.6: Genotype frequencies in diabetic & non-diabetic subjects, NPHSII v UDACS

	ε2+	ε3ε3	ε4+	P
NPHSII	319 (14.3%)	1333 (59.8%)	577 (25.9%)	
UDACS	95 (12.8%)	495 (66.7%)	153 (20.5%)	0.003
UDACS: Men	62 (13.7%)	300 (66.1%)	92 (20.2%)	0.03

χ^2 test was used to compare the genotype distribution between UDACS and NPHSII.
Comparing NPHSII to all UDACS subjects χ^2=11.9, p=0.003. When the NPHSII genotype distribution was compared to men only from UDACS, the difference remained significant (χ^2=7.3, P=0.03)

4.1.5 Discussion

This work demonstrates an association between the common *APOE* gene variants with lipid fractions, plasma TAOS and parameters of LDL-oxidation. In line with previous studies (Kalina *et al.* 2002), ε2+ subjects had lower plasma total cholesterol, LDL and higher plasma Tg concentration compared to ε3ε3 and ε4+ subjects. The increased Tg in ε2+ subjects can be explained by the fact that receptor binding activity is defective in subjects with apoE2, being between 1-2% of the binding activity of apoE3 and apoE4 (Weisgraber *et al.* 1982). Homozygosity for ε2 predisposes to the development of type III hyperlipoproteinaemia due to delayed metabolic clearance of apoE containing lipoproteins. The result is that there is an accumulation of chylomicron and VLDL remnants in the plasma (Weisgraber *et al.* 1982). This increase in plasma Tg level may also account for the increased CRP observed in these subjects, as Tg may be proinflammatory (in UDACS: CRP and Tg were correlated $r=0.20$ $p<0.001$).

Before stratifying by smoking status, in those free from CHD, Ox-LDL was significantly higher in ε4+ subjects compared to ε2+/ε3ε3. This persisted after adjustment for plasma LDL and Tg. Of note, LDL was also higher in these subjects and hence much of the Ox-LDL may be a reflection of the higher LDL level. With respect to Ox-LDL:LDL, these levels were similar in the ε2+ and ε4+ subjects, which were higher than ε3ε3. This remained unchanged after adjusting for LDL-PPD. For absolute Ox-LDL, ε4+ subjects showed a linear increase from never to ex and to current smokers, with ε4+ current smokers having Ox-LDL approximately 25% higher than never smokers. No difference was observed in the ε2+ or ε3ε3 subjects by smoking status. There was a significant

interaction between the ε4 allele and smoking to increase Ox-LDL. With respect to Ox-LDL:LDL, the same effect was observed between ε4+ subjects and smoking, such that ε4+ smokers had levels approximately 28% higher than ε4+ never smokers. Again a significant interaction was observed between the ε4 allele and smoking to increase Ox-LDL:LDL. These results therefore suggest that in ε4+ subjects, there is an increased susceptibility of LDL-oxidation.

Of interest, in the never smokers, ε2+ subjects had higher Ox-LDL:LDL than the ε3ε3/ε4 subjects. One possible explanation for this would be that these subjects have higher Tg compared to the other genotypes. The result would be increased formation of sd-LDL, which would be more prone to oxidation. However after adjustment for LDL-PPD, the result remained unchanged. Alternatively, it may that in the presence of increased plasma LDL levels (associated with the ε4 allele) and smoking the overall effect of LDL oxidation is increased because of the higher baseline LDL levels. This suggests that in an unstressed environment (smoking absence), that ε2+ may be associated with increased Ox-LDL:LDL, but with smoking there is no increased susceptibility to oxidation. In the ε4+ subjects, there is increased susceptibility to oxidation with smoking, so that not only does absolute Ox-LDL increase but also Ox-LDL:LDL. This is relevant, since in the NPHSII study, risk was greatest in ε4+ smokers, however in the ε4+ ex-smokers, risk was considerably lower (by more than three fold), than for ε3ε3 ex-smokers (NPHSII OR: ε4+ smokers 3.17, ε3ε3 smokers 1.38, ε2+ smokers 1.18, ε4+ ex-smoker 0.84, ε3ε3 ex-smokers 1.74, ε2+ ex-smokers 0.48; all compared to never smokers).

Further indirect support for the increased susceptibility of the ε4 allele to oxidation being associated with LDL levels, comes from the 4S study. Subjects with the ε4+ had a 1.8-fold higher CHD risk, however these subjects also showed the greatest benefit from statin treatment (Gerdes *et al.* 2000). Therefore, the increased susceptibility to LDL oxidation and its effect on risk may be associated with the higher LDL levels seen in the ε4+ subjects, however in never smokers (an unstimulated state), the ε2+ allele is associated with higher Ox-LDL per LDL, since they have lower LDL and higher Tg compared to ε4+ subjects.

Further analysis following stratification by LDL-size, showed that the effect of ε4+ to increase Ox-LDL and Ox-LDL:LDL in smokers, was more so in those with smaller LDL particle size. The numbers in this analysis are small, and the results need to be interpreted with caution. This observation suggests that in the presence of a genotype associated with higher LDL, smokers with smaller LDL size have an increased susceptibility to oxidation. This observation is relevant, as *in vitro* studies have demonstrated that sd-LDL is more prone to oxidation by free radicals (such as those in cigarette smoke) resulting in Ox-LDL (Ayaori *et al.* 1997; Makimattila *et al.* 1999). Moreover, sd-LDL is associated with higher levels of IgM antibodies against malondialdehyde-modified LDL than larger LDL (Jansen *et al.* 1995). This is in line with current thinking proposing that sd-LDL is more susceptible to oxidation.

With respect to plasma TAOS, ε2+ subjects had increased levels following exposure to cigarette smoke, suggesting that ε2+ has an antioxidant role in the face of a stimulus.

Therefore, in the face of a pro-oxidant stimulus, apoE may have a general plasma antioxidant role which is unrelated to lipid effects. Different isoforms of apoE possess different antioxidant activity. Studies have reported differing effects on susceptibility to oxidation in plasma, as well as specifically in relation to LDL. *In vitro*, the antioxidant efficacy of apoE is apoE2>apoE3>apoE4 (Miyata *et al.* 1996; Jolivalt *et al.* 2000). These studies showed allele-specific protection from hydrogen peroxide cytotoxicity. Furthermore, serum lipid peroxides are higher in ε4+ subjects (Smith *et al.* 1998), and in subjects with Alzheimer's disease, products of lipid peroxidation in plasma and urine are lower in ε2+ compared to ε4+ (Fernandes *et al.* 1999). The antioxidant effect associated with the ε2 allele may be related to the fact that apoE2 has 2 free –SH groups, apoE3 1 – SH group and apoE4 none. Moreover specific effects have also been observed with respect to LDL-oxidation, which may be related to unidentified effects of apoE isoforms on the physico-chemical properties of lipoproteins that promote or protect from oxidation. ε4+ subjects have the lowest plasma apoE levels. Compared to ε3ε3 subjects, macrophages from ε4ε4 subjects have less efficient net cholesterol efflux, resulting in greater accumulation of LDL, and subsequent foam cell formation. Moreover, plasma lipoproteins from apoE-deficient mice are more prone to *in vitro* oxidation than lipoproteins from wild-type mice (Hayek *et al.* 1994). This effect may also be reversed with supplementation with vitamin E (Palinski *et al.* 1994). Smoking increases the rate of oxidation of lipoprotein particles, and therefore as demonstrated in this chapter, ε4+ subjects who smoke have a higher propensity to LDL oxidation, and subsequently an increased risk of atherosclerosis than ε4+ who do not smoke or have those with other genotypes, as demonstrated in the NPHSII.

When the genotype distribution for the *APOE* gene variants was compared between the diabetic subjects and the healthy subjects from NPHSII, there was significantly lower percentage of subjects with the ε4+ genotype in the diabetic subjects (even when men only subjects were included). One possible explanation for this could be that that in combination, the cardiovascular risk associated with this genotype and diabetes results in reduced life expectancy and hence the lower observed frequency. Although these two samples may partly differ in their environmental background there are close similarities. Both samples are of Caucasian origin and recruited in the UK. The UDACS sample was recruited from an inner London teaching hospital, where there is considerable migration from other parts of the UK. The NPHSII cohort was recruited from 9 general medical practices throughout the UK. Within the NPHSII sample there was no evidence of genetic heterogeneity for the common *APOE* gene variant between subjects recruited from London and outside London (p=0.09).

In summary, this section of work has shown a genotype dependent effect of *APOE* on both a plasma marker of oxidation in the circulation and specifically in relation to LDL-oxidation. ε4 is associated with increased LDL-oxidation, whilst ε2 is associated with a higher plasma TAOS. These effects may be related to both lipid-specific effects (apoE4) and direct plasma antioxidant effects (apoE2). *APOE* is a key lipid-regulating gene, however as described, it is also associated with and contributes to oxidative stress and these are influenced by an environmental pro-oxidant state of smoking.

4.2 Novel mutations in the *APOE* gene identified during genotyping

4.2.1 Introduction

In this section, three unusual heteroduplex patterns observed during *APOE* genotyping of the subjects in UDACS were further explored.

4.2.2 Aim

To further genotypically characterise the aberrant DNA heteroduplex pattern observed in 3 subjects within UDACS. These subjects were further characterised biochemically and phenotypically to understand and to explore any changes associated with the aberrant heteroduplex patterns.

4.2.3 Methods

This analysis was performed on 3 subjects from UDACS with aberrant heteroduoplex patterns. Two of these were Caucasian and 1 Afro-Caribbean. Further genetic analysis was performed by sequencing (chapter 2), and phenotypic characterisation by apoE isoelectric focusing (IEF) and the measurement of plasma apoE levels (chapter 2).

4.2.4 Results

4.2.4.a APOE genotyping & sequencing of mutations

Three new variant heteroduplex patterns were identified in the 964 (all race) subjects. All were identified in males with T2DM. The expected heteroduplex patterns for the *APOE* gene variants are shown in Chapter 2. All three new variants, were seen as the presence of an additional band on heteroduplex gel electrophoresis, as shown in figure 4.5. This

pattern was confirmed on repeat PCR and heteroduplex genotyping. Subject 1 was genotyped as ε2ε2 + 1 extra band, subject 2 as ε3ε3 + 1 extra retarded band and subject 3 as ε3ε3 + 2 extra bands. In view of this, the three samples were sequenced. Three novel mutations were identified and are summarised in figure 4.6 and table 4.7. Subject 1 and 3 had single base changes that were predicted to alter amino acids Arg150His and Arg114Pro respectively, however subject 2 had a frame-shift mutation predicted to result in premature termination of the gene product, and hence a change in plasma apoE concentration. These mutations were heterozygous in all three subjects.

Figure 4.5: Heteroduplex patterns of *APOE* mutations identified

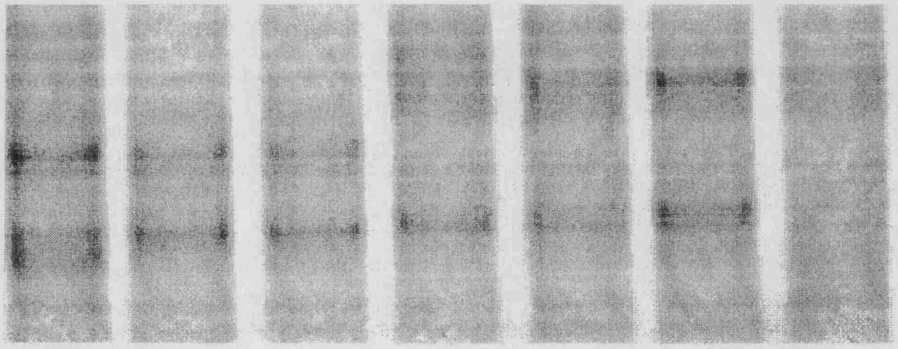
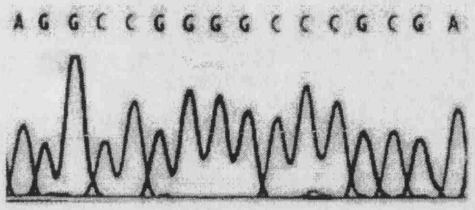
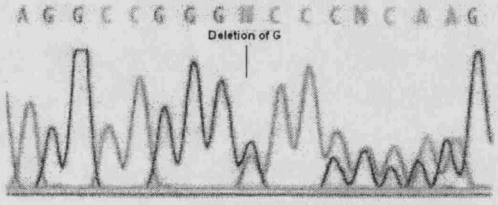


Figure 4.6: Sequencing of ApoE mutations

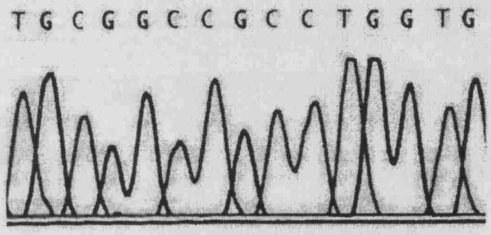
Normal sequence



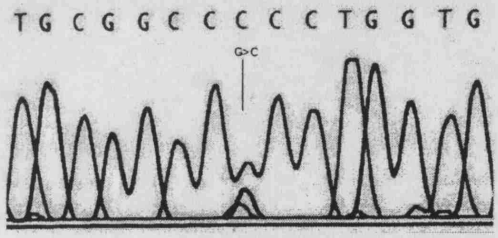
166 DEL G
Frame-shift mutation



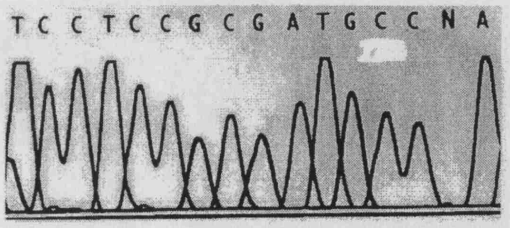
Normal sequence



CGC>CCC at position 395
Arg 114 Pro



Normal sequence



CGC>CAC at position 503
Arg 150 His

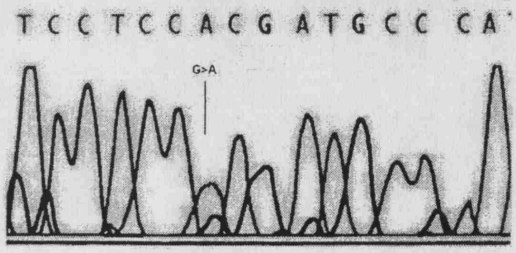


Table 4.7: ApoE genotypes and phenotypes in subjects

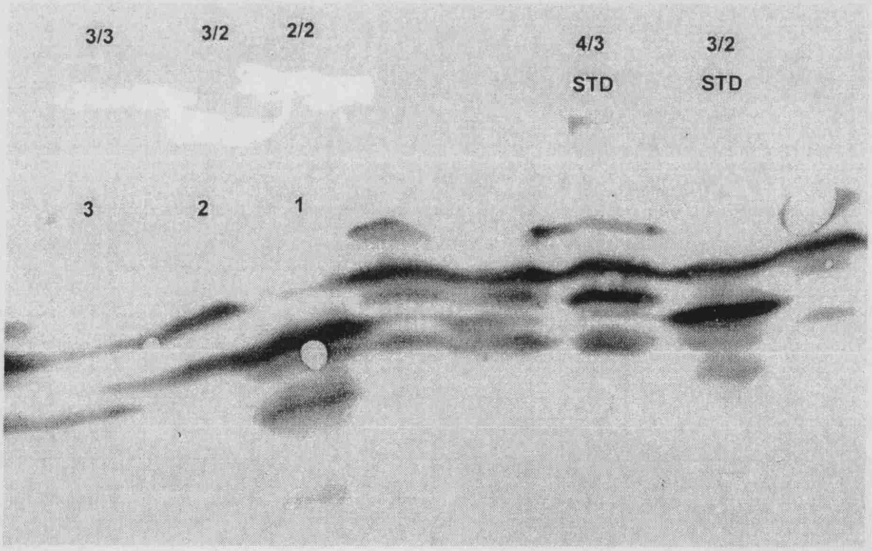
Subject	1	2	3
Predicted *APOE* variant by Heteroduplex pattern	ε2ε2+ 1 Extra band	ε3ε3+ 1 Extra retarded band	ε3ε3+ 2 Extra band
Predicted ApoE genotype by sequencing	ε2ε2	ε3ε3	ε3ε3
Base change*	503 G>A	550-G	395 G>C
Amino acid change	Arg150His	Premature termination after 166	Arg114Pro
Predicted ApoE variant by IEF	E2E2	E2E3	E3E3
ApoE conc (mg/l)	72.6	19.8	21.7
Reported ApoE conc (mg/l) (Smit *et al.* 1988)	138.0 ± 20.0	44.0 ± 20.0	44.0 ± 20.0

*Bases were numbered from the sequence where A of the initiator methionine is +1

4.2.4.b Isoelectric focusing

To study the phenotypes of these mutations further, IEF was performed, the results of which are shown in table 4.7 (figure 4.7). In subjects 1 and 3 the *APOE* genotype as determined by heteroduplex analysis (ε2ε2, ε3ε3 respectively) corresponded to that determined by isoelectric focusing. In subject 2, the IEF phenotype was E2E3 with a genotype of ε3ε3. Interestingly in this subject, none of the smaller protein fragments were observed which usually appear below the main bands. When the genotype was determined by sequencing, subject 1 was an ε2ε2, subject 2 and ε3ε3, and subject 3 an ε3ε3.

Figure 4.7: Isoelectric focusing gel for the subjects with the newly identified mutations



4.2.4.c Plasma ApoE concentration

As shown in table 4.7, subject 2 with the frame-shift mutation (166 Del G) had a plasma apoE concentration of 19.8mg/l. The respective plasma apoE concentrations for subject 1 (Arg150His) and subject 3 (Arg114Pro) were 72.6mg/l and 21.7mg/l respectively. As shown in table 4.7, the concentration of apoE in the plasma has been shown previously to range from 50-70mg/l (Smit *et al.* 1988) and by genotype to range from 138 (± 38)mg/l in ε2ε2, 55 (± 20)mg/l in ε3ε3 to 44 (± 20)mg/l in ε4ε4 subjects.

4.2.4.d Other clinical associations

Other routine clinic measures for the 3 subjects are shown in table 4.8. Subject 1 (Arg150His) had a total plasma cholesterol of 3.0mmol/l on the first visit and currently has a total cholesterol of 5.0mmol/l, LDL of 2.3mmol/l and Tg of 1.4mmol/l. Subject 2, (frame-shift mutation) is 56 years of age, and to date has not had a clinically detectable cardiovascular event. There was no apparent family history of CHD events. This subject had the highest current plasma Tg concentration of the three subjects (1.8mmol/l), but this would not be considered elevated at a population level. The baseline clinical measures before treatment showed subject 2 to have a total cholesterol of 4.5mmol/l and a high HDL of 1.5mmol/l. Subject 3 (Arg114Pro) had the highest baseline plasma total cholesterol concentration of 6.8mmol/l. Interestingly following treatment with a relatively low dose of simvastatin (10mg/day), his recent cholesterol was 2.0mmol/l (with an LDL of 0.6mmol/l and Tg of 0.8mmol/l).

Table 4.8: Clinical & biochemical measures in the novel *APOE* mutations

Subject	1(Arg150His)	2 (166 –G)	3 (Arg114Pro)
Current clinical & biochemical measures			
Age (years)	64.7	56.3	70.4
Duration DM (years)	16	9	18
Ethnic origin	Afro	Cauc	Cauc
Family history of CHD	No	No	Yes
CHD	No	No	Yes
Statin therapy	No	No	Simvastatin 10mg/day
Diabetes therapy	Insulin	Metformin	Insulin and Metformin
BMI (Kg/m^2)	29.4	28.5	31.6
Glucose (mmol/l)	16.8	9.6	8.6
HbA1c (%)	9.4	7.2	6.6
Cholesterol (mmol/l)	5.0	4.2	2.0
LDL (mmol/l)	2.9	2.3	0.6
HDL (mmol/l)	1.5	1.1	1.0
Tg (mmol/l)	1.4	1.8	0.8
Creatinine (mmol/l)	106	110	113
Proteinuria	N	N	Y
CRP (mg/l)	10.29	1.84	4.52
Baseline measures before therapy (first clinic measurements)			
Cholesterol (mmol/l)	3.0	4.5	6.8
HDL (mmol/l)	1.1	1.5	1.1

4.2.5 Discussion

This section reports the identification of three novel mutations in the *APOE* gene in a sample of 964 subjects (743 with T2DM), one of which is a frame-shift mutation resulting in approximately 70% lower plasma apoE concentration compared to normal. In this subject, there was a discrepancy between apoE phenotype as determined by IEF and genotype as determined by heteroduplex and sequencing.

ApoE contains two important structural domains: an amino-terminal (residues 1-164) and a carboxyl-terminal domain (residues 201-299). The amino-terminal has been extensively studied and this contains the receptor-binding domain of apoE. This domain encompasses residues 130-150 and contains nine positively charged amino acids, six of which face outwards. These positively charged residues interact with the negative charges present in the ligand domain of the LDL and LDL-receptor-related protein (LRP) receptors (Mahley 1988) and hence facilitate the uptake and removal of apoE containing lipoproteins (VLDL and IDL remnants) from the circulation. The carboxyl-terminal of apoE has a strong α-helical character containing a heparin binding domain (De Loof *et al.* 1986). This domain plays an important role in the interaction of apoE with proteoglycans on the arterial wall, anchoring the lipoprotein particle to the endothelium, thus allowing the enzyme lipoprotein lipase (LpL) to hydrolyse triglycerides to free fatty acids and glycerol. The frame-shift mutation seen in subject 2, despite reducing plasma apoE concentration to a low level, did not appear to alter the total cholesterol or LDL markedly. This deletion is outside the receptor binding domain for LDL and therefore, even though we would expect apoE levels to be reduced due to premature termination of

protein synthesis, we *might* expect receptor binding activity to be within normal limits. This may explain the relatively normal total cholesterol and LDL levels in this subject. Interestingly, this subject did however have the highest Tg concentration of the three subjects, but this would not be considered elevated at a population level. Since this mutation would result in premature termination of apoE synthesis, and loss of the carboxyl-terminal domain, there may be loss of interaction between proteoglycans and apoE on the endothelium and hence subsequently reduced LpL hydrolysis of plasma Tg. With such a mutation one might expect this subject to be at higher cardiovascular risk, but at 56 years of age, has had no such clinically manifest events.

The Arg150His amino acid change observed in subject 1 would not be expected to result in a significant change in amino acid charge (both Arg and His being hydrophilic positively charged polar molecules) in the amino-terminal. This mutation would therefore not be expected to alter the IEF of the protein, which was E2E2, the same as that determined by heteroduplex and sequencing. In addition, receptor binding and hence plasma cholesterol, LDL and Tg levels would be expected to be unchanged. The plasma cholesterol level at the first clinic visit in this subject was 3.0mmol/l, (currently cholesterol 5mmol/l, LDL 2.3mmol/l and triglycerides 1.4mmol/l).

The Arg114Pro amino acid substitution would result in a change from a hydrophilic positively charged, polar amino acid on the amino-terminal domain (residues 1-164) to a hydrophobic non-polar amino acid. This might then affect receptor binding. This subject had the highest plasma total cholesterol level (at first visit 6.8mmol/l). Interestingly,

following treatment with a relatively low dose of a statin (simvastatin 10mg/day), his cholesterol dropped markedly to 2.0mmol/l (LDL 0.6mmol/l, Tg 0.8mmol/l). This subject (now 70 years of age) has clinical evidence of CHD, and is also an ex-smoker with a family history of CHD in a first degree relative.

The pattern of apoE isoforms observed on IEF gels are due to variation in charge as a result of the amino acid sequence encoded at the *APOE* gene locus. Non-enzymatic post-translational modification may also alter isoform mobility (Zannis *et al.* 1981). With respect to apoE the usual degree of migration towards the anode is E2 (Cys112/Cys158)> E3 (Cys112/Arg158)> E4 (Arg112/Arg158), as Arg has a positive charge and Cys is uncharged. This pattern of migration is therefore dependent on the charge of the amino acid at positions 112 and 158. With respect to the frame-shift mutation, the apoE phenotype as determined by IEF was E2E3. This subject would have had an abnormal protein after position 166 due to the frame shift mutation (analysis of the sequence after this point revealed the presence of a premature stop codon at position 232). Due to the heterozygous nature of this mutation, it is therefore not surprising that one of the alleles as determined by IEF, is the same as that determined by heteroduplex analysis and sequencing (ε3). The other allele as determined by IEF was that of an ε2 allele. This may represent the mutant allele, since if transcription was interrupted resulting in an abnormal and smaller protein then this would migrate further on the IEF gel and may run to a similar position to E2.

With respect to the Arg150His change, the heteroduplex pattern (ε2ε2) was similar to the IEF phenotype (E2E2). This mutation would not result in a change in amino acid charge, nor size. Therefore, as expected, the *APOE* genotype as determined by IEF and heteroduplex were the same.

In the subject with the Arg114Pro mutation, the heteroduplex pattern was of an ε3ε3 individual (with 2 extra bands) and the IEF of an E3E3 subject. The Arg114Pro change would result in a loss of a charge, however this did not appear to alter the IEF significantly.

Previously, a discrepancy between apoE phenotype as determined by IEF and genotype has been described in subjects with diabetes (Snowden *et al.* 1991). One possible explanation for this, is that in diabetic subjects, sialic acid derivatives present in the plasma result in post-translational modification and non-enzymatic glycosylation of apoE (Curtiss *et al.* 1985; Eto *et al.* 1986; Black *et al.* 1990). Even though this may occur, in our current study the expected IEF phenotypes in the samples appear to be identified by the described mutations.

In summary, three new mutations were identified in 964 subjects (743 with type 2 diabetes) screened for the common *APOE* gene variants. LDL-PPD, and Ox-LDL levels were not available on these subjects. Family studies would be desirable in these subjects, but no relatives are available. Each of these three mutations are of course rare and we would not advocate their routine screening when genotyping for the common *APOE* gene

variants. The frequency of newly identified mutations in our sample of subjects with T2DM is higher than detected in healthy subjects using the same heteroduplex method (from the Whitehall-II Study of UK subjects, the frequency of new mutations was 1 in 7900 subjects (SE Humphries and E Brunner-unpublished observation). Dyslipidaemia is common in subjects with diabetes (particularly T2DM/metabolic syndrome) and further screening for common *APOE* variants in subjects at risk of dyslipidaemia, such as those with the metabolic syndrome, T2DM and a family history of premature CHD, may reveal abnormal heteroduplex patterns and uncover further mutations in this important lipid-regulating gene.

CHAPTER FIVE

VARIATION IN THE HUMAN GLUTATHIONE S-TRANSFERASE GENE AND ASSOCIATIONS WITH MARKERS OF OXIDATIVE STRESS

CONTENTS OF CHAPTER FIVE

5.1 Introduction

As described in Chapter 1, Glutathione S-transferases (GSTs) play a major role in cellular antioxidant defense mechanisms, by catalysing the reduction of potentially harmful peroxides (Berliner *et al.* 1995). GSTs are a family of enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (Mannervik *et al.* 1988; Ketterer *et al.* 1992). These enzymes catalyse the interaction of glutathione with a wide variety of organic compounds including potential atherogenic substances to form thioesters, a reaction that may be the first step in the detoxification process (Rebbeck 1997).

Polymorphic deletion variants in the *GSTM1* and *GSTT1* genes produce either a functional protein (non-deletion alleles or heterozygous deletion, *GSTM1-1* and *GSTT1-1*) or result in the complete absence of the protein (homozygous deletion alleles, *GSTM1-0* and *GSTT1-0*) (Pemble *et al.* 1994). Considerable interest has focused on the association of these gene variants in relation to tobacco-related cancers (Rebbeck 1997), since *GSTM1* and *GSTT1* enzymes detoxify not only products of oxidative stress but also carcinogenic compounds such as polycyclic aromatic hydrocarbons, a major constituent of tobacco smoke (Rebbeck 1997). Several epidemiological studies have suggested that *GSTM1-0* and *GSTT1-0* are associated with increased risk of smoking-related cancers including lung, bladder, ovarian and colorectal cancers (Bell *et al.* 1993; Chenevix-Trench *et al.* 1995; McWilliams *et al.* 1995; Brockmoller *et al.* 1996; Coughlin *et al.* 2002). In contrast to these observations, their role in CHD risk and the pathophysiology of atherosclerosis, particularly in relation to exposure to cigarette smoke, remains unclear

(Li *et al.* 2000; Wilson *et al.* 2000; de Waart *et al.* 2001; Li *et al.* 2001; Wang *et al.* 2002; Masetti *et al.* 2003; Olshan *et al.* 2003).

5.2 Aims

The purpose of the work described in this chapter was twofold. Firstly, to examine the association between the gene variants of *GSTM1* and *GSTT1* with markers of oxidative stress and inflammation in patients with diabetes mellitus, a condition associated with premature atherosclerosis, increased oxidative stress and inflammatory burden (Brownlee 2001), and secondly to examine the association and potential interaction between these common gene variants and cigarette smoking in relation to CHD risk.

5.3 Methods

This work was performed on those subjects recruited into UDACS. Genotyping was performed as described in chapter 2. Analysis focused on the association of genotype with the measures of oxidative stress and LDL-oxidation, already described in chapter 3. In the analysis, raw and adjusted values for markers of oxidative stress are shown (as described in chapter 3: TAOS adjusted for Tg and glucose, Ox-LDL for Tg and LDL, Ox-LDL:LDL for LDL-PPD).

The genotype distribution for non-diabetic subjects from NPHSII was also made available for analysis of the results described in section 5.4.e.

5.4 Results

5.4.a GST genotype distribution and clinical data

Seven hundred and seventy three (97%) of the 799 Caucasian subjects were successfully genotyped for the *GST* variants in UDACS. The baseline characteristics of the subjects (by CHD status) for whom genotyping was successful are summarised in table 5.1. Of the 773 diabetic Caucasian subjects, CHD was present in 157 individuals (20.3%). There was no significant difference in GST genotype distribution between those with and without CHD (*GSTM1* p=0.43, *GSTT1* p=0.74).

Table 5.1: Baseline differences in subjects by CHD status in UDACS

Trait	No CHD (n=616)	CHD (n=157)	P Value
Age (years)	59.1 (14.0)	68.0 (10.3)	<0.001
Duration (years)$^{+}$	11 (5-21)	11 (6-18)	0.89
Systolic blood pressure (mmHg)*	139 (20)	138 (22	0.93
Diastolic blood pressure (mmHg)*	80 (11)	77 (12)	0.002
Body mass index (kg/m^2)*	28.3 (5.5)	29.5 (5.4)	0.02
HbA_{1c} (%)*	7.8 (1.6)	7.6 (1.5)	0.14
LDL-Cholesterol (mmol/l)	2.90 (0.93)	2.4 (0.88)	<0.001
HDL-Cholesterol (mmol/l)*	1.4 (0.4)	1.2 (0.4)	<0.001
Tg (mmol/l)*	1.6 (1.0)	1.9 (1.0)	0.006
CRP (mg/l)*	1.53 (1.32)	1.81 (1.57)	0.03
TAOS (%)	42.57 (13.1) (n=595)	41.4 (13.1) (n=152)	0.28
Ox-LDL (U/l)*	45.8 18.2) (n=396)	43.9 (20.1) (n=106)	0.36
Ox-LDL:LDL (U/mmol)*	16.8 (7.4) (n=396)	19.0 (10.0) (n=106)	0.002
LDL-MPD (nm)	26.90 (0.86) (n=405)	26.70 (0.789) (n=106)	0.04
LDL-PPD (nm)	26.72 (1.00) (n=405)	26.53 (0.92) (n=106)	0.07
Sex (F/M)	251/365 (40.7%/59.3%)	50/107 (32.1/67.9%)	0.05
Never/Ex/Current smokers	317/190/109 (51.5/30.8/17.7%)	67/71/19 (42.9/45.5/11.7%)	0.002
GSTM1 (M1-0/M1-1)	308/308 (50.0/50.0%)	73/84 (46.5/53.5%)	0.43
GSTT1 (T1-0/T1-1)	125/491 (20.3/79.7%)	30/127 (19.1/80.9%)	0.74
ACEI (No/Yes)	355/261 (57.7/42.3%)	69/88 (43.9/56.1%)	0.002
Aspirin (No/Yes)	383/233 (62.2/37.8%)	40/117 (25.2/74.8%)	<0.001
Insulin (No/Yes)	334/282 (54.2/45.8%)	101/56 (64.3/35.7%)	0.02
Statin (No/Yes)	505111 (82.0/18.0%)	62/95 (39.7/60.3%)	<0.001

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

5.4.b GST genotype, lipids & measures of oxidative stress

As shown in Table 5.2, the *GSTT1-1* compared to the *GSTT1-0* genotype was associated with significantly higher plasma CRP (p=0.003). This association remained significant after adjustment for age, BMI and glucose (p=0.02). The *GSTT1-1* genotype was also associated with significantly higher Ox-LDL compared to the *GSTT1-0* genotype (p=0.008). Again this association remained significant after adjustment for LDL and Tg (p=0.02). Consistent with these results, Ox-LDL:LDL was also higher in *GSTT1-1* compared to *GSTT1-0* (p=0.05, after adjustment for LDL-PPD this was no longer significant p=0.21). Furthermore, *GSTT1-1* subjects had more small dense LDL than *GSTT1-0* subjects, with a lower LDL-MPD (p=0.01) and LDL-PPD (p=0.01). No difference was seen in plasma TAOS by genotype.

With respect to *GSTM1*, there was a non-significant trend for plasma TAOS to be lower in those with the *GSTM1-0* genotype compared to the *GSTM1-1* genotype (P=0.08). No significant difference was observed by *GSTM1* genotype with CRP, Ox-LDL, LDL-MPD and LDL-PPD (table 5.2).

Table 5.2: Glutathione-S-Transferase genotype by measures of oxidative stress & inflammation in all subjects

Trait	*GSTT1-0* (n=155)	*GSTT1-1* (n=618)	P	*GSTM1-0* (n=381)	*GSTM1-1* (n=392)	P
Total Cholesterol (mmol/l)	5.0 (1.1)	5.1 (1.1)	0.11	5.0 (1.1)	5.0 (1.1)	0.33
LDL (mmol/l)	2.7 (.0)	2.8 (0.9)	0.28	2.8 (1.0)	2.8 (0.9)	0.76
TC:HDL*	3.5 (1.1)	3.7 (1.3)	0.01	3.7 (1.2)	3.7 (1.2)	0.67
Tg (mmol/l)*	1.5 (0.9)	1.7 (1.0)	0.03	1.7 (1.0)	1.7 (1.0)	0.45
CRP (mg/l)*	1.31 (1.12)	1.66 (1.44)	0.003	1.62 (1.48)	1.55 (1.28)	0.49
Adjusted CRP (mg/ml)*	1.64 (1.29)	1.97 (1.65)	0.02	1.93 (1.66)	1.87 (1.49)	0.59
TAOS (%)	42.6 (13.8) (n=148)	42.4 (12.9) (n=599)	0.78	41.6 (13.9) (n=370)	43.2 (12.7) (n=377)	0.08
Adjusted TAOS (%)	42.4 (27.6)	42.5 (25.6)	0.97	40.7 (26.6)	44.2 (25.3)	0.07
Ox-LDL (U/l)*	41.2 (17.3) (n=101)	46.5 (18.9) (n=400)	0.008	44.6 (20.9) (n=261)	46.2 15.5) (n=240)	0.35
Adjusted Ox-LDL (U/l)*	42.3 (16.8)	46.5 (17.8)	0.02	44.6 (19.6)	46.8 (14.7)	0.11
Ox-LDL:LDL (U/mmol)*	16.0 (7.5) (n=101)	17.6 (8.1) (n=400)	0.05	16.8 (8.5) (n=261)	17.7 (7.3) (n=240)	0.69
Adjusted Ox-LDL:LDL (U/mmol)*	16.5 (7.6)	17.6 (7.9)	0.14	17.0 (8.3)	17.8 (7.2)	0.61
MPD (nm)	27.04 (0.88) (n=105)	26.81 (0.83) (n=409)	0.01	26.85 (0.88) (n=266)	26.86 (0.81) (n=248)	0.86
PPD (nm)	26.90 (0.93) (n=105)	26.63 (0.98) (n=409)	0.01	26.63 (0.98) (n=266)	26.75 (0.93) (n=248)	0.18

*Log transformed, +Square root transformed for analysis. Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data Median and interquartile range shown for +duration of diabetes

Table 5.3: Glutathione-S-Transferase genotype by measures of oxidative stress & inflammation in subjects without CHD

Trait	*GSTT1-0* (n=125)	*GSTT1-1* (n=491)	P	*GSTM1-0* (n=308)	*GSTM1-1* (n=308)	P
Total Cholesterol (mmol/l)	5.1 (1.1)	5.2 (1.0)	0.15	5.2 (1.0)	5.2 (1.1)	0.52
LDL (mmol/l)	2.8 (0.9)	2.9 (0.9)	0.39	2.9 (1.3)	2.9 (0.9)	0.88
TC:HDL*	3.5 (1.1)	3.7 (1.3)	0.03	3.7 (1.2)	3.7 (1.3)	0.76
Tg (mmol/l)*	1.5 (0.90)	1.7 (1.1)	0.04	1.6 (1.0)	1.6 (1.0)	0.99
CRP (mg/l)*	1.27 (1.11)	1.50 (1.37)	0.008	1.58 (1.46)	1.49 (1.19)	0.39
Adjusted CRP (mg/ml)*	1.63 (1.30)	1.94 (1.66)	0.04	1.92 (1.67)	1.83 (1.39)	0.49
TAOS (%)	43.1 (14.3) (n=118)	42.6 (12.8) (n=477)	0.80	41.8 (13.3) (n=300)	43.6 (12.9) (n=295)	0.09
Adjusted TAOS (%)	43.2 (28.7)	42.8 (25.2)	0.89	41.1 (26.2)	44.7 (25.5)	0.08
Ox-LDL (U/l)*	40.3 (17.5) (n=82)	47.3 (18.0) (n=314)	0.001	44.9 (20.6) (n=208)	46.8 (14.7) (n=188)	0.32
Adjusted Ox-LDL (U/l) *	41.1 (17.1)	47.2 (17.1)	0.004	44.9 (19.8)	46.9 (13.6)	0.88
Ox-LDL:LDL (U/mmol)*	15.3 (7.2) (n=82)	17.2 (7.4) (n=314)	0.03	16.4 (8.0) (n=208)	17.2 (6.5) (n=188)	0.92
Adjusted Ox-LDL:LDL (U/mmol)*	15.9 (7.5)	17.4 (7.4)	0.10	16.7 (8.1)	17.5 (6.6)	0.77
MPD (nm)	27.11 (0.90) (n=84)	26.83 (0.84) (n=321)	0.008	26.90 (0.89) (n=209)	26.89 (0.82) (n=196)	0.90
PPD (nm)	26.98 (0.94) (n=84)	26.65 (0.98) (n=321)	0.007	26.66 (0.99) (n=209)	26.78 (0.96) (n=196)	0.20

*Log transformed, +Square root transformed for analysis. Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data Median and interquartile range shown for +duration of diabetes

5.4.c Interaction between GST genotype and smoking

Since cigarette smoking is both pro-inflammatory and pro-oxidant, the association was further examined between *GST* genotypes and measures of oxidative stress and LDL-oxidation, in subjects by smoking status. Since CHD may itself be pro-inflammatory and is associated with increased plasma markers of oxidative stress and LDL-oxidation (as described in chapter 3), the focus of this analysis was on those subjects without CHD. As shown in table 5.3, the associations between genotypes with lipid parameters and measures of oxidative stress were similar to that for all subjects (regardless of CHD status).

In those without CHD, the *GSTT1-1* genotype, when compared to the *GSTT1-0* genotype, was associated with a significantly higher plasma CRP (1.50±1.37mg/l v 1.27 ±1.11mg/l, p=0.008; p=0.04 after adjustment of CRP for the correlates of age, BMI and glucose). As shown in figure 5.1, this difference by genotype was observed exclusively in the never and ex-smokers (in never/ex-smokers grouped, *GSTT1-0* v *GSTT1-1*: 1.19 ±1.04 v 1.60 ±1.39, p=0.003, after adjustment p=0.03). However, in current smokers, as expected, CRP was higher, but with no difference by genotype (*GSTT1-0* v *GSTT1-1*: 1.51 ±1.34 v 1.64 ±1.25, p=0.68, after adjustment p=0.64). No significant interaction between genotype and smoking status in determining CRP (p=0.24).

In addition, as shown in table 5.3, Ox-LDL was also significantly higher in *GSTT1-1* compared to the *GSTT1-0* subjects (40.3±17.5U/l v 47.3±18.0U/l, p=0.001; p=0.004 after adjustment of Ox-LDL). This difference was most pronounced in current smokers, where

GSTT1-1 had a mean plasma Ox-LDL 33% higher than GSTT1-0, p=0.03 (after adjustment p=0.02), figure 5.2. As shown, in *GSTT1-1* subjects there was a linear non-significant increase in plasma Ox-LDL from never smokers to ex-smokers to current smokers (ANOVA p=0.27, linearity p=0.11, after adjustment ANOVA p=0.14, linearity p=0.04). The interaction between genotype (*GSTT-1* v *GSTT-0*) and smoking status (never v ex v current) in determining Ox-LDL was not statistically significant (p=0.18 and p=0.06 after adjustment of Ox-LDL for, LDL-C and Tg).

Furthermore, with respect to Ox-LDL:LDL, levels were higher in *GSTT1-1* compared to *GSTT1-0* (16.0±7.5U/mmol v 17.6±8.1U/mmol, p=0.05), however after adjustment for LDL-PPD, this no longer remained significant (p=0.14). Again, the difference was most pronounced in current smokers, where *GSTT1-1* had a mean level 37% greater than *GSTT1-0*, p=0.04 (after adjustment p=0.08). As shown in figure 5.3, in *GSTT1-1* subjects there was a significant linear increase in plasma Ox-LDL:LDL from never smokers to ex-smokers to current smokers (ANOVA p=0.04, linearity p=0.04, after adjustment ANOVA p=0.04, linearity p=0.04). The interaction between genotype and smoking in determining Ox-LDL:LDL was not significant (p=0.11 and p=0.11 after adjustment).

GSTT1-1 subjects compared to *GSST1-0* subjects had a lower LDL-PPD diameter (26.65±0.87nm v 26.98±0.94nm, p=0.007) and LDL-MPD (26.33±0.84 v 27.11±0.90, p=0.008). As shown in figure 5.4, no significant interaction was observed between genotype and smoking status (p=0.32) in determining LDL particle size.

No significant difference was observed in TAOS by *GSTT1* genotype (*GSTT1-0* v *GSTT1-1*: 43.1±14.3% v 42.26±12.8%, p=0.70; p=0.90 after adjustment of TAOS) nor after stratifying by smoking status (figure 5.5).

Of note, in subjects with CHD, no significant association was observed between *GSTT1* genotypes and CRP, Ox-LDL, TAOS or PPD in smokers and non-smokers (data not shown). With respect to the *GSTM1* genotype, no significant association was observed with CRP, Ox-LDL, LDL-PPD (or LDL-MPD) after stratifying by CHD status. Moreover no interactions were observed by genotype and smoking in determining plasma markers of lipid peroxidation of inflammation.

Figure 5.1: CRP by *GSTT1* genotype & smoking status in subjects without CHD

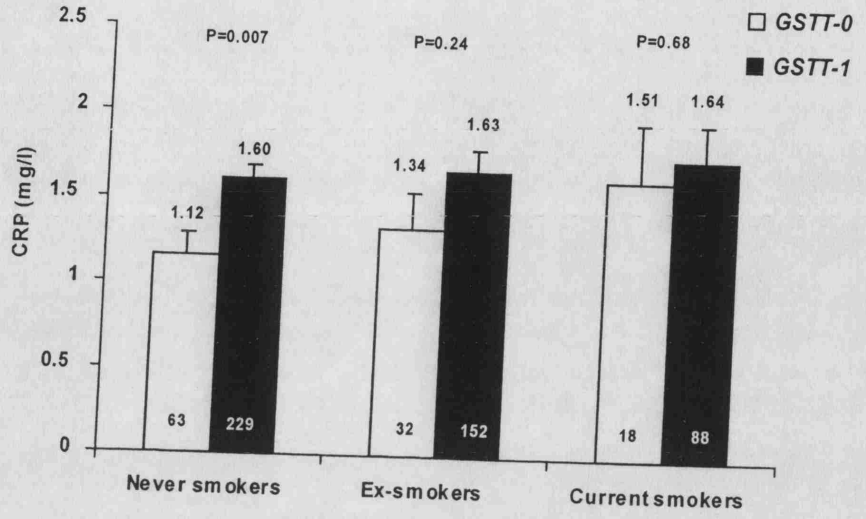


Figure 5.2: Ox-LDL by *GSTT1* genotype & smoking status in subjects without CHD

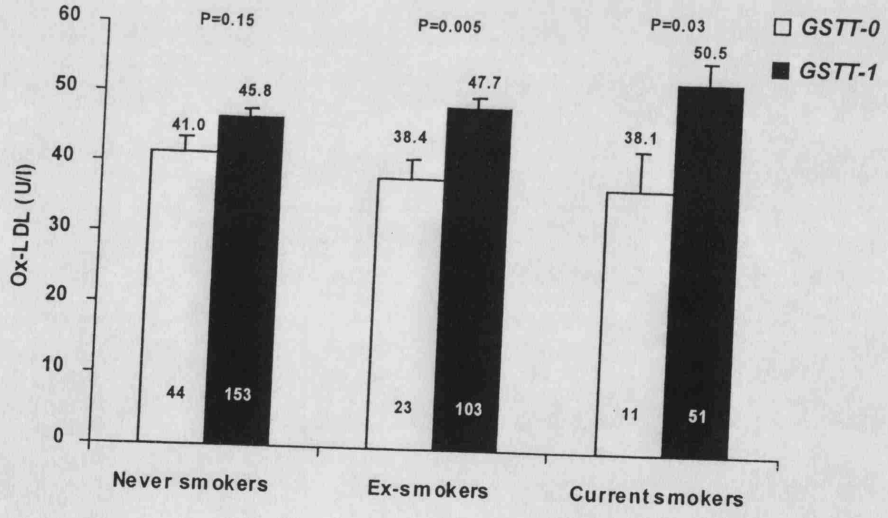


Figure 5.3: Ox-LDL:LDL by *GSTT1* genotype & smoking status in subjects without CHD

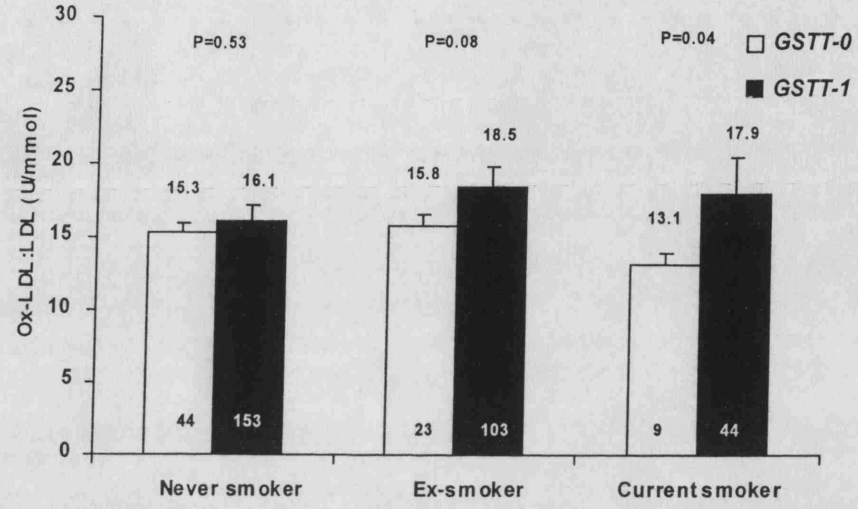


Figure 5.4: LDL-PPD by *GSTT1* genotype & smoking status in subjects without CHD

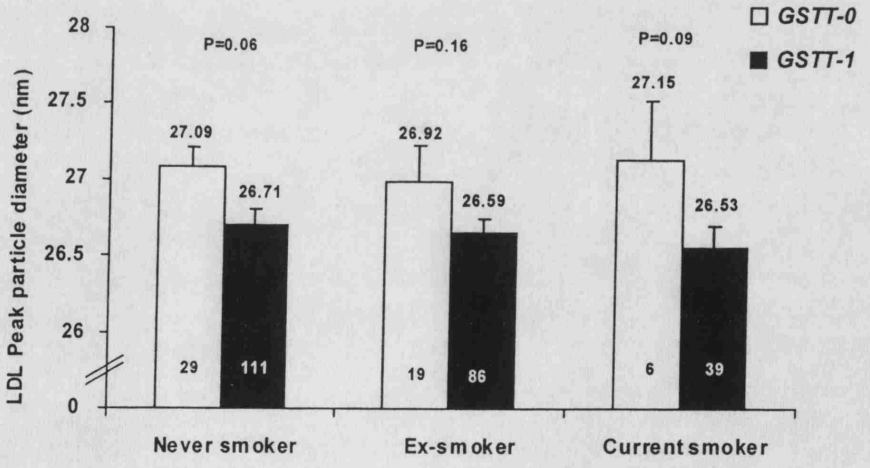
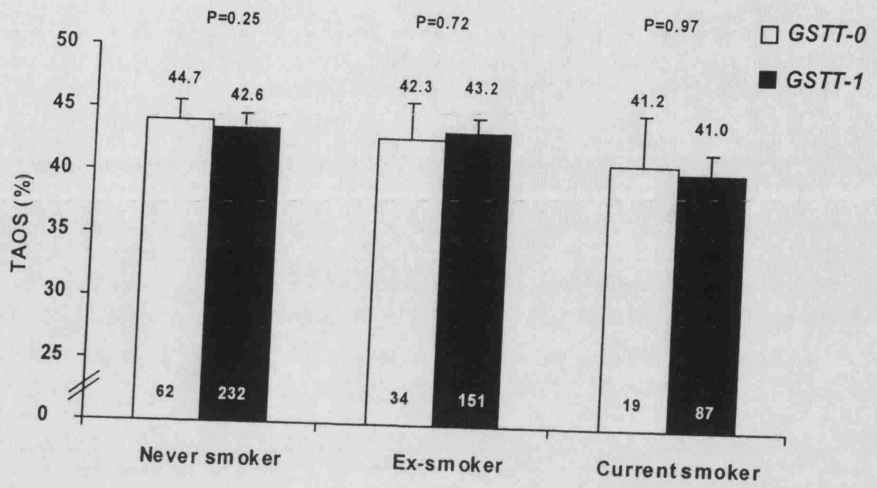


Figure 5.5: TAOS by *GSTT1* genotype & smoking status in subjects without CHD

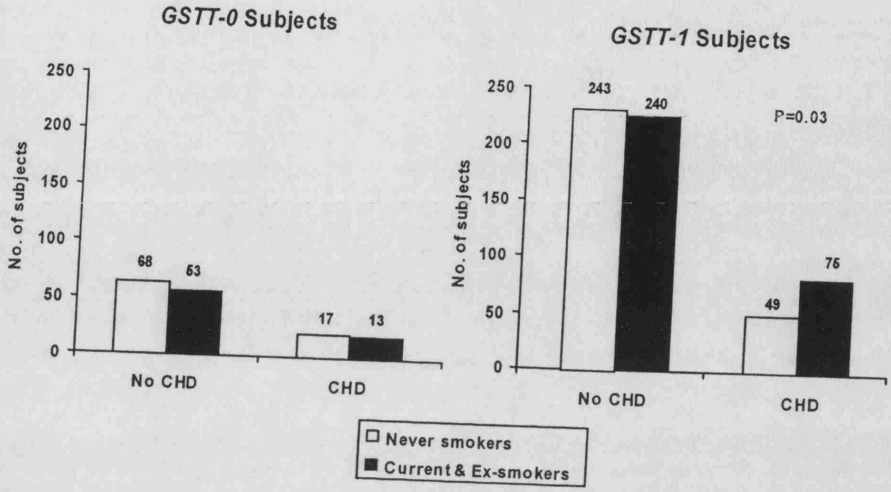


5.4.d GSTT genotype, smoking history and CHD status

Figure 5.6 shows all of the UDACS subjects, grouped by *GSTT1* genotype and stratified by CHD and smoking status. Current and ex-smokers have been combined, as CHD risk is increased in the presence of previous exposure to cigarette smoke. In subjects with the *GSTT1-1* genotype, there was a significantly higher proportion of current/ex-smokers with CHD compared to never smokers (χ^2=4.61, p=0.03). In subjects with the *GSTT1-0* genotype, no such difference was observed (χ^2=0.002, p=0.96).

No association was observed between *GSTM1* genotype, smoking history and CHD status.

Figure 5.6: *GSTT1* genotype, smoking history and CHD status



5.4.e Genotype distribution difference compared to the non-diabetic NPHS sample

Genotype data for the *GST* gene variants (unpublished) were also available on the non-diabetic NPHSII subjects. The genotype distribution between this sample (at relatively low risk for CHD) was compared with the high risk sample of subjects with diabetes from UDACS. The baseline characteristics of the subjects in the NPHSII study grouped by CHD status, are shown in table 5.4. There was no significant difference in genotype distribution (for *GSTT1* or *GSTM1*) by CHD status. No interaction was observed between smoking status and *GSTT1* genotype in determining CHD risk (p=0.89).

Of the UDACS (n=773) sample, 80% were *GSTT1-1* and 20% were *GSTT1-0* (table 5.5). When the genotype distribution was compared to that of NPHSII, in which 84% of the 2592 non-diabetic subjects were *GSTT1-1* and 16% were *GSTT1-0*, there was a

significant difference (χ^2=6.6, p=0.01). Since the NPHSII sample is all male, the genotype distribution was also compared between NPHSII and men only (n=471) from UDACS. The genotype distributions remained significantly different (χ^2=9.02, p=0.002).

Table 5.4: Baseline differences in subjects by CHD status in NPHSII

Trait	No CHD (n=2399)	CHD (n=193)	P Value
Age (years)	56.0 (3.4)	56.3 (3.5)	0.18
Systolic blood pressure (mmHg)*	136.6 (18.8)	87.9 (11.6)	0.0003
Diastolic blood pressure (mmHg)*	84.3 (11.3)	87.9 (11.6)	0.0003
Body mass index (kg/m^2)*	26.2 (3.3)	26.7 (3.4)	0.03
Current smoking % (n)	27.5% (660)	38.2% (74)	<0.0001
Cholesterol (mmol/l)	5.70 (1.00)	6.07 (1.01)	<0.0001
Triglyceride (mmol/l)*	1.77 (0.93)	2.09 (1.12)	<0.0001
GSTT1 (T1-0/T1-1)	392/2007 (16.3/83.7%)	30/163 (15.5/84.5%)	0.77
GSTM1 (M1-0/M1-1)	1142/1257 (47.6/52.4%)	88/105 (45.6/54.4%)	0.59

Mean (SD) shown. Geometric mean and approximate SD shown for systolic blood pressure, body mass index and triglyceride. *Log transformed.

Table 5.5: Genotype frequencies in diabetic & non-diabetic subjects

Study group	*GSTT1* genotype		P Between groups
	T1-0	*T1-1*	
NPHSII	422 (16%)	2170 (84%)	0.01
UDACS	155 (20%)	618 (80%)	

χ^2 test was used to compare the genotype distribution between UDACS and NPHSII. Comparing NPHSII to all UDACS subjects χ^2=6.6, p=0.01. When the NPHSII genotype distribution was compared to men only from UDACS, the difference remained significant (UDACS men only *GSTT1-1* 366 [78%]; *GSST1-0* 103 [22%], χ^2=9.02, P=0.002)

5.5 Discussion

The work described in this chapter demonstrates an association between the *GSTT1* gene variant with markers of lipid peroxidation and inflammation, in a sample of subjects with diabetes, a group at high risk of CHD. In subjects free from CHD, the *GSTT1-1* genotype was associated with smaller LDL particle size compared to the *GSTT1-0* genotype, and this association was independent of smoking status. Furthermore, smoking history appeared to influence the effect of the *GSTT1* variant on measures of LDL-oxidation. In the *GSTT1-1* subjects, current smokers had higher Ox-LDL compared to ex-smokers, who in turn had higher levels compared to never smokers. In the *GSTT1-0* subjects, no additive effect was seen by smoking history on plasma Ox-LDL. These observations are interesting and complement each other, as sd-LDL is more prone to oxidation by free radicals (such as those in cigarette smoke) resulting in Ox-LDL, which is independently associated with increased atherosclerotic burden and increased CHD risk (Weinbrenner *et al.* 2003).

Plasma CRP was also significantly higher in those with the *GSTT1-1* genotype compared to the *GSTT1-0* subjects. Cigarette smoking is a well established pro-inflammatory stimulus and an important risk factor for CHD. In the *GSTT1-1* subjects, plasma CRP was significantly higher than in the *GSTT1-0* group and furthermore, in the *GSTT1-1* subjects, smoking did not result in a further increase in plasma CRP. Conversely, in the *GSST1-0* subjects there was a linear increase in plasma CRP from never smokers to ex-smokers to current smokers. This observation suggests that not only is the *GSTT1-1* genotype associated with greater inflammation, but also that the *GSTT1-0* genotype may be anti-

inflammatory, but this effect is lost in the face of a stimulus, such as smoking. These associations of the *GSTT1* genotype with markers of lipid peroxidation and inflammation were confined to subjects free from clinically manifest CHD, and were not apparent in those subjects with CHD. This may be explained by the fact that in the presence of established CHD, oxidative stress and inflammation is increased as a result of the atherosclerotic process (Harrison *et al.* 2003) and this might overwhelm the modest effect of genotype. In UDACS, plasma TAOS and Ox-LDL was not however significantly different between subjects with and without CHD. This may be due to the fact that those with CHD are being treated with agents such as statins and aspirin, which again may overwhelm any genotype effect. Of note, subjects with CHD had a higher plasma CRP, smaller dense LDL and increased Ox-LDL:LDL.

The results suggest firstly that the functional variant of the *GSTT1* gene (i.e. *GSTT1-1*), is associated with an increase in plasma markers associated with CHD and atherosclerosis. Secondly, *GSST1-1* in the presence of another risk factor (i.e. cigarette smoking) is associated with an additive deleterious effect. This is also supported by the observation that in subjects with the *GSTT1-1* genotype, there were a significantly higher proportion of current/ex-smokers with CHD compared to never smokers.

Diabetes is also a major risk factor for CHD and is associated with increased oxidative stress and lipid peroxidation (Brownlee 2001). When we compared the genotype distribution for the *GSTT1* variant between the diabetic subjects in UDACS and the healthy subjects in NPHSII, there was significantly lower prevalence of subjects with the

GSTT1-1 genotype in the diabetic subjects (even when men only subjects were included). One possible explanation for this could be that that in combination, the cardiovascular risk associated with this genotype and diabetes results in reduced life expectancy and hence the lower observed frequency. As described in chapter 4, although these two samples may partly differ in their environmental background there are close similarities, as described in chapter 4. Within the NPHSII sample there was no evidence of genetic heterogeneity for the *GST* variants between subjects recruited from London and outside London (*GSTT1* p=0.39, *GSTM1* p=0.28).

Several epidemiologic studies suggest that *GSTM1-0* and *GSTT1-0* are associated with increased risk of smoking-related cancers, including lung, bladder, ovarian and colorectal cancers (Chenevix-Trench *et al.* 1995; McWilliams *et al.* 1995; Coughlin *et al.* 2002). In contrast, their role in CHD risk and the pathophysiology of atherosclerosis, particularly in relation to exposure to cigarette smoke, remains unclear (Li *et al.* 2000; Wilson *et al.* 2000; de Waart *et al.* 2001; Li *et al.* 2001; Wang *et al.* 2002; Masetti *et al.* 2003; Olshan *et al.* 2003). With respect to *GSTM1*, no associations with markers of inflammation or lipid peroxidation, or with CHD risk were observed. Previously, Li *et al,* in a sample of smokers showed that *GSTM1-0* subjects compared to *GSTM1-1* had a higher risk of CHD but not of lower extremity arterial disease (Li *et al.* 2000; Li *et al.* 2001). Stalenhoef *et al,* showed that smokers with *GSTM1-0* had increased carotid atherosclerosis, measured by carotid intimal thickness (IMT) compared with *GSTM1-1* smokers over a 2-year period (de Waart *et al.* 2001). However, Wilson *et al,* showed a reduced risk of acute myocardial infarction in *GSTM1-0* smokers in a UK Caucasian (Wilson *et al.* 2000) and a UK sample

of subjects with a South Asian origin (Wilson *et al.* 2003). Similarly, the literature remains unclear with respect to the role of the *GSTT1* variant in relation to CHD risk. The "Atherosclerosis Risk In Communities" (ARIC) study, showed *GSTT1-1* smokers to be at an increased risk of CHD, lower extremity arterial disease and carotid artery atherosclerosis (Li *et al.* 2000; Li *et al.* 2001; Olshan *et al.* 2003). However, two other published studies showed an association between the *GSTT1-0* variant and CHD risk (Masetti *et al.* 2003; Park *et al.* 2003).

To understand, the complex association of the GST enzymes with CHD risk and the pathophysiology of atherosclerosis, we must consider that these enzymes may result in both harmful and the protective actions. The positive association between *GSTT1-1* genotype, smoking and CHD is contrary to that of most previous cancer studies where *GSTT1-1* has been found to be the protective genotype against smoking related cancers (Chenevix-Trench *et al.* 1995; Coughlin *et al.* 2002). However, there has also been some contrary evidence suggesting that the *GSTT1-1* may activate some carcinogens and increase the risk of cancers (Bruning *et al.* 1997). The association between *GSTT1* genes and CHD or cancers in smokers might be either positive or negative, depending on whether the transferases activate or detoxify chemicals in cigarette smokers. In the case of CHD risk, smokers with *GSTT1-1* may have lower risk if the functional proteins detoxify the relevant chemicals in cigarette smoke or higher risk if the functional proteins *activate* the relevant chemicals and increase oxidative stress and inflammation, as seems to be the case in our study. Therefore, in *GSTT1-1* subjects, the risk of CHD would be increased (compared to those with homozygous deletion alleles) if the functional proteins

had an effect to *activate* the relevant toxic chemicals in cigarette smoke. Conversely, if the functional proteins detoxify the relevant chemicals in cigarette smoke then these subjects would be at a lower risk (Pemble *et al.* 1994; Rebbeck 1997)

In summary, this section demonstrates an association between the *GSTT1-1* variant and markers of inflammation and lipid peroxidation. Furthermore this variant interacts with smoking to increase inflammation and lipid peroxidation. Further work is required to determine the underlying mechanism.

CHAPTER SIX

VARIATION IN THE HUMAN *UCP 2* GENE, MARKERS OF OXIDATIVE STRESS AND CHD RISK

CONTENTS OF CHAPTER SIX

6.1 The *UCP2* -866G>A gene variant and markers of oxidative stress

6.1.1 Introduction

As described in chapter 1, the mitochondrial electron transport chain plays a major role in cellular ROS production. Much of the previous work relating to variation in the *UCP2* gene has focused on the -866G>A promoter variant, described in chapter 1. This work has studied associations with energy expenditure, obesity and insulin secretion. However, UCP2 may have a 'major' effect on the mitochondrial proton gradient, and hence in allowing the neutralisation of superoxide. A valid hypothesis would be that this variant may alter cellular ROS production and thus UCP2 may play an important antioxidant role and protect against CHD.

6.1.2 Aims

The purpose of the work described in this section, was primarily to study the effects of the -866G>A variant in relation to plasma TAOS and measures of LDL-oxidation. Further analysis was also performed to study gene-environment interaction with smoking. Since UCP2 may have a role as an anti-oxidant, analysis in the presence of a pro-oxidant state such as smoking, or in the presence of CHD, might reveal differences otherwise not observed in a 'normal' state.

6.1.3 Methods

This work was performed on those samples obtained from UDACS. *UCP2* -866G>A genotyping was performed as described in chapter 2. Analysis focused on the association of genotype with the plasma measures of oxidative stress and LDL-oxidation already

described in chapter 3. In the analysis, raw and adjusted values for markers of oxidative stress are shown (as described in chapter 3: TAOS adjusted for Tg and glucose, Ox-LDL for Tg and LDL, Ox-LDL:LDL for LDL-PPD).

6.1.4 Results

6.1.4.a UCP2 -866G>A genotype distribution and clinical data

Seven hundred and sixty three (95.5%) of 799 Caucasian subjects were successfully genotyped for the *UCP2* -866G>A variant. Within the sample the genotype distribution was in Hardy Weinberg equilibrium (GG/GA/AA: 315/365/89, χ^2=1.17, p=0.28). The rare (A) allele frequency was 0.353 (95%CI: 0.33-0.38), similar to that reported for healthy controls (Esterbauer *et al.* 2001). The baseline characteristics of the subjects (by CHD status) for whom genotyping was successful are summarised in table 6.1. Of the 763 diabetic Caucasian subjects, CHD was present in 154 individuals (20.2%). There was no significant difference in UCP2 genotype distribution between those with and without CHD (-866G>A: p=0.22).

Table 6.1: Baseline characteristics of subjects by CHD status

Trait	No CHD (n=609)	CHD (n=154)	P Value
Age (years)	61.0 (14.0)	67.8 (10.3)	<0.001
Duration (years)$^{+}$	11 (5-21)	11 (6-18)	0.80
Systolic blood pressure (mmHg)*	139 (20)	139 (22)	0.99
Diastolic blood pressure (mmHg)*	80 (11)	77 (12)	0.003
Body mass index (kg/m^2)*	28.3 (5.6)	29.5 (5.4)	0.02
HbA_{1c} (%)*	7.8 (1.6)	7.6 (1.5)	0.14
LDL-Cholesterol (mmol/l)	2.9 (0.9)	2.5 (0.90)	<0.001
HDL-Cholesterol (mmol/l)*	1.4 (0.5)	1.2 (0.4)	<0.001
Tg (mmol/l)*	1.6 (0.9)	1.9 (1.0)	0.01
CRP (mg/l)*	1.53 (1.33)	1.82 (1.58)	0.03
TAOS (%)	42.7 (13.2) (n=588)	41.5 (13.1) (n=149)	0.32
Ox-LDL (U/l)*	45.8 (18.3) (n=395)	44.3 (19.3) (n=103)	0.48
Ox-LDL:LDL (U/mmol)*	16.8 (7.4) (n=395)	19.0 (9.8) (n=103)	0.002
LDL-MPD (nm)	26.89 (0.85) (n=402)	26.70 (0.79) (n=104)	0.03
LDL-PPD (nm)	26.72 (0.97) (n=402)	26.52 (0.93) (n=104)	0.06
Sex (F/M)	240/350 (41.2/58.8%)	49/104 (32.0/68.0%)	0.04
Never/Ex/Current smokers	316/188/105 (51.9/30.8/17.3%)	67/68/19 (43.7/44.4/11.9%)	0.006
UCP2 -866G>A	255/279/75 (41.9/45.8/12.3%)	58/82/14 (37.7/53.2/9.1%)	0.22
ACEI (No/Yes)	353/256 (58.0/42.0%)	68/86 (44.2/55.8%)	0.002
Aspirin (No/Yes)	382/227 (62.8/37.2%)	40/114 (25.7/74.3%)	<0.001
Insulin (No/Yes)	331/278 (54.3/45.7%)	98/56 (63.6/36.4%)	0.04
Statin (No/Yes)	498/111 (81.7/18.3%)	62/92 (40.5/659.5%)	<0.001

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

6.1.4.b UCP2 -866G>A genotype, lipids & measures of oxidative stress

As shown in table 6.2, no significant difference was observed in plasma TAOS by genotype (for GA/GG v AA, p=0.33). With respect to absolute Ox-LDL, AA subjects having the lowest levels (for GG/GA v AA, p=0.03, after adjustment p=0.04). With respect to plasma Ox-LDL:LDL, the same trend was observed, with AA subjects having the lowest levels (GG/GA v AA, p=0.05, after adjustment p=0.06).

After stratifying by CHD status, in those subjects with CHD, a significant association was observed between genotype and plasma TAOS (GG v GA v AA: 41.1±12.5% v 43.2 ±12.7% v 33.3±15.3%, p=0.03, after adjustment of plasma TAOS p=0.04). As shown in figure 6.1, this association was recessive in nature, with AA subjects having a mean plasma TAOS approximately 22% lower than GG/GA subjects (GG/GA v AA: 42.3±12.5% v 33.3±15.3%, p=0.01, unchanged after adjustment). As shown in figure 6.1, no association was observed in those without CHD (for GG/GA v AA p=0.94). There was a significant interaction between genotype (GG, GA, AA) and CHD status in determining plasma TAOS (p=0.02, after adjustment of plasma TAOS p=0.03). After stratifying by gender, the above association was present in the males, but not in the females (figure 6.2). In males, the interaction between genotype (GG v GA v AA) and CHD status (No CHD v CHD) in determining plasma TAOS was not significant (p=0.06). When the interaction model contained (GG/GA v AA) as the genotype variables, the interaction was significant (p=0.03, after adjustment p=0.04). No interaction was observed in females between genotype (GG v GA v AA) and CHD status p=0.12 (for GG/GA v AA and CHD status, p=0.60).

Table 6.2: *UCP2* -866 genotype, measures of oxidative stress & inflammation in all subjects

Trait	GG (n=315)	GA (n=365)	AA (n=89)	P
Total Cholesterol (mmol/l)	5.2(1.1)	5.1 (1.1)	5.1 (1.1)	0.61
LDL (mmol/l)	2.9 (0.9)	2.8 (1.0)	2.9 (0.9)	0.49
TC:HDL*	3.8 (1.4)	3.6 (1.2)	3.7 (1.0)	0.39
Tg (mmol/l)*	1.7 (1.0)	1.7 (1.0)	1.76 (0.9)	0.53
CRP (mg/l)*	1.57 (1.33)	1.61 (1.45)	1.53 (1.22)	0.90
Adjusted CRP (mg/ml)*	1.88 (1.51)	1.93 (1.67)	1.83(1.38)	0.85
TAOS (%)	43.3 (12.8) (n=302)	42.0 (13.7) (n=349)	41.2 (12.2) (n=86)	0.32
Adjusted TAOS (%)	44.4 (25.4)	41.5 (26.9)	39.9 (24.0)	0.24
Ox-LDL (U/l)*	47.0 (18.2) (n=202)	45.5 (18.1) (n=236)	40.7 (20.4) (n=60)	0.06
Adjusted Ox-LDL (U/l)*	46.7 (21.8)	45.5 (17.1)	40.8 (19.1)	0.10
Ox-LDL:LDL (U/mmol)*	17.4 (7.2) (n=202)	17.6 (8.1) (n=236)	15.4 (8.6) (n=60)	0.35
Adj Ox-LDL:LDL (U/mmol)*	17.5 (7.2)	17.7 (8.1)	15.6 (8.6)	0.36
MPD (nm)	26.88 (0.88) (n=209)	26.82 (0.84) (n=236)	26.89(0.73) (n=61)	0.76
PPD (nm)	26.66 (0.98) (n=209)	26.67 (0.97) (n=236)	26.78 (0.91) (n=61)	0.71

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

Figure 6.1: Plasma TAOS in relation to *UCP2* -866G>A genotype in all subjects

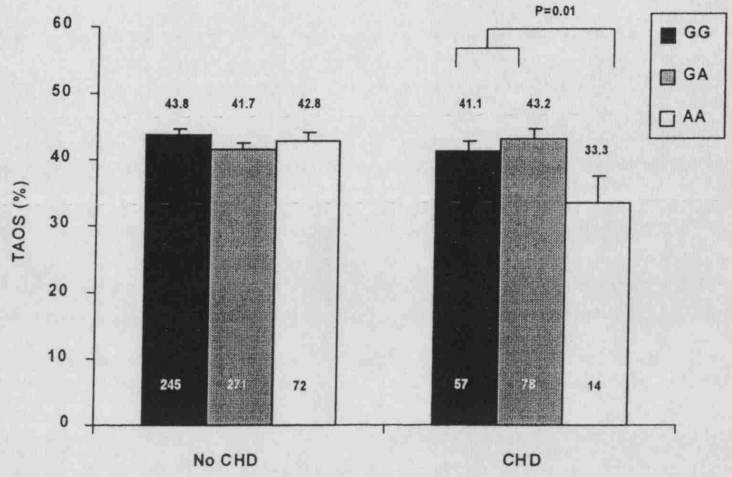
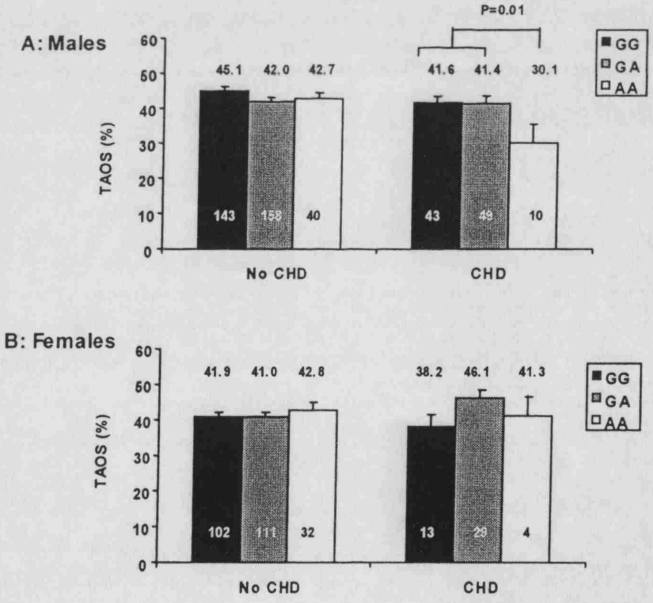


Figure 6.2: Plasma TAOS in relation to *UCP2* -866G>A genotype in all subjects by gender



No such association or interaction was observed with respect to Ox-LDL (figure 6.3) and Ox-LDL:LDL (figure 6.4). Further stratification by gender was not possible with these

measures as there was only 1 AA female with CHD where plasma Ox-LDL was measured.

Figure 6.3: Plasma Ox-LDL in relation to *UCP2* -866G>A genotype in all subjects

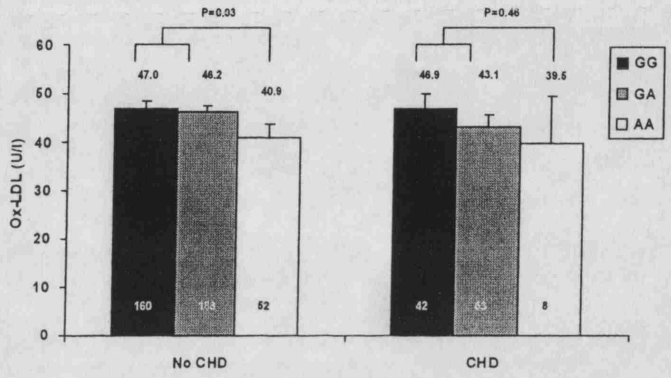
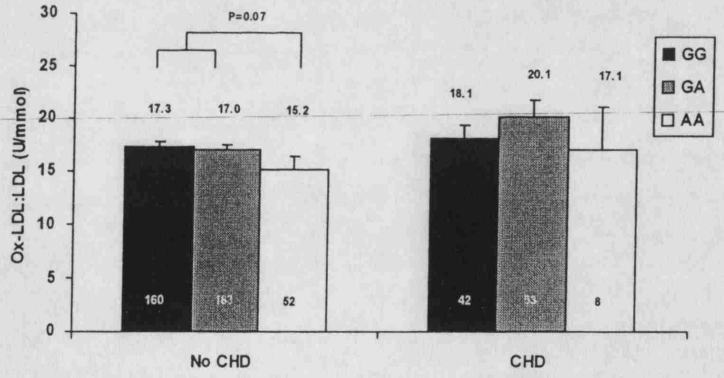


Figure 6.4: Plasma Ox-LDL:LDL in relation to *UCP2* -866G>A genotype in all subjects



6.1.4.c UCP2 -866G>A genotype and F_2-isoprostane sub-study

To further corroborate the association between the AA genotype and plasma TAOS observed in male subjects with CHD, plasma esterified F_2-isoprostane was measured in twenty male subjects from UDACS. These were selected for homozygosity for the *UCP2* -866G>A variant and closely matched for baseline characteristics (table 6.3).

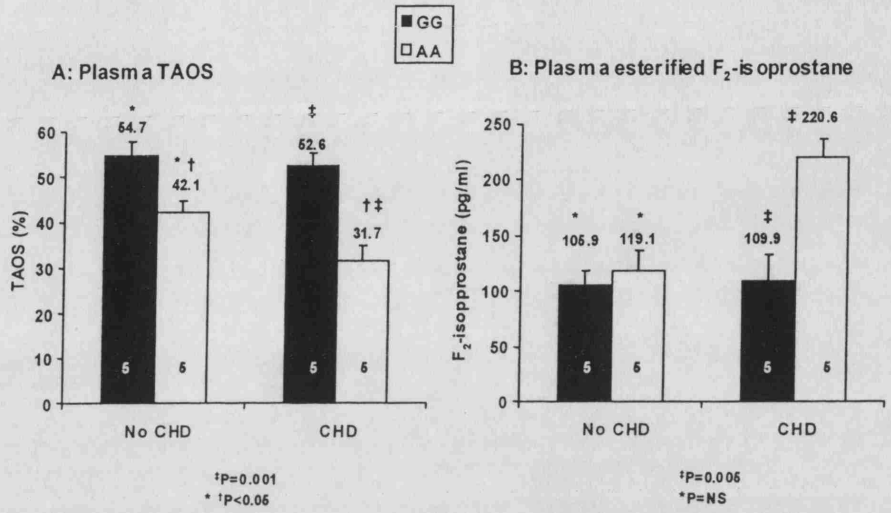
Overall, in those subjects with CHD, plasma TAOS was not significantly different compared to those without CHD (CHD v No CHD: 42.2±12.7% v 48.4±12.9%, p=0.22). Those with CHD had higher plasma F_2-isoprostanes (CHD v No CHD: 168.3±74.2pg/ml v 111.7±79.1pg/ml, p=0.05). By genotype, there was a highly significant difference in plasma TAOS in all subjects (AA v GG: 36.9±8.4% v 53.6±10.5%, p<0.0001), in those without CHD (AA v GG: 42.1±6.1% v 54.7±7.0, p=0.02) and in those with CHD (AA v GG: 31.7±7.3% v 52.6±6.3%, p=0.001). AA homozygotes with CHD had the lowest TAOS of all groups (figure 6.5A). In accordance with this, plasma F_2-isoprostane concentrations were significantly higher in AA homozygotes both overall (AA v GG: 175.4±64.5pg/ml v 104.6±72.8pg/ml, p=0.011) and in those with CHD (AA v GG: 220.6±37.2pg/ml v 109.9±51.1pg/ml, p=0.005), but not significantly in AA homozygotes without CHD (AA v GG: 119.1±40.2pg/ml v 105.9±27.5pg/ml, p=0.58; figure 6.5B). There was evidence of a significant interaction between genotype (GG v AA) and CHD status in determining F_2-isoprostanes (p=0.01), but not with respect to plasma TAOS (p=0.19).

Table 6.3: Plasma TAOS and esterified F_2-isoprostanes in relation to *UCP2* - 866G>A genotype and CHD status in the UDACS sub-study

	No CHD			CHD		
	GG (n=5)	AA (n=5)	P	GG (n=5)	AA (n=5)	P
Age (years)	65.4 (5.3)	64.6 (4.4)	0.80	65.1 (7.2)	64.0 (6.2)	0.79
BMI ($kg.m^{-2}$)	29.6 (4.8)	32.7 (6.9)	0.43	36.2 (19.0)	30.34 (3.7)	0.52
Duration DM (years)	6 (4-12)	6 (0.5-20.5)	0.78	10 (8-17)	12 (7.5-21)	0.64
HbA1c (%)	7.5 (1.3)	6.5 (0.7)	0.19	8.6 (1.5)	8.4 (2.1)	0.88
Glucose (mmol/l)*	8.3 (1.7)	9.4 (5.1)	0.68	10.5 (4.3)	10.0 (5.2)	0.87
Cholesterol (mmol/l)	5.5 (1.0)	5.1 (0.8)	0.50	4.6 (0.9)	4.5 (0.3)	0.90
LDL (mmol/l)	3.4 (1.0)	2.8 (0.6)	0.28	2.4 (0.5)	2.2 (0.6)	0.65
HDL (mmol/l)	1.4 (0.3)	1.3 (0.4)	0.93	1.2 (0.1)	1.1 (0.2)	0.27
Tg (mmol/l)	2.3 (1.5)	2.1 (0.6)	0.81	2.2 (1.7)	3.3 (1.4)	0.30
TAOS (%)	54.68 (6.99)	42.06 (6.12)†	0.02	52.6 (6.25)	31.70 (7.27) †	0.001
F_2 isoprostanes (pg/ ml)	105.9 (27.48)	119.1 (40.2) ††	0.58	109.9 (51.1)	220.6 (37.2) ††	0.005
Systolic BP (mmHg)	145 (12)	141 (12)	0.63	148 (27)	136 (20)	0.46
Diastolic BP (mmHg)	80.4 (8.1)	81.2 (8.4)	0.88	82 (7)	81 (9)	0.57
% Proteinuria	40% (n=4)	30 % (n=3)	0.64	60 % (n=3)	60 % (n=3)	1.00
% Smokers	20 % (n=1)	20 % (n=1)	1.00	20% (n=1)	0 % (n=0)	0.29
% on insulin	20 % (n=1)	20 % (n=1)	1.00	60 % (n=3)	40 % (n=2)	0.53
% on Aspirin	40 % (n=2)	20 % (n=1)	0.49	80 % (n=4)	60 % (n=3)	0.49
% on Statin	0 % (n=0)	20 % (n=1)	0.29	80 % (n=4)	100 % (n=5)	0.29
% on ACEI	20 % (n=1)	40 % (n=2)	0.49	80 % (n=4)	60 % (n=3)	0.49

Mean (SD) shown or Median (IQR). *Geometric mean and approximate SD shown for glucose
Genotype groups were closely matched for baseline characteristics.
Comparing AA (No CHD v CHD)
†P<0.05
††P<0.01

Figure 6.5: Plasma TAOS and F_2-isoprostane in men from UDACS selected for homozygosity for *UCP2* -866G>A genotype



6.1.4.d Interaction between UCP2 -866G>A genotype and smoking

Since cigarette smoking is both pro-inflammatory and pro-oxidant, I further examined the association between *UCP2* -866G>A variant with measures of oxidative stress and LDL-oxidation in subjects by smoking status. As described in chapter 3, since CHD may itself be pro-inflammatory and is associated with increased plasma markers of oxidative stress and LDL-oxidation, the focus of this analysis was on those subjects without CHD. As shown in table 6.4, the associations between genotype with lipid parameters and measures of oxidative stress were similar to that for all subjects (regardless of CHD

status). Due to the above differences observed in plasma TAOS by gender, males and females were considered together and separately.

Table 6.4: *UCP2* -866 genotype, measures of oxidative stress & inflammation in all subjects without CHD

Trait	GG (n=255)	GA (n=279)	AA (n=75)	P
Total Cholesterol (mmol/l)	5.2 (1.0)	5.2 (1.1)	5.2 (1.1)	0.96
LDL (mmol/l)	2.9 (0.9)	2.8 (1.0)	2.8 (0.9)	0.98
TC:HDL*	3.7 (1.3)	3.7 (1.2)	3.6 (1.0)	0.91
Tg (mmol/l)*	1.7 (1.1)	1.6 (1.0)	1.5 (0.9)	0.44
CRP (mg/l)*	1.50 (1.28)	1.56 (1.39)	1.52 (1.28)	0.86
Adjusted CRP (mg/ml)*	1.83 (1.47)	1.91 (1.62)	1.83 (1.43)	0.81
TAOS (%)	43.8 (12.8) (n=245)	41.7 (13.9) (n=271)	42.8 (11.0) (n=72)	0.19
Adjusted TAOS (%)	45.2 (25.5)	40.8 (27.4) (n=269)	42.9 (21.5) (n=72)	0.16
Ox-LDL (U/l)*	47.0 (17.5) (n=160)	46.2 (18.1) (n=183)	40.9 (19.2) (n=52)	0.08
Adjusted Ox-LDL (U/l)*	46.9 (16.7)	45.9 (17.2)	41.0 (17.9)	0.09
Ox-LDL:LDL (U/mmol)*	17.3 (7.2) (n=160)	17.0 (7.2) (n=183)	15.2 (8.3) (n=52)	0.52
Adj Ox-LDL:LDL (U/mmol)*	17.4 (7.1)	17.2 (7.4)	15.5 (8.5)	0.67
MPD (nm)	26.94 (0.90) (n=167)	26.83 (0.85) (n=183)	26.93 (0.70) (n=52)	0.43
PPD (nm)	26.72 (0.97) (n=167)	26.83 (0.85) (n=183)	26.84 (0.87) (N=52)	0.63

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

No overall (males and females combined) association was observed between the -866G>A genotype and plasma TAOS (table 6.4). With respect to smoking, plasma TAOS was lower in current smokers compared to ex and never smokers, but this was not

statistically significant (never smokers v ex-smokers v current smokers: 42.9±12.8% v 43.1±12.9% v 41.1±14.7%, linearity between groups p=0.32, ANOVA p=0.40). As shown in figure 6.6, there was a dominant effect of the A allele to lower plasma TAOS in current and ex-smokers (in current smokers GA/AA v GG p=0.09, after adjustment p=0.05; in ex-smokers GA/AA v GG p=0.05, after adjustment p=0.05). A significant interaction was observed overall between genotype (GG v GA v AA) and smoking status (never, ex, current) in determining plasma TAOS, p=0.03, after adjustment of plasma TAOS, p=0.02.

When only male subjects were considered, a non-significant trend was seen between genotype and plasma TAOS (GG: 45.1±12.8% v GA 42.0±14.1% v AA 42.7±10.0% ANOVA p=0.12, linearity p=0.09, after adjustment p=0.07, linearity p=0.06). There was evidence of a dominant effect of the A allele, with GA/AA subjects having lower TAOS compared to GG (p=0.04, after adjustment p=0.02). With respect to smoking status, there was a non-significant trend in plasma TAOS by smoking history (never smokers v ex-smokers v current smokers: 44.5±12.0% v 43.5±13.0% v 41.1±15.7%, linearity between groups p=0.08, ANOVA p=0.20, after adjustment ANOVA p=0.22, linearity p=0.10). Similar results were observed when current smokers were compared to never-smokers (p=0.08) and when current smokers were compared to ex- and never-smokers grouped together (p=0.09). As shown in figure 6.7A, there was a dominant effect of the A allele to lower plasma TAOS in current (GA/AA v GG p=0.04, after adjustment p=0.02) and ex-smokers (ex-smokers GA/GG v GG p=0.04, after adjustment p=0.04). In current smokers, GA/AA subjects had a mean plasma TAOS 18% lower than GG, whilst in ex-

smokers, this difference was 10%. In the males without CHD, there was a highly significant interaction between genotype (GG v GA v AA) and smoking status (never, ex, current) in determining plasma TAOS, p=0.004 (after adjustment of TAOS p=0.003). This association remained if the interaction model contained current smokers compared to never smokers (p=0.01, after adjustment p=0.009) and if ex- and never-smokers were grouped together (p=0.06, adjusted p=0.04). When the model contained GG v GA/AA subjects and smoking status (never, ex, current), the interaction was highly significant p=0.005 for TAOS (after adjustment p=0.003).

In the females, as shown in figure 6.7B, no interaction was observed between genotype (GG v GA v AA) and smoking status (never, ex, current) in determining plasma TAOS, p=0.86.

Figure 6.6: Plasma TAOS in relation to *UCP2* -866G>A genotype in all subjects by smoking status

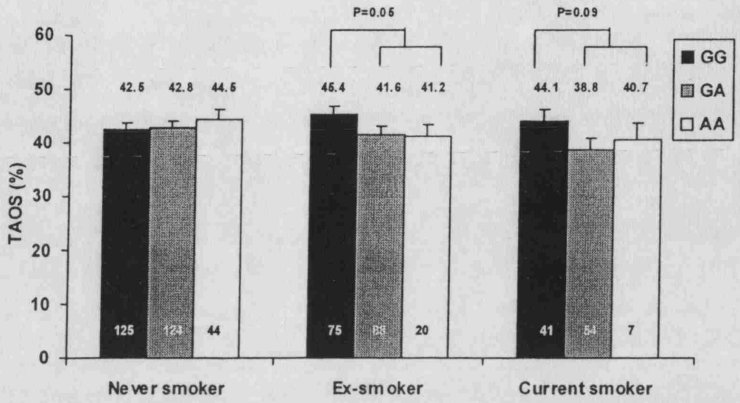
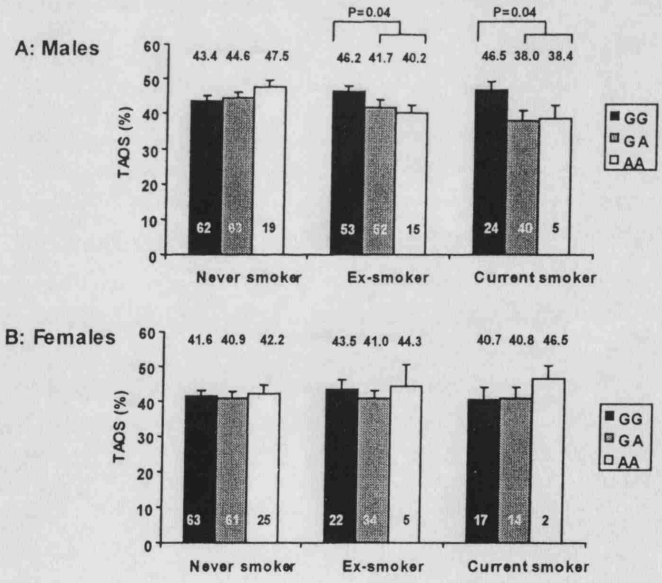


Figure 6.7: Plasma TAOS in relation to *UCP2* -866G>A genotype in all subjects by smoking status



With respect to Ox-LDL and Ox-LDL:LDL in subjects free from CHD, gender stratification was not possible as there was only 1 female AA current smoker with ox-LDL measured. Figure 6.8 shows the association between genotype and smoking status in determining absolute plasma Ox-LDL. As shown in figure 6.8, and previously in table 6.3, there was a non-significant linear trend across genotype for Ox-LDL to be lower in AA subjects, suggesting a co-dominant genotype effect (linear trend for GG v GA v AA, p=0.06; for GG/GA v AA, p=0.02, after adjustment p=0.07). As shown in figure 6.8, a no significant associations were observed by genotype in never smokers (p=0.09) and ex-smokers (p=0.22). No significant interaction was observed between genotype and smoking status in determining Ox-LDL (p=0.27).

With respect to Ox-LDL:LDL, in line with the above observation for Ox-LDL, there was a non-significant linear trend across genotype (table 6.4) for Ox-LDL:LDL to be lower in AA subjects (linear trend for GG v GA v AA, p=0.011; for GG/GA v AA, p=0.07, after adjustment p=0.10). As shown in figure 6.9, in current smokers, AA subjects had significantly lower Ox-LDL:LDL compared to GG/GA subjects, p=0.02, after adjustment p=0.05. There was no evidence of interaction, p=0.61 between genotype (GG v GA v AA) and smoking (current, ex, never) in determining Ox-LDL:LDL. Furthermore, no interaction was observed when GG/GA compared to AA by smoking status, p=0.25.

Figure 6.8: Plasma Ox-LDL in relation to *UCP2* -866G>A genotype in all subjects by smoking status

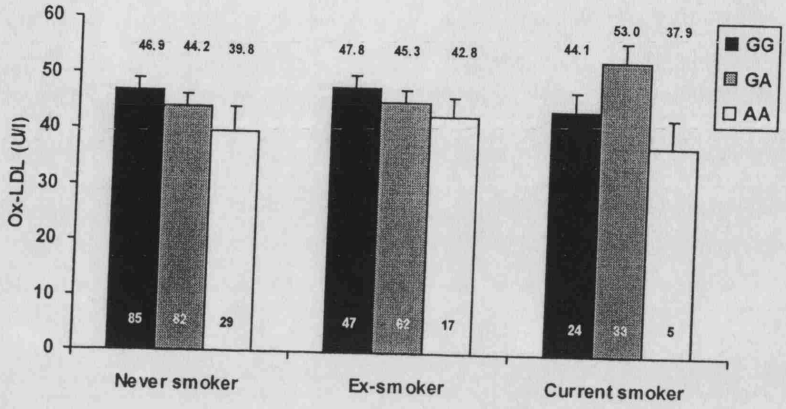
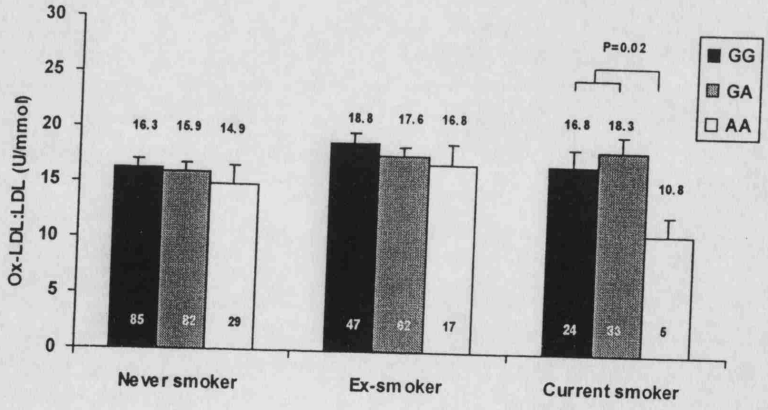


Figure 6.9: Plasma Ox-LDL:LDL in relation to *UCP2* -866G>A genotype in all subjects by smoking status



6.1.4.e UCP2 -866G>A genotype and F_2-isoprostane smoking sub-study

To further corroborate the association between the AA genotype and plasma TAOS observed in those current smokers, plasma total F_2-isoprostane was measured in twenty male subjects from UDACS, free from CHD. These were selected for homozygosity for the *UCP2* -866G>A variant and closely matched for baseline characteristics (table 6.5). Total F_2-isoprostane, as opposed to esterified F_2-isoprostane, was measured in this sub-study. This was performed to replicate the association with genotype by another measure of F_2-isoprostane.

No difference was observed in plasma TAOS by smoking status (smokers v non-smokers: 44.0±11.8% v 47.9±8.3%, p=0.44). Plasma total F_2-isoprostane was significantly higher (56% greater) in smokers compared to non-smokers (smokers v non-smokers: 693.9±380.6pg/ml v 446.2±124.5pg/ml, p=0.04). By genotype, plasma TAOS was not different by genotype (AA v GG: 42.1±9.9% v 49.3±9.6%, p=0.13). In non-smokers, no difference was observed in plasma TAOS by genotype (AA v GG: 48.7±6.2% v 47.2±10.4%, p=0.82), however in current smokers a significant difference was observed (AA v GG: 36.8±9.5% v 51.4±9.5%, p=0.04). AA homozygous smokers had the lowest TAOS of all groups (figure 6.10A). In accordance with this, plasma F_2-isoprostane concentration was elevated in AA homozygotes both overall (AA v GG: 697.8±417.7pg/ml v 464.0±128.1pg/ml, p=0.07) and in smokers (AA v GG: 1133.6±701.2pg/ml v 500.8±64.7pg/ml, p=0.04), but not significantly in non-smokers (AA v GG: 463.5±96.3pg/ml v 432.7±150.5pg/ml, p=0.74; figure 6.10B). There was

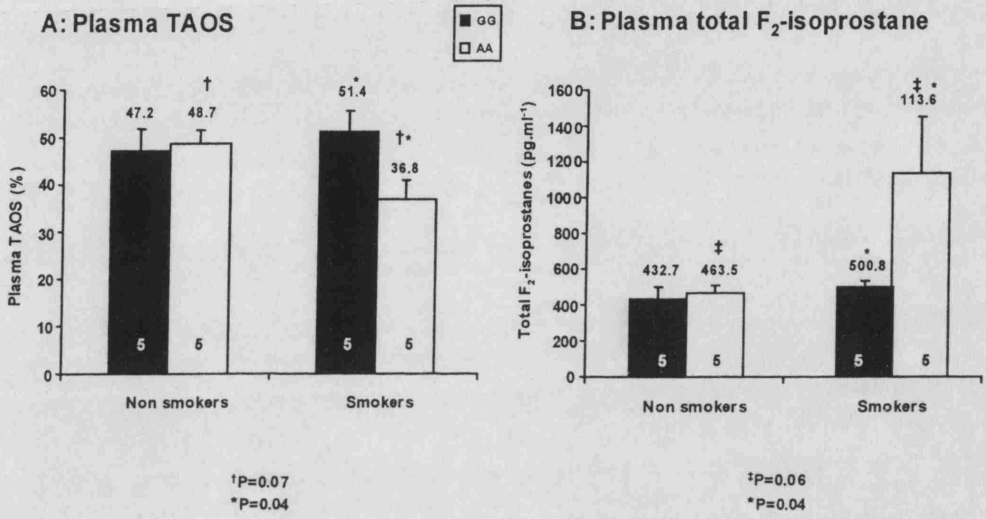
evidence of a borderline interaction between genotype and smoking status in determining F_2-isoprostanes ($p=0.11$) and plasma TAOS ($p=0.07$).

Table 6.5: Plasma TAOS and total F_2-isoprostanes in relation to *UCP2* -866G>A genotype and smoking status in the UDACS sub-study

	Non Smoking			Smoking		
	GG (n=5)	AA (n=5)	P	GG (n=5)	AA (n=5)	P
Age (years)	63.0 (3.1)	62.5 (9.2)	0.91	64.6 (3.8)	63.6 (4.7)	0.72
BMI (kg/m^2)	29.9 (4.1)	32.9 (8.7)	0.51	26.9 (3.8)	30.5 (3.5)	0.16
Duration DM (years)$^+$	9 (7-14)	9 (2-27)	0.58	11 (5-23)	6 (3-23)	0.93
HbA1c (%)	8.9 (0.8)	7.4 (1.1)	0.04	7.7 (1.1)	7.3 (1.0)	0.54
Glucose (mmol/l)	10.3 (4.1)	8.6 (0.9)	0.43	9.2 (6.7)	11.1 (6.1)	0.65
Cholesterol (mmol/l)	5.0 (0.9)	4.9 (0.9)	0.88	5.3 (1.5)	5.4 (0.7)	0.88
LDL (mmol/l)	3.0 (0.9)	2.7 (1.45)	0.69	2.4 (0.8)	3.0 (0.7)	0.29
HDL (mmol/l)	1.4 (0.3)	1.3 (0.4)	0.97	1.4 (0.4)	1.4 (0.3)	0.92
Tg (mmol/l)	1.5 (0.6)	2.0 (1.4)	0.46	3.7 (4.9)	2.1 (0.8)	0.48
TAOS (%)	47.2 (10.4)	48.7 (6.2)‡	0.82	51.4 (9.5)	36.8 (9.5)‡	0.04
F_2-isoprostane (pg/ml)*	432.7 (150.5)	463.5 (96.3)‡	0.74	500.8 (64.7)	1133.6 (701.2)‡	0.04
Systolic BP (mmHg)	141 (10)	144 (18)	0.78	136 (19)	136 (13)	1.00
Diastolic BP (mmHg)	82.0 (5.0)	88.0 (16.7)	0.48	80 (9)	82 (7)	0.70
% Microalbuminuria	40% (n=2)	50 % (n=2)	0.76	40 % (n=2)	40 % (n=2)	1.00
% on insulin	60 % (n=3)	25 % (n=1)	0.29	60 % (n=3)	25 % (n=1)	0.29
% on Aspirin	60 % (n=3)	50 % (n=2)	0.76	0 % (n=0)	40 % (n=2)	0.11
% on Statin	0 % (n=0)	0 % (n=0)	-	40 % (n=2)	20 % (n=1)	0.49
% on ACEI	55.6 % (n=3)	50.0 % (n=2)	0.76	60 % (n=3)	80 % (n=4)	0.49

Mean (SD) shown or Median (Interquartile range) shown. *Log transformed $^+$Square root transformed.
The geometric mean and approximate standard deviation is shown for plasma total F_2-isoprostane concentration. ‡Comparing AA: Non smokers v current smokers, p=0.07 for TAOS, p=0.06 for F_2-isoprostanes.

Figure 6.10: Plasma TAOS and total F_2-isoprostane in men free from CHD matched for baseline characteristics and selected for homozygosity for *UCP2* -866G>A genotype

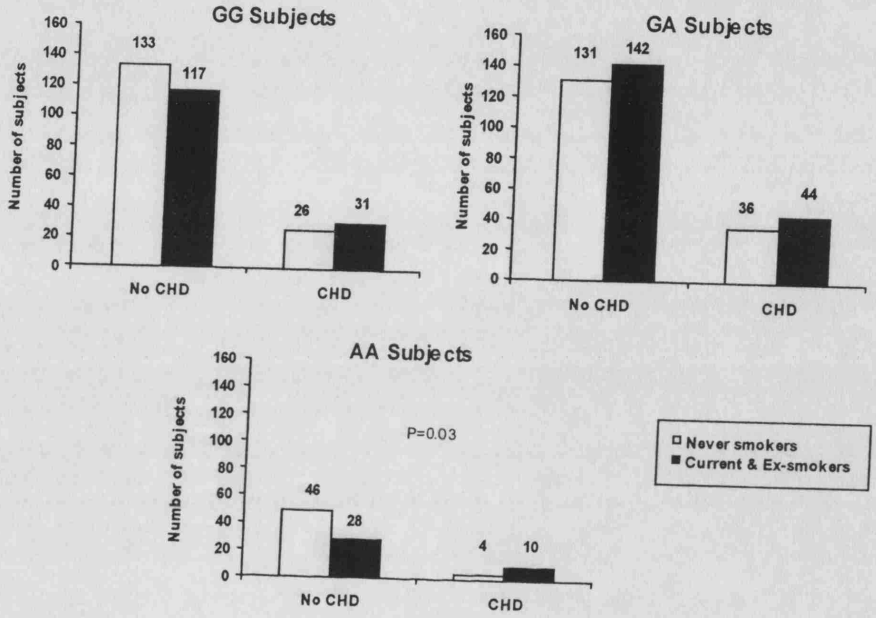


6.1.4.f UCP2 -866G>A genotype, smoking history and CHD status

Figure 6.11 shows all of the UDACS subjects grouped by *UCP2* genotype and stratified by CHD and smoking status. As described in chapter 4, current and ex-smokers have been combined as CHD risk is increased in the presence of previous exposure to cigarette smoke. In subjects with the AA genotype, there were a significantly higher proportion of current/ex-smokers (26.3%) with CHD compared to never smokers (8.0%), χ^2=4.61, p=0.03. In subjects with the GG and GA genotype, no such difference was observed (for GG current/ex-smokers with CHD v never smokers with CHD: 20.9% v 16.4%, χ^2=1.07,

p=0.30; for GA current/ex-smokers with CHD v never smokers with CHD: 23.7% v 21.6%, χ^2=0.22, p=0.64).

Figure 6.11: *UCP2* -866G>A genotype, smoking history and CHD status



6.2 The *UCP2* -866G>A gene variant and prospective CHD risk

6.2.1 Introduction

The work described in section 6.1, suggets an association between the *UCP2* -866A allele and increased plasma markers of oxidative stress (TAOS and F_2-isoprostane). No difference in genotype frequency was observed in UDACS by CHD status. This is however a cross-sectional study, which, as such, cannot calculate prospective risk. Therefore to evaluate any possible association with prospective CHD risk, samples were genotyped from NPHSII. If the observations described in section 6.1 are correct, an increase in prospective CHD risk *might* be observed in subjects homozygous for the A allele. This *might* help establish that increased oxidative stress plays a role in atherogenesis, in line with the observations described in chapter 3.

6.2.2 Aims

The aim of this work was firstly to study the association between the -866G>A variant and prospective CHD risk, and secondly to look at the interaction of this genotype with established risk factors in determining risk.

6.2.3 Methods

This work was performed on the NPHSII sample, described in chapter 2. *UCP2* -866G>A genotyping was performed as previously described. Analysis focused on the association of genotype with prospective risk, and on gene-environment interaction, specifically between the -866G>A variant and those factors associated with increased oxidative stress. Insufficient plasma was available to measure and study measures of TAOS and

LDL-oxidation, but as described in chapter 3, an association was observed between plasma TAOS and prospective CHD risk. In the analysis, the hazard ratio (HR) will be presented after adjustment for age and practice, and also for other risk factors where described.

6.2.4 Results

6.2.4.a UCP2 -866G>A genotype distribution and clinical data

Genotypes were obtained in 2695 (97.1%) of the 2775 subjects with available DNA from the NPHSII sample (table 6.6). At follow up, subjects with CHD had the expected baseline risk factors associated with risk. Genotype distribution was in Hardy Weinberg equilibrium (GG/GA/AA: 1088/1245/362, χ^2=0.04, p=0.85) and not different from that of UDACS (χ^2=1.85, p=0.40). The rare (A) allele frequency of 0.37 (95% CI 0.35- 0.38) were similar to that of healthy controls previously reported (Esterbauer *et al.* 2001) and to the UDAC sample (χ^2=0.78, p=0.38).

Homozygosity for the A allele was associated with a significant elevation in baseline diastolic blood pressure (84.2±11.2mmHg, 84.4±11.4mmHg, 86.2±11.1mmHg for GG, GA, AA respectively, p=0.003 for AA v G allele carriers). Men who were AA genotype also tended to have increased plasma levels of CRP, measured in 721 subjects at random (GG v GA v AA: 1.25 ±1.48mg/l v 1.25 ±1.37mg/l v 1.53 ±1.76mg/l, p=0.09 for AA v G allele carrier). All other baseline characteristics were independent of *UCP2* genotype.

Table 6.6: Baseline characteristics by CHD event status for 2695 men from NPHSII genotyped for the *UCP2* -866G>A gene variant

Trait	Controls No CHD event n = 2491	Cases CHD event n = 204	P†
Age (years)	56.0 (3.4)	56.6 (3.5)	0.008
Systolic Blood Pressure (mmHg)	138.0 (19.1)	143.8 (20.1)	<0.0001
Diastolic Blood Pressure (mmHg)	84.3 (11.3)	87.8 (11.6)	<0.0001
Body Mass Index (kg/m^2)	26.4 (3.5)	27.1 (3.4)	0.004
Current smoking % (n)	27.5% (685)	38.2 % (78)	<0.0001
Diabetes % (n)	2.1% (51)	6.4% (13)	<0.0001
Cholesterol (mmol/l)	5.70 (1.00)	6.07 (1.01)	<0.0001
Triglyceride (mmol/l)*	1.77 (0.93)	2.09 (1.12)	<0.0001
CRP (mg/l)* ; n=721	1.20 (1.33) N=623	4.65 (5.07) N=98	<0.0001
Fibrinogen (g/l)*	2.70 (0.51)	2.84 (0.50)	<0.0001

Data are mean (SD) unless otherwise stated.
*geometric mean (approximate SD).
† P values from Cox proportional hazard model

6.2.4.b UCP2 -866G>A variant and prospective CHD Risk

CHD risk was elevated amongst those with the *UCP2* -866AA genotype (HR 2.22 [1.53-3.22] for AA v GG genotype; p=0.0002; table 6.7). The hazard ratios demonstrated a recessive effect of the A allele (HR 2.08 [1.49–2.86]; p<0.0001 for AA v GA+GG). The doubling in risk remained highly statistically significant even after adjustment for accepted risk factors (excluding CRP) in the study group overall (HR 1.86 [1.33–2.59];

p<0.0001 for AA v GG; table 3) and in the 721 subjects in whom CRP was also measured (HR 2.05 [1.28–3.26]; p=0.003 for AA v GA+GG; table 3).

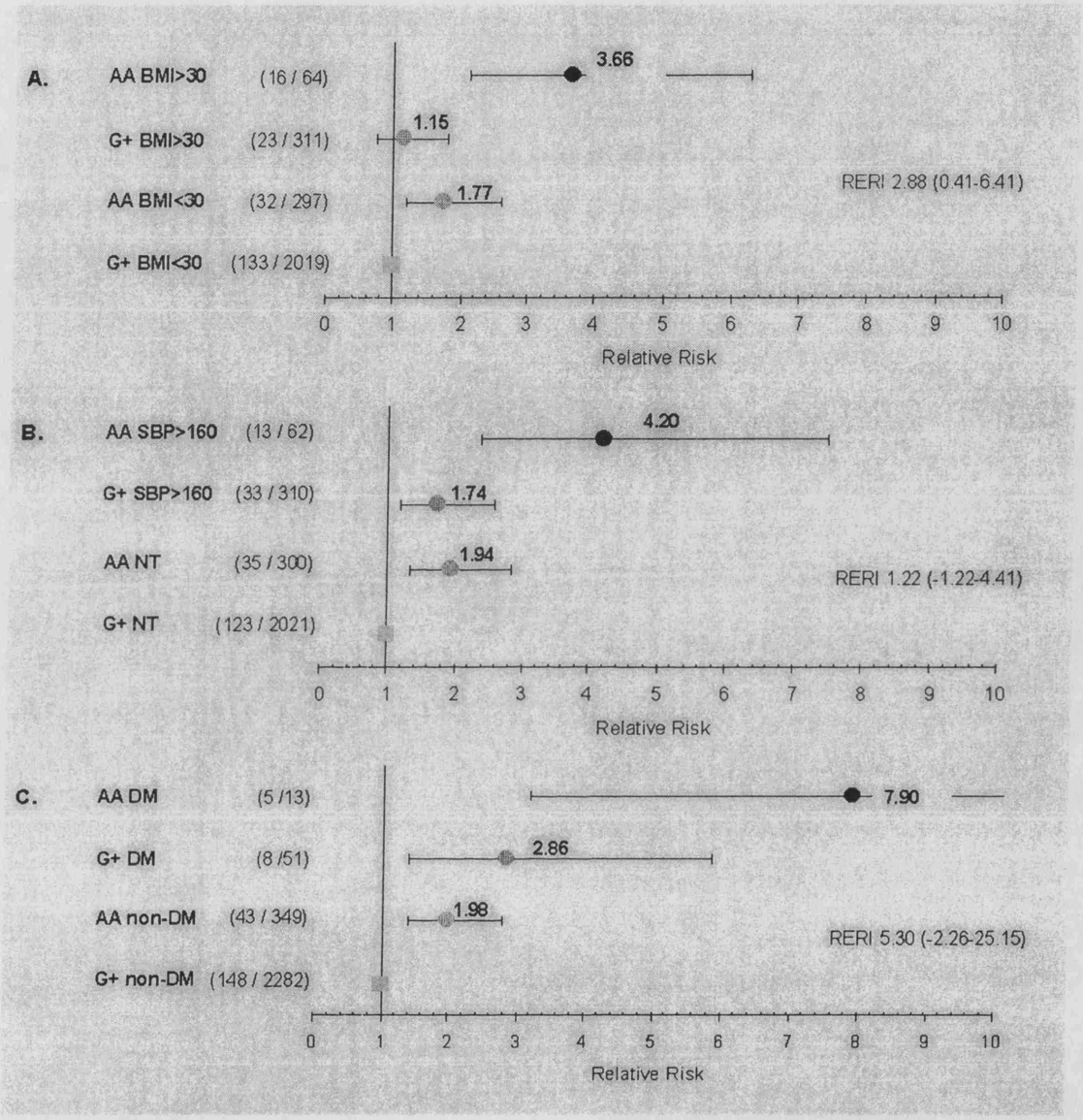
Even in those AA homozygous men without accepted risk factors for CHD (described below), risk was elevated, being 77% higher in non-obese subjects (defined as BMI<30Kg/m^2; figure 6.12A), 94% higher in non-hypertensives (systolic BP<160mmHg; figure 6.12B) and 98% higher in non-diabetics (figure 6.12C). Overall, obesity increased CHD risk by 45% (HR 1.45 [1.01-2.06], p=0.04) in line with previous observations (Kannel *et al.* 2002), but obese subjects with the G allele were relatively protected (figure 6.12A). The impact of genotype and obesity on CHD risk was greater than additive (as shown by the RERI estimate being higher than unity; figure 6.12A). The presence of diabetes carried an approximate 3.5-fold increase in CHD risk (HR 3.38 [1.91-5.98], p<0.0001), similar to previous reports (Garcia *et al.* 1974). Amongst the small group of diabetics, 38.5% (5/13) of the *UCP2* -866AA homozygotes had a CHD event (HR 4.00 [1.55–10.31], p=0.004 for diabetic AA v non-diabetic AA; figure 6.12C).

Table 6.7: CHD risk by *UCP2* -866G>A genotype in NPHSII

UCP2 -866G>A Genotype	Number with CHD events / total number (rate per 1000 py)	Hazard Ratio* (95% CI)	Adjusted Hazard Ratio † (95% CI)	Fully Adjusted Hazard Ratio § (95% CI)
GG	69 / 1088 (6.7)	1	1	1
GA	87 / 1245 (7.4)	1.13 (0.82 – 1.55)	1.14 (0.83 – 1.56)	1.15 (0.71 – 1.87)
AA	48 / 362 (14.9)	2.22 (1.53 - 3.22)	1.99 (1.37 – 2.90)	2.22 (1.29 – 3.82)
Probability		0.0002	0.002	0.01
GG + GA	156 / 2333 (7.1)	1	1	1
AA	48 / 362 (14.9)	2.08 (1.49 – 2.86)	1.86 (1.33 – 2.59)	2.05 (1.28 – 3.26)
Probability		<0.0001	<0.0001	0.003

* Adjusted for age, practice.
† Adjusted for age, practice, BMI, DBP, smoking, cholesterol, triglycerides, fibrinogen and diabetes.
§ Adjusted for age, practice, BMI, DBP, smoking, cholesterol, triglycerides, fibrinogen, diabetes and CRP (n=721).

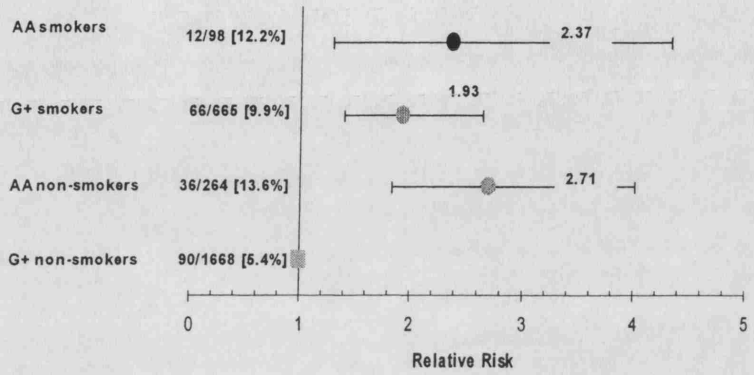
Figure 6.12: Relative CHD risk in NPHSII by *UCP2* -866G>A genotype according to presence or absence of accepted risk factors



Numbers in parentheses are CHD cases / total number of subjects in group; G +, G allele carriers; BMI, body mass index; SBP, systolic blood pressure; NT, normotensive; DM, diabetes mellitus; RERI, relative excess risk due to interaction.

The association with smoking is shown in figure 6.13. In G allele carriers, the risk associated with smoking was almost twice that in G allele non smokers. This is in line with current estimates that predict that smoking doubles the CHD risk. In the AA non smokers, risk was greater than that for smokers with the G allele. Of interest, in AA smokers the risk was not increased further. The interaction between genotype (GG/GA v AA) and smoking history was significant in determining risk, p=0.03. At first sight this may suggest that risk associated with the AA genotype is independent of smoking status, however in the whole sample, the proportion of AA smokers was considerably lower than that of AA non smokers (AA smokers: 3.6% (98/2695) v AA non smokers: 9.8% (264/2695)). This observation *might* be explained by the possibility that the risk associated with AA smokers was so great, that life expectancy in these subjects was lower than the age of recruitment into the study (approximately 56 years).

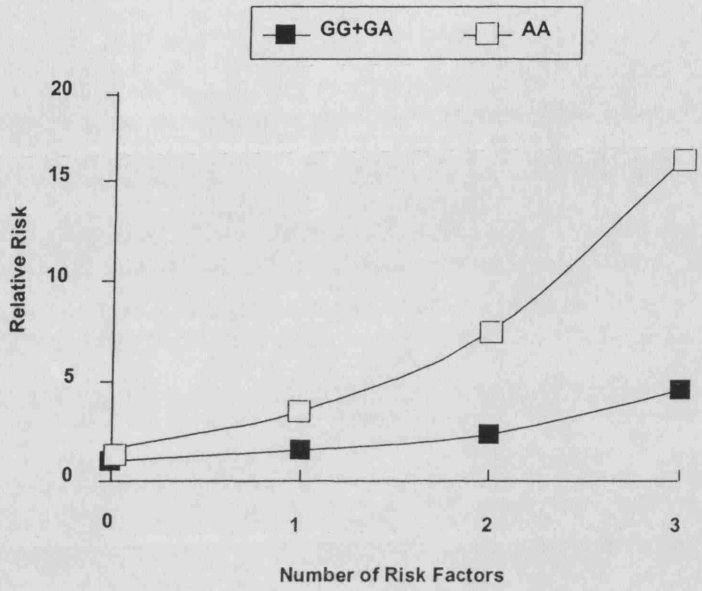
Figure 6.13: Relative CHD risk in NPHSII by *UCP2* -866G>A genotype by smoking history



6.2.4.c UCP2 -866G>A genotype and interaction with other risk factors

Figure 6.14, summarises the association between gene-environment interaction for the *UCP2* -866G>A variant. Within NPHSII, 14% of subjects had a BMI>30Kg/m^2, 14% a systolic BP>160mmHg and 2.3% had diabetes at baseline. As shown, with respect to these risk factors, all three show an additive interaction with the AA genotype ($p<0.05$). The relative risk for CHD with respect to a one-risk factor increase was 2.08 [1.43-3.04] in AA subjects compared to 1.63 [1.06-2.51] in GG/GA subjects.

Figure 6.14: Interaction between the *UCP2* -866G>A variant and risk factors



The prevalence of risk factors was no risk factors 74%, 1 risk factor 22.4%, 2 or more risk factors 3.8%.

6.3 Discussion

6.3.a Association of the -866G>A variant with plasma markers of oxidative stress

The work described in this section is the first to describe the association between the *UCP2* -866G>A gene variant and plasma markers of oxidative stress. Diabetes and CHD are both associated with an increased burden of oxidative stress (Cai *et al.* 2000; Brownlee 2001; Evans *et al.* 2002), and thus lower plasma TAOS (Sampson *et al.* 2002).

In keeping with a causal role for oxidative stress in atherosclerosis (as described in chapter 3), plasma TAOS was significantly lower in diabetic men with CHD compared to those without CHD, and in the NPHSII sample, lower plasma TAOS was associated with increased CHD risk. Both CHD and the *UCP2* -866A allele were associated with a modest reduction in plasma TAOS (and hence increased oxidative stress), however in combination, the effect was greatly enhanced. This is reflected by the fact that in UDACS, males with CHD had lower plasma TAOS, but this was dependent on genotype, with subjects homozygous for the *UCP2* -866A allele having the lowest plasma levels. This suggests that the *UCP2* genotype has an influence on the burden of oxidative stress. This association was recessive in nature and was apparent only in males. In a subset of men with CHD matched for baseline characteristics (including age and treatment), AA homozygosity was associated with 40% lower TAOS, mirrored by a 100% increase in F_2-isoprostane concentrations compared to GG homozygosity.

This study provides evidence for an interaction between the *UCP2* -866G>A gene variant and cigarette smoking in determining plasma levels of oxidative stress. Like CHD,

cigarette smoking is associated with increased oxidative stress. Both smoking and the *UCP2* -866A allele were associated with a modest reduction in plasma TAOS, however in combination, the effect was greatly enhanced. This effect was also confined to male subjects, and in this group, where analysis focused on those subjects without CHD, the A allele appeared to have a dominant effect on plasma TAOS in ex- and current smokers. In the males, mean plasma TAOS was lowest in current smokers, intermediate in ex-smokers and highest in never-smokers. Subjects with the *UCP2* -866A allele, who were current smokers, had the lowest plasma TAOS of all groups tested (approximately 18% lower than GG current-smokers). This association was also observed in the ex-smokers, except that the difference in plasma TAOS by genotype was of a smaller magnitude (10% difference). In the never smokers, no significant difference was observed by genotype on plasma TAOS. This data is further supported by the analysis of the subset of men without CHD, matched for baseline characteristics (including age, glycaemic control and treatment). *UCP2* AA homozygosity was associated with 30% lower plasma TAOS mirrored by a 125% increase in total F_2-isoprostane concentrations. Even though, total as opposed to esterified F_2-isoprostane was measured, the result was the same increase in the plasma measurement associated with the AA genotype, thus re-enforcing the above observation with respect to esterified F_2-isoprostane in the CHD group of subjects.

With respect to plasma Ox-LDL and Ox-LDL:LDL, these were lower in AA subjects (in all and those without CHD) compared to GG and GA subjects. This was independent of smoking status and CHD and is contradictory to expectation, since the A allele is associated with lower plasma TAOS and higher F_2-isoprostane. One possible explanation

for this paradoxical effect may relate to lipoprotein oxidation. The oxidation of lipoproteins is generally considered to occur in the vessel wall (Berliner *et al.* 1996; Steinberg 1997), which in the case of LDL (being converted into Ox-LDL) may then either lead to foam cell and subsequent plaque formation or undergo back diffusion into the plasma (which may then be measured in the plasma as Ox-LDL). Previously, stable isotope kinetic studies have demonstrated that the subendothelial retention time of LDL particles is directly associated with the number of circulating Ox-LDL particles (Pietzsch *et al.* 2000). Therefore, in the presence of increased plasma oxidative stress associated with the *UCP2* -866A allele, it may be that more Ox-LDL is retained in the subendothelial space and this is reflected in the lower plasma levels. This hypothesis is plausible since increased plasma oxidative stress is associated with non-lipid pathophysiological process with deleterious effects on the vasculature (described in chapter 1). These include altered expression of cell adhesion molecules, vasomotion and also induction of pro-inflammatory mediators via altered NF-κB expression. Interestingly, increased levels of CRP in atherosclerotic plaques are associated with increased binding of Ox-LDL and increased uptake into plaque macrophages (Torzewski *et al.* 1998).

6.3.b Association of the -866G>A variant with CHD risk

In the second section of this chapter the association of the *UCP2* -866G>A variant with prospective CHD risk was examined.

In prospectively studied middle-aged men from NPHSII, CHD risk was doubled amongst those homozygous for the *UCP2* -866AA allele, even amongst the normotensive, lean, non-smokers and non-diabetics. This effect was also independent of other biochemical risk factors. However, the risk associated with genotype was substantially increased in the presence of conventional CHD risk factors known to be associated with oxidative stress, such as hypertension, obesity and diabetes (Cai *et al.* 2000; Chisolm *et al.* 2000; Brownlee 2001; Witztum *et al.* 2001; Evans *et al.* 2002; Harrison *et al.* 2003). Indeed, the risk of CHD was elevated almost four-fold in obese AA compared to non-obese G allele carriers. There was also a significant interaction between genotype (GG/GA v AA) and smoking history in determining risk, p=0.03. As described, at first sight it appeared that risk associated with the AA genotype was independent of smoking status, however in the whole sample the proportion of AA smokers was considerably lower than that of AA non smokers (AA smokers: 3.6% (98/2695) v AA non smokers: 9.8% (264/2695)). This *might* be explained by the possibility that the risk associated with AA smokers was so great, that these subjects life expectancy was lower than the age of recruitment into the study (approximately 56 years).

The lack of any significant difference in genotype distribution between those with and without CHD in UDACS may appear to conflict with the prospectively-derived data from NPHSII. However, this may not be the case. Firstly, prospective gene-association studies are more powerful than case-control studies (Humphries *et al.* 2003). Secondly, increased obesity, increased oxidative stress, inflammation, and hyperglycaemia might all overwhelm the *UCP2* genotype 'strength of signal' in diabetes. Thirdly, case-control

cross-sectional studies are prone to intrinsic bias, for example due to altered rates of disease progression, subsequent progression of secondary phenotypes, or genotype associations with death or treatment changes. Indeed, the presence of the A allele might be associated with both earlier disease presentation and earlier death in some, subsequently balanced by more aggressive secondary prevention strategies. Such influences are well-recognised confounders (Risch 2000; Humphries *et al.* 2003; Sing *et al.* 2003).

The number of diabetic men recruited to NPHSII is small. However, the substantially-increased CHD risk amongst diabetic men with the *UCP2* -866AA genotype in NPHSII is congruent with increased markers of oxidative stress found in the plasma of AA subject from UDACS.

6.3.c Conclusion and explanation for the observations

This work suggests a role for UCP2 (and hence the mitochondrial electron transport chain) in the regulation of oxidative stress, and highlights its potential impact upon CHD risk. Previously, *in vitro* studies have shown that *UCP2* gene expression is induced by both extra- and intra-mitochondrial sources of ROS (Pecqueur *et al.* 2001; Echtay *et al.* 2002), whilst selective down-regulation of UCP2 increases endothelial cell ROS generation (Duval *et al.* 2002). Therefore, UCP2 appears to have an important intrinsic anti-oxidant role. This may explain the dependence of the observed genotypic effect on the presence/absence of CHD or smoking status. Thus, under conditions of increased oxidative stress (e.g. smoking, diabetes, CHD), increased UCP2 expression should prove

vasculo-protective (Arsenijevic *et al.* 2000; Echtay *et al.* 2002). In support of this, UCP2 protects against atherosclerosis in LDL-receptor deficient mice (Blanc *et al.* 2003).

An interesting observation was that the association of the *UCP2* -866G>A variant with plasma TAOS was observed in males and not in females. To my knowledge, there is no information on the effect of sex hormones on UCP2 expression, and no differences have been observed between males and females in respect to UCP2 expression (Millet *et al.* 1997; Oberkofler *et al.* 1998). No sex hormone response elements have been identified in the promoter region of the UCP2 gene. The association of this gene variant with both plasma markers of oxidative stress and CHD risk needs to be further evaluated in a sample of females.

The *UCP2* -866A promoter variant may be directly functional in regulating *UCP2* gene induction in response to oxidative stress or be in LD with one or more variants, possibly within the coding regions of the gene, hence conferring differences in protein function. This will be explored in the next chapter investigating the role of the *UCP2* 45bp D/I variant in the 3'UTR. Although promoter constructs of the -866A allele are associated with greater transcriptional activity in pancreatic β cells, they are associated with greater *repression* of transcription in somatic non-β cells (Krempler *et al.* 2002). It maybe, therefore, that the *UCP2* -866A allele is associated with lower inducible UCP2 expression within the vasculature or circulating immune cells. As such, one would anticipate the A allele to be associated with increased OS and higher risk of CHD as

demonstrated in these studies. This will be explored later in chapters 7 and 8 of this thesis.

CHAPTER SEVEN

THE HUMAN *UCP2* D/I GENE VARIANT AND MARKERS OF OXIDATIVE STRESS

CONTENTS OF CHAPTER SEVEN

7.1 The UCP 2 45bp D/I gene variant and markers of oxidative stress

7.1.1 Introduction

In chapter 6, an association was observed between the *UCP2* -866G>A gene variant and plasma markers of oxidative stress and LDL-oxidation. Another commonly studied variant is the 45 base pair deletion/insertion (D/I) variant in the 3' untranslated region in exon 8. Association studies with respect to this variant have focused on the risk of obesity, and have yielded conflicting results (Dalgaard *et al.* 2001; Esterbauer *et al.* 2001; Wang *et al.* 2004). This variant is reported to be in moderate LD with the -866G>A variant, with D' ranging from 0.74-0.77 (Esterbauer *et al.* 2001; Wang *et al.* 2004).

7.1.2 Aims

The purpose of the work described in this section, was to study the association of the D/I variant with plasma TAOS and measures of LDL-oxidation. Further analysis was also performed to study gene-environment interaction with smoking. In the second part of this chapter, I will also explore the possible role that this variant may have in combination with the -866G>A variant.

7.1.3 Methods

This work was performed on UDACS. *UCP2* D/I genotyping was performed as described in chapter 2. Analysis focused on the association of genotype with the plasma measures of oxidative stress and LDL-oxidation already described in chapter 3. In the analysis, raw and adjusted values for markers of oxidative stress are shown (as described in chapter 3:

TAOS adjusted for Tg and glucose, Ox-LDL for Tg and LDL, Ox-LDL:LDL for LDL-PPD).

7.1.4 Results

7.1.4.a UCP2 D/I genotype distribution and clinical data

Seven hundred and sixty three (95.5%) of the 799 Caucasian subjects were successfully genotyped for the *UCP2* D/I variant. Within the sample the genotype distribution was in Hardy Weinberg equilibrium (DD/DI/II: 395/323/45, χ^2=1.17, p=0.06). The rare (I) allele frequency was 0.27 (95%CI: 0.27-0.29), which is in line with other samples (I allele frequency: 0.29 (Dalgaard *et al.* 2001)-0.32 (Wang *et al.* 2004)). The baseline characteristics of the subjects (by CHD status) for whom genotyping was successful is identical to that described for the *UCP2* -866G>A variant summarised in table 6.1, chapter 6. There was no significant difference in *UCP2* D/I genotype distribution between those with and without CHD (No CHD v CHD: 317/255/37 (52.1/41.9/6.1%) v 78/68/08 (50.6/44.2/5.2%) p=0.84).

7.1.4.b UCP2 D/I genotype, lipids & measures of oxidative stress

As shown in table 7.1, no significant difference in plasma TAOS was observed by genotype, but II subjects had a mean plasma TAOS 6% lower relative to DD/DI subjects (for DD/ID v II, p=0.24). With respect to absolute Ox-LDL, there was no significant difference by genotype, and again II subjects had 6% lower levels compared to DD/ID (p=0.39, after adjustment p=0.41). With respect to plasma Ox-LDL:LDL, the same trend

was observed, with levels being 13% lower in the II group (DD/ID v II, p=0.11, after adjustment p=0.08).

Table 7.1: *UCP2* D/I genotype, measures of oxidative stress & inflammation in all subjects

Trait	DD (n=395)	DI (n=323)	II (n=45)	P
Total Cholesterol (mmol/l)	5.1 (1.1)	5.1 (1.0)	5.2 (1.0)	0.83
LDL (mmol/l)	2.9 (1.0)	2.8 (0.9)	3.0 (0.9)	0.18
TC:HDL*	3.8 (1.3)	3.6 (1.1)	3.8 (1.0)	0.14
Tg (mmol/l)*	1.7 (1.1)	1.7 (1.0)	1.5 (0.8)	0.35
CRP (mg/l)*	1.54 (1.31)	1.61 (1.45)	1.8 (1.42)	0.51
Adjusted CRP (mg/ml)*	1.86 (1.53)	1.92 (1.6)	2.01 (1.47)	0.76
TAOS (%)	42.5 (13.5) (n=383)	42.7 (12.7) (n=311)	40.1 (13.6) (n=43)	0.49
Adjusted TAOS (%)	42.6 (26.8)	43.0 (24.9)	37.5 (26.7)	0.42
Ox-LDL (U/l)*	46.6 (17.4) (n=263)	44.4 (19.8) (n=203)	42.8 (19.2) (n=32)	0.32
Adjusted Ox-LDL (U/l)*	46.5 (16.5)	44.4 (18.5)	42.9 (18.6)	0.52
Ox-LDL:LDL (U/mmol)*	17.6 (7.7) (n=263)	17.1 (8.5) (n=203)	15.2 (6.9) (n=32)	0.23
Adj Ox-LDL:LDL (U/mmol)*	17.7 (7.5)	17.3 (8.5)	15.2 (6.5)	0.22
MPD (nm)	26.85 (0.87) (n=270)	26.86 (0.81) (n=204)	26.82 (0.82) (n=32)	0.96
PPD (nm)	26.68 (1.00) (n=270)	26.68 (0.90) (n=204)	26.70 (1.10) (n=32)	1.00

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

After stratifying by CHD status (figure 7.1), in contrast to the -866G>A variant, no significant difference was observed in plasma TAOS by genotype (DD v DI v II: No CHD ANOVA p=0.62; CHD ANOVA p=0.71). As shown in figure 7.1, no significant interaction was observed between genotype (DD, DI, II) and CHD status in determining plasma TAOS (p=0.93, after adjustment of plasma TAOS p=0.92). Further stratification by gender was not possible as there was only 1 II female with CHD. In the males, the pattern of results was similar to that observed for all subjects (figure 7.2).

As shown in figures 7.3 and 7.4, no association was observed between genotype and Ox-LDL (No CHD group ANOVA p=0.44; CHD group ANOVA p=0.29) or Ox-LDL:LDL (No CHD group ANOVA p=0.43; CHD group ANOVA p=0.34). Further stratification by gender was not possible with these measures as there was only 1 II female with CHD where plasma Ox-LDL was measured.

Figure 7.1: Plasma TAOS by the *UCP2* D/I variant in all subjects

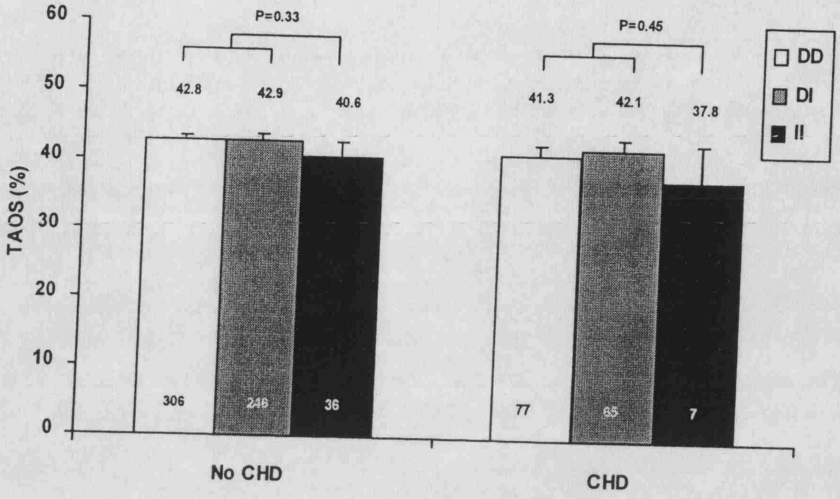


Figure 7.2: Plasma TAOS by the *UCP2* D/I variant in males

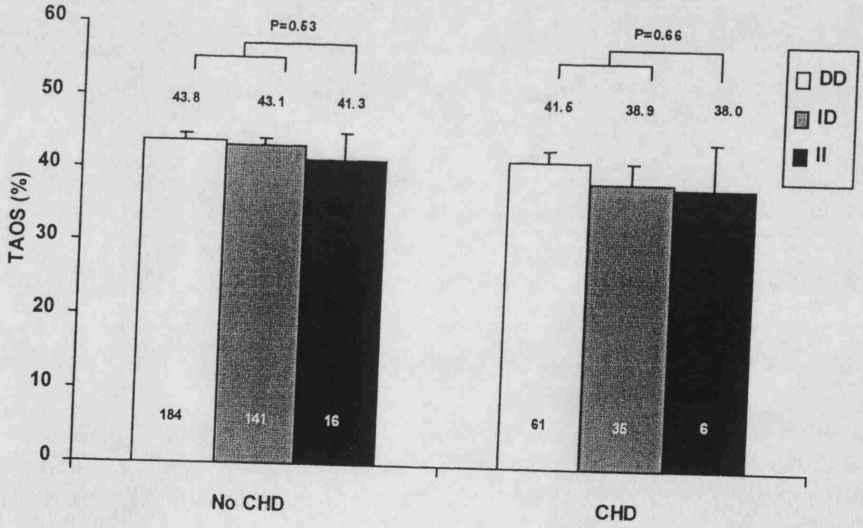


Figure 7.3: Plasma OX-LDL in by the *UCP2* D/I variant in all subjects

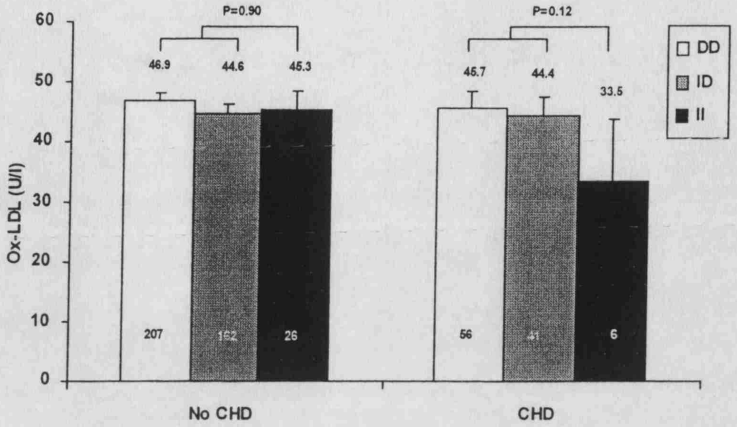
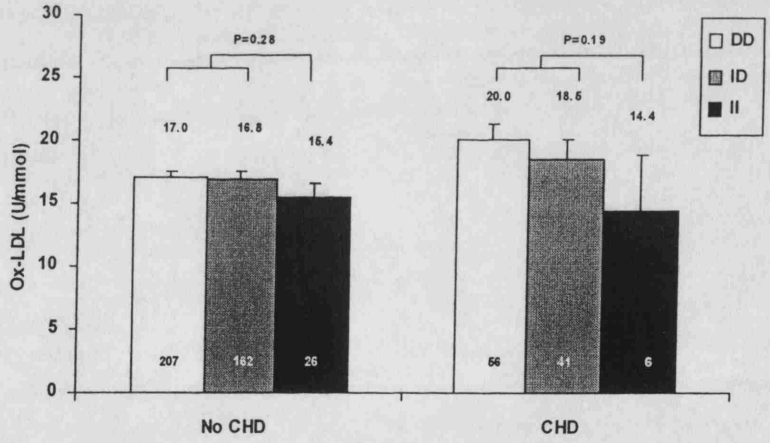


Figure 7.4: Plasma Ox-LDL:LDL by the *UCP2* D/I variant in all subjects



7.1.4.c Interaction between UCP2 D/I genotype and smoking

As described in the previous chapters, since cigarette smoking is both pro-inflammatory and pro-oxidant, further analysis was performed to look for an association between *UCP2* D/I variant with measures of oxidative stress and LDL-oxidation in subjects by smoking status. The focus of this analysis was on those subjects without CHD. As shown in table 7.2, the associations between genotype with lipid parameters and measures of oxidative stress were similar to that for all subjects (regardless of CHD status). Due to the differences observed in plasma TAOS by gender, described in chapter 6 for the -866G>A variant, males and females were considered together and separately.

With respect to the D/I variant, due the low frequency of the II genotype, stratification by gender and smoking status was not possible for DD/DI/II (stratification resulted in 3 current II smokers:1 male and 2 females). Therefore, in this analysis, II subjects were excluded and the focus was on comparing DD and DI subjects.

Table 7.2: *UCP2* D/I genotype, measures of oxidative stress & inflammation in subjects without CHD

Trait	DD (n=317)	DI (n=255)	II (n=37)	P
Total Cholesterol (mmol/l)	5.2 (1.0)	5.2 (1.0)	5.3 (1.0)	0.57
LDL (mmol/l)	2.9 (0.9)	2.8 (0.9)	3.1 (0.8)	0.14
TC:HDL*	3.7 (1.3)	3.6 (1.2)	3.8 (1.1)	0.36
Tg (mmol/l)*	1.7 (1.0)	1.6 (1.0)	1.5 (0.8)	0.28
CRP (mg/l)*	1.46 (1.24)	1.58 (1.40)	1.85 (1.10)	0.22
Adjusted CRP (mg/ml)*	1.80 (1.47)	1.93 (1.61)	2.07 (1.61)	0.46
TAOS (%)	42.8 (13.8) (n=306)	42.9 (12.3) (n=246)	40.6 (13.6) (n=36)	0.62
Adjusted TAOS (%)	43.1 (17.6)	43.4 (24.1)	38.3 (26.6)	0.54
Ox-LDL (U/l)*	46.9 (16.8) (n=207)	44.5 (19.9) (n=162)	45.3 (15.7) (n=26)	0.44
Adjusted Ox-LDL (U/l)*	46.6 (16.4)	44.4 (18.5)	45.3 (14.5)	0.63
Ox-LDL:LDL (U/mmol)*	17.0 (7.1) (n=207)	16.8 (8.1) (n=162)	15.4 (5.9) (n=26)	0.43
Adj Ox-LDL:LDL (U/mmol)*	17.3 (7.1)	17.1 (8.2)	15.3 (5.5)	0.28
MPD (nm)	26.93 (0.88) (n=214)	26.88 (0.83) (n=162)	26.69 (0.81) (n=26)	0.40
PPD (nm)	26.76 (1.01) (n=214)	26.69 (0.89) (n=162)	26.62 (1.10) (n=26)	0.70

*Log transformed, $^{+}$Square root transformed for analysis
Mean and SD shown
Geometric mean and approximate SD shown for *Log transformed data
Median and interquartile range shown for $^{+}$duration of diabetes

No overall (males and females combined) association was observed between smoking status and plasma TAOS (never smokers v ex-smokers v current smokers: 42.9±12.8% v 43.1±12.9% v 41.1±14.7%, linearity between groups p=0.32, ANOVA p=0.40). Furthermore, as shown in figure 7.5, no difference was observed in plasma TAOS between the DD and DI genotypes within each smoking category (DD v DI: never

smokers p=0.56, ex-smokers p=0.92, current smokers p=0.55) or by genotype across each smoking category. No interaction was observed between genotype and smoking status in determining plasma TAOS

When only male subjects were considered (figure 7.6A), no statistically significant difference in plasma TAOS was observed by smoking status (never smokers v ex-smokers v current smokers: 44.5±12.0% v 43.5±13.0% v 41.1±15.7%, linearity between groups p=0.09, ANOVA p=0.20). No difference was observed between ID and DD within each smoking category (never smokers p=0.62, ex-smokers p=0.68, current smokers p=0.32) or by genotype across each smoking category. Similarly in females, no association or interaction was observed (figure 7.6B).

Figure 7.5: Plasma TAOS by the *UCP2* D/I variant by smoking status in all subjects without CHD

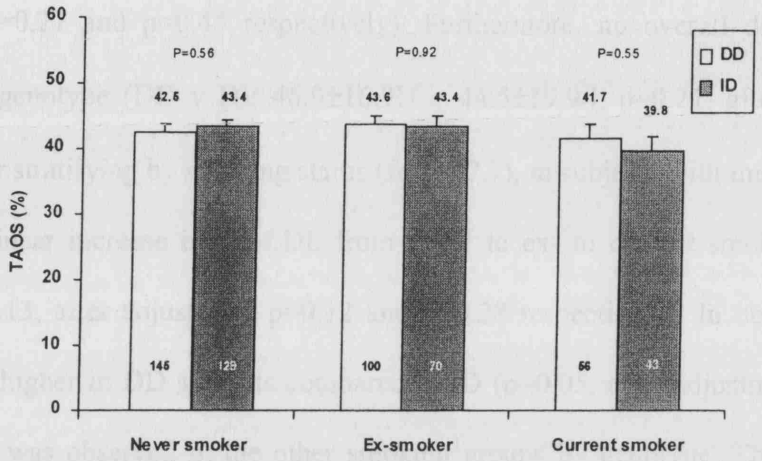
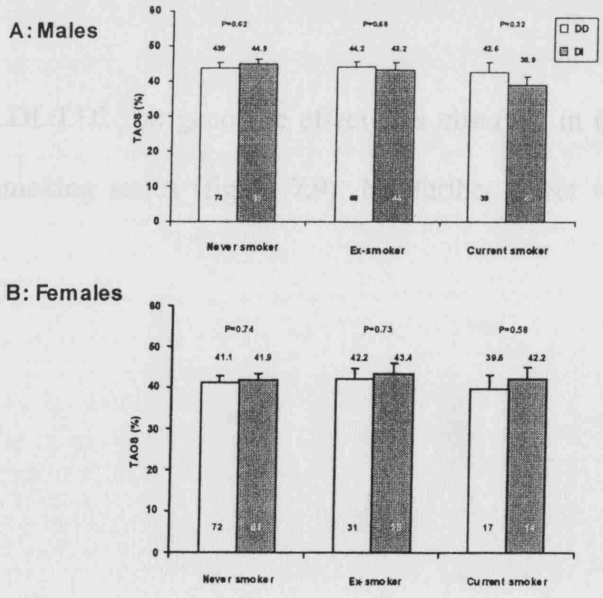


Figure 7.6: Plasma TAOS by the *UCP2* D/I variant by gender



No overall (males and females combined) association was observed between smoking status and plasma Ox-LDL (never smokers v ex-smokers v current smokers: 44.6±19.6U/l v 45.9±15.5U/l v 48.0±18.4U/l linearity p=0.21, ANOVA p=0.45, after adjustment p=0.27 and p=0.44 respectively). Furthermore, no overall difference was observed by genotype (DD v DI: 46.9±16.9U v 44.5±19.9/l, p=0.21, after adjustment p=0.38). After stratifying by smoking status (figure 7.7), in subjects with the ID genotype there was a linear increase in Ox-LDL from never to ex- to current smokers (p=0.05, ANOVA p=0.13, after adjustment p=0.12 and p=0.28 respectively). In never smokers, Ox-LDL was higher in DD subjects compared to ID (p=0.05, after adjustment p=0.13). No difference was observed in the other smoking groups by genotype. The interaction between genotype (ID v DD) and smoking status was significant in determining plasma Ox-LDL (p=0.04, after adjustment p=0.16). This pattern was observed in both males and females after stratifying by gender (figure 7.8).

With respect to Ox-LDL:LDL, no genotype effect was observed in the whole group or after stratifying by smoking status (figure 7.9). No further effect was observed after stratifying by gender.

Figure 7.7: Plasma Ox-LDL in relation to *UCP2* D/I genotype in all subjects by smoking status

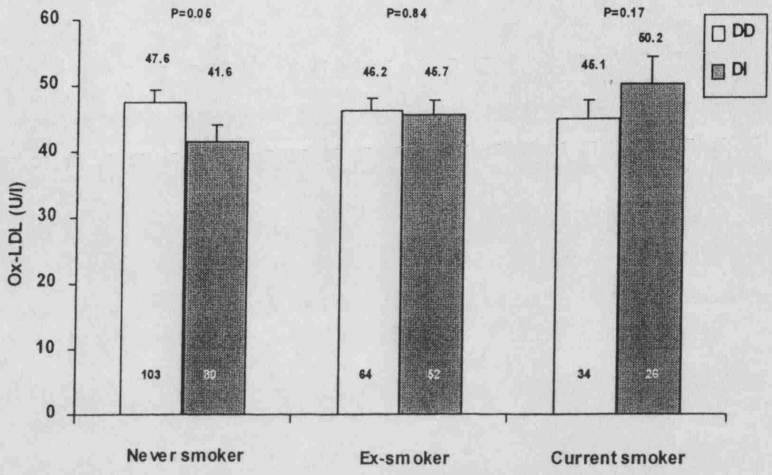


Figure 7.8: Plasma Ox-LDL in relation to *UCP2* D/I genotype by gender

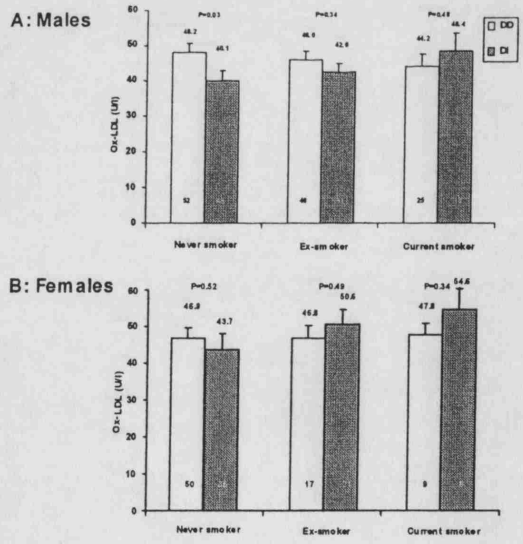
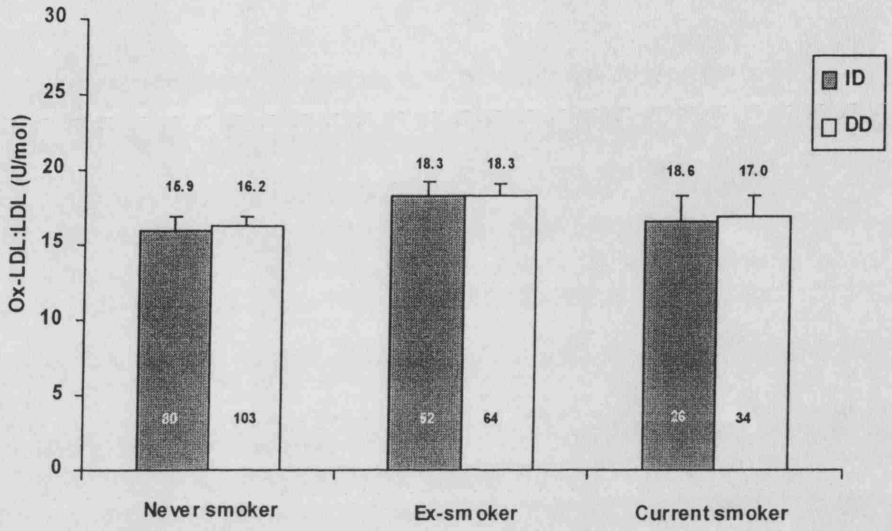


Figure 7.9: Plasma Ox-LDL:LDL in relation to *UCP2* D/I genotype in all subjects by smoking status



7.2 Combined effect of the *UCP2* -866G>A and 45bp D/I variants in determining plasma TAOS

7.2.1 Introduction

As described in section 7.1 and in chapter 6, plasma TAOS appears to be mainly under the influence of the -866G>A variant compared to the D/I variant. Previously, the -866G>A variant has been suggested account for 71% of the variance in the D/I transcript ratio (Esterbauer *et al.* 2001). *In vitro,* studies using human adipose tissue, showed that the -866A allele was associated with increased mRNA transcription and reduced mRNA stability compared to the -866G allele. Furthermore, in human adipose, the D allele was associated with increased RNA stability compared to the I allele. (Esterbauer *et al.* 2001; Wang *et al.* 2004). However, *in vivo*, haplotype analysis of the -866G>A and D/I variant showed results consistent with a single-locus and supported the association of obesity with the -866G>A site. The combined effects of these gene variants have not been previously explored in relation to plasma markers of oxidative stress.

7.2.2 Aims

The aim of this section was to explore the combined effect of the -866G>A and D/I variant in determining plasma TAOS.

7.2.3 Results

7.2.3.a Linkage Disequilibrium between the UCP2 -866G>A and 45bp D/I gene variants

The LD between the 2 gene variants is demonstrated in table 7.3. The D' between the variants was 0.71 ($p<0.001$), being similar to that of previous studies (0.74-0.77 (Esterbauer *et al.* 2001; Wang *et al.* 2004)), suggesting a moderate to high degree of LD.

Table 7.3: LD amongst *UCP2* -866G>A and 45bp D/I gene variants in all subjects

		UCP2 -866		
		GG	GA	AA
UCP2 45bp D/I	DD	263 (34.5%)	115 (15.1%)	17 (2.2%)
	ID	44 (5.8%)	235 (30.8%)	44 (5.8%)
	II	6 (0.8%)	11 (1.4%)	28 (3.7%)

Table 7.4, shows the frequencies of the different haplotype combinations of the two variants. In those subjects with an A allele, 62% also had an I allele present and conversely for those with a G allele, 92% also had a D allele present.

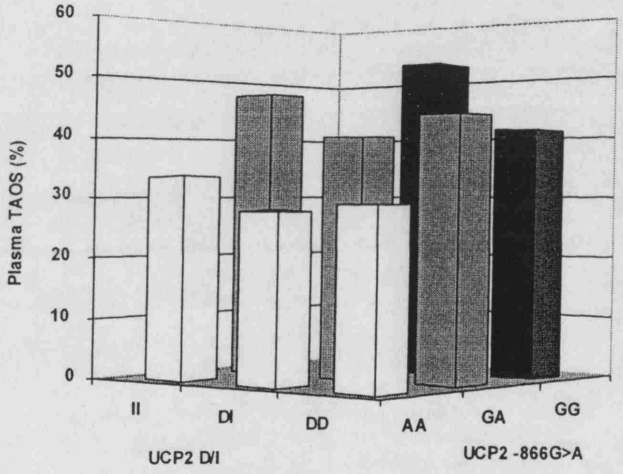
Table 7.4: Haplotype frequency for the *UCP2* gene variants in UDACS

Haplotype	G/D	A/D	G/I	A/I
Frequency	0.595	0.134	0.05	0.219

7.2.3.b Combined effects on plasma TAOS by CHD status

Since the association between the *UCP2* -866G>A variant and plasma TAOS was present in males with CHD, the combined effects of this and the *UCP2* D/I variant was studied in this group of subjects. Figure 7.10, shows plasma TAOS in these subjects by genotypes.

Figure 7.10: Combined *UCP2* -866G>A and D/I genotype effect on plasma TAOS in males with CHD



	GG	GA	AA
DD	41	19	1
DI	2	28	19
II	0	2	4

Number of subjects in groups

As shown in figure 7.10, and in line with results described previously in chapter 6, AA subjects had the lower plasma TAOS (irrespective of the D/I genotype). No clear effect can be observed with respect to the D/I variant.

Further haplotype analysis was performed with the use of the *Thesias* statistical package, under the guidance of Emma Hawe (Centre statistician). This looks at haplotype effects in unrelated individuals and assumes an additive effects model. As shown in table 7.5, no significant differences were observed on plasma TAOS by haplotype. However, plasma TAOS was lowest in A carriers (A/D and A/I) and therefore, in line with the previous results homozygosity for the A allele would be associated with lower TAOS.

Table 7.5: Plasma TAOS by haplotype in males with CHD

Haplotype	Frequency	Plasma TAOS Mean (95% CI)*	P
G/D	0.638	21.2 (19.1-23.2)	Reference haplotype
G/I	0.023	30.9 (0.2-61.7)	0.53
A/D	0.131	18.9 (13.5-24.3)	0.46
A/I	0.207	16.8 (13.3-20.3)	0.06
Global p=0.15			

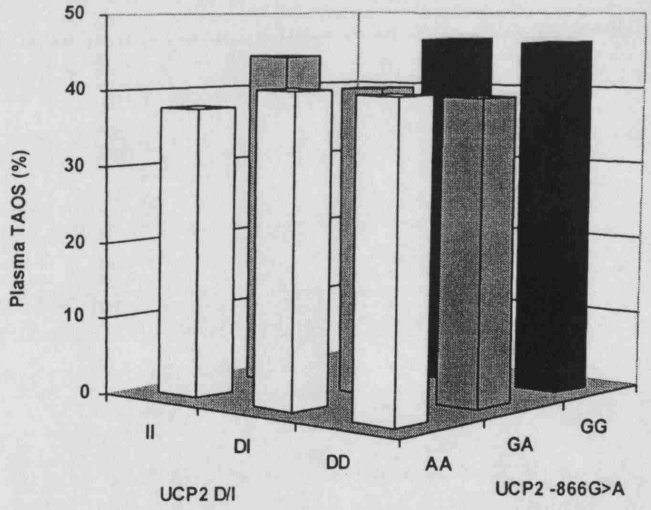
*Mean shown for haplotype that would be on one chromosome. This is an additive model, therefore these values may be added according to the haplotype combination. The global P-value is derived from the likely hood ratio test.

7.2.3.c Combined effects on plasma TAOS by smoking status

The combined effects of the *UCP2* -866G>A and the *UCP2* D/I variant were also examined in male smokers (current and ex) without CHD. As described in chapter 6, in these subjects, the A allele was associated with lower plasma TAOS.

As shown in figure 7.11, and in line with results described previously in chapter 6, GA/AA subjects had the lower plasma TAOS (irrespective of the D/I genotype). No clear effect can be observed with respect to the D/I variant.

Figure 7.11: Combined -866G>A and D/I genotype effect on plasma TAOS in male current and ex-smokers without CHD



	GG	GA	AA
DD	65	38	4
DI	12	50	11
II	0	4	5

Number of subjects in groups

Further haplotype analysis was performed with the use of the *Thesias* statistical package. As shown in table 7.6, plasma TAOS was lower in A allele carriers and this effect was

independent of that of the D/I variant, with both A/D and A/I having lower TAOS (p=0.07) compared to the common haplotype.

Table 7.6: Plasma TAOS by haplotype in male current and ex-smokers without CHD

Haplotype	Frequency	Plasma TAOS Mean (95% CI)*	P
G/D	0.60	22.7 (20.9-24.4)	Reference haplotype
G/I	0.05	24.1 (16.5-31.7)	0.72
A/D	0.16	18.5 (14.8-22.1)	0.07
A/I	0.19	18.6 (15.0-22.2)	0.07
Global p=0.15			

*Mean shown for haplotype that would be on one chromosome. This is an additive model, therefore these values may be added according to the haplotype combination. The global P-value is derived from the likely hood ratio test.

7.3 Discussion

The work described in this chapter has shown no robust associations between the *UCP2* D/I gene variant and plasma markers of oxidative stress and LDL-oxidation. Trends were observed for plasma TAOS, Ox-LDL and Ox-LDL:LDL to be lower in II subjects. No significant associations were observed between genotype and CHD or smoking to influence plasma TAOS or LDL-oxidation. Analysis was partly confounded by the small number of II subjects, which limited stratification of data. Previous studies looking at the D/I variant in relation to obesity have provided conflicting results (Dalgaard *et al.* 2001; Esterbauer *et al.* 2001; Marti *et al.* 2004). In a large prospective study of 544 obese Danish men and 872 healthy controls no association was observed respect to BMI and changes in body weight (Dalgaard *et al.* 1999).

In line with previous observations, the -866G>A and D/I variant are in moderate to high LD. In those subjects with an A allele, 62% also had an I allele present and conversely for those with a G allele, 92% also had a D allele present. Similar to previously reported results (Esterbauer *et al.* 2001) in relation to BMI, the results of the haplotype analysis are consistent with a single-locus and support the association of the -866G>A variant with lower plasma TAOS, independently of the D/I variant. Previously, *in vitro,* studies using adipose human tissue, have shown the -866A allele to be associated with increased mRNA transcription and reduced mRNA stability compared to the -866G allele. Furthermore, the D allele is associated with increased RNA stability compared to the I allele in subjects homozygous for the -866G allele. (Esterbauer *et al.* 2001; Wang *et al.* 2004). The results in this section fail to support any protective effect of the D allele in

relation to plasma oxidative stress and do not support any influence of the D allele in conferring any effect of the -866G>A variant.

In the following chapter, further analysis will focus on the -866G>A variant and its association with cellular ROS, in unstimulated and stimulated cells. The influence of RNA expression will also be investigated. No further work will be performed in relation to the D/I gene variant.

CHAPTER EIGHT

IN VITRO STUDIES EXPLORING THE ASSOCIATION BETWEEN THE *UCP2* -866G>A VARIANT AND CELLULAR OXIDATIVE STRESS

CONTENTS OF CHAPTER EIGHT

8.1 The *UCP2* -866G>A genotype and cellular oxidative stress

8.1.1 Introduction

The work in this section will focus on *in vitro* studies to further explore the association between the *UCP2* -866A allele and increased plasma markers of oxidative stress in diabetic males and with increased prospective CHD risk in healthy men, described in chapter 6. Since monocytes play an important role in inflammation, infection and atherogenesis, *in vitro* stimulation studies were performed to study cellular oxidative stress in monocytes from subjects selected by homozygosity for the -866G>A variant.

8.1.2 Aims

The primary aim of the work described in this section was to examine cellular ROS production in monocytes from subjects selected by homozygosity for the -866A and -866G allele, and secondly to examine the response in cellular oxidative stress by genotype, within environments associated with increased cellular 'stress'.

8.1.3 Cellular oxidative stress by genotype in 'stress' environments

8.1.3a Methods

Ethical approval was obtained for this study from the UCL/UCLH ethics committee and all subjects gave informed consent. Ten healthy age-matched male volunteers (aged between 22-30 years), were selected by homozygosity for the -866G>A gene variant. All subjects were non-smokers, non-diabetic and were free from any clinically apparent infection and inflammation at the time of venous sampling. Monocyte extraction from whole blood, cell culture, measurement of cellular oxidative stress and preparation of cellular 'stressors' was performed as described in chapter 2.

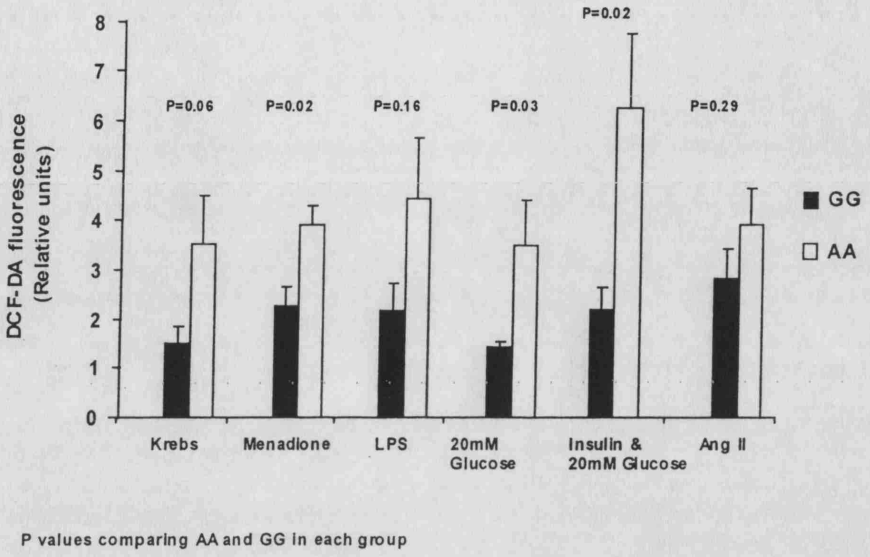
Initially, cellular DCF-DA fluorescence was measured in the basal culture with Krebs buffer (containing 5mM glucose) and in the presence of 5 different 'stressor' environments (Menadione, bacterial lipopolysaccharide-LPS, 20mM glucose, insulin and 20mM glucose, and angiotensin II).

8.1.3.b Results

Figure 8.1 shows the cellular DCF-DA fluorescence in the presence of the different 'stressors'. These are the results of two experiments performed in duplicate for each subject (5 AA, 5 GG). As shown, a difference in DCF-DA fluorescence by genotype was not only observed in the presence of basal Krebs reagent (2.3 fold higher in AA), but also with all the 'stressors' used. However, genotype difference in DCF-DA fluorescence was only significant with menadione (1.8 fold higher in AA), 20mM glucose (2.5 fold higher in AA), and insulin+glucose (2.9 fold higher in AA). Although there were difference by genotype in DCF-DA fluorescence for menadione, and 20mM glucose, the overall increase in DCF-DA fluorescence was not different to that of Krebs reagent.

In the further experiments in this chapter, investigations were performed in Krebs buffer (5mM glucose), and Krebs buffer supplemented with 20mM glucose, insulin+glucose, and menadione (as described in chapter 2). The above experiments were repeated on a further two occasions (in the experiments to follow) and the difference in DCF-DA fluorescence by genotype was consistently observed in these environments.

Figure 8.1: Cellular oxidative stress in monocytes by the *UCP2* -866G>A variant in 'stress' environments



This data therefore demonstrates a difference in cellular oxidative stress by genotype, not only in a basal state (Krebs with 5mM glucose) but also in the presence of 'stressors', specifically with 20mM glucose, insulin+glucose and menadione. As described in chapter 1, glucose may increase cellular oxidative stress via both intra- and extra-mitochondrial mechanisms. Menadione directly increases mitochondrial superoxide generation by increasing electron transfer through the respiratory chain. Monocytes from GG subjects had lower cellular oxidative stress in both of these environments. This data therefore supports an antioxidant role for the *UCP2* -866G allele.

8.1.4 Reducing extra-mitochondrial ROS with a NADPH oxidase inhibitor

8.1.4.a Methods

Diphenyleneiodonium chloride (DPI) is a NADPH oxidase inhibitor. Therefore, this compound would lower extra-mitochondrial oxidative stress and should therefore lower the overall oxidative cellular burden. Incubation was therefore performed in the presence and absence of DPI, to firstly assess whether the 'stressor' effects of 20mM glucose, insulin+glucose, and menadione were dependent on NADPH oxidase (i.e. extra-mitochondrial), and secondly to assess the difference in DCF-DA fluorescence by genotype in the presence of a lower oxidative cellular burden. In these studies, DPI was added to the culture medium at 10μM for one hour. These are the results of two experiments performed in duplicate for each subject (5 AA, 5GG) after adjusting for wells with DCF-DA and no cells. The methods are further detailed in chapter 2.

8.1.4.b Results

As shown in figure 8.2A and 8.2B, in the presence of Krebs buffer or 20mM glucose, the difference in DCF-DA fluorescence was abolished by the addition of DPI, suggesting that the observed difference in cellular oxidative stress by genotype in these environments is dependent on extra-mitochondrial sources of oxidative stress, and further supports a modulating effect by genotype in response to the oxidative stress in the cell as a whole. This is further supported by the results obtained in culture with insulin+glucose. As described in section 8.1.3, insulin+glucose was associated with the highest DCF-DA fluorescence and furthermore the greatest difference by genotype. As shown in figure 8.2.C, in the presence of DPI, the genotype-dependent difference decreased from a 2.7-

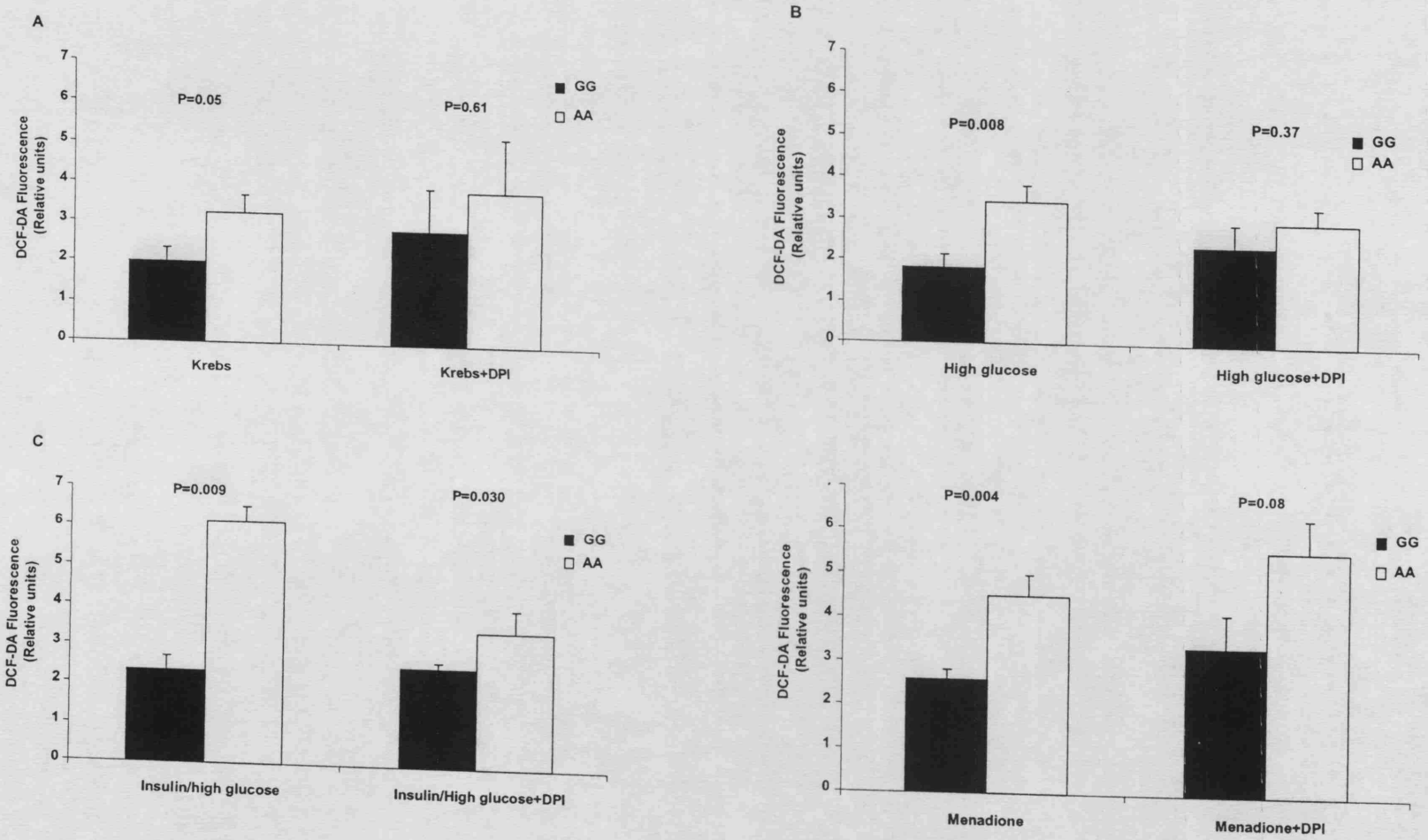
fold increase in AA subjects to a 1.4-fold increase (p=0.05). No change was observed in DCF-DA fluorescence in GG subjects.

With respect to menadione, the difference in DCF-DA fluorescence by genotype essentially remained unchanged after the addition of DPI. Menadione is a specific and direct mitochondrial stimulant of superoxide production. Therefore, DPI, which essentially blocks extra-mitochondrial sources of oxidative stress, would not be expected to alter the difference in DCF-DA fluorescence by genotype.

This data therefore suggests that 'stressors' which increase oxidative stress, by extra- or intra-mitochondrial mechanisms are associated with a difference in cellular oxidative burden by genotype. The difference in cellular oxidative burden by genotype is present if an extra-mitochondrial 'stressor' is present, or if an intra-mitochondrial 'stressor' is present (even in the absence or a reduced extra-mitochondrial source.)

In summary, this data suggests an antioxidant role for UCP2 and a differential effect of the -866G>A variant in modulating cellular oxidative burden in response to both extra- and intra-mitochondrial 'stressors' associated with increased oxidative stress.

Figure 8.2: Reducing extra-mitochondrial ROS with a NADPH oxidase inhibitor



8.1.5 Studies with *UCP2* oligonucleotide antisense

8.1.5.a Methods

As described in chapter 2, a *UCP2* antisense oligonucleotide was developed complementary to the sequence at the start of the *UCP2* transcription site. The expected result of this would be to inhibit/reduce transcription and subsequently protein synthesis. Incubation was therefore performed in the presence and absence of antisense oligonucleotide, to firstly assess whether this would influence cellular DCF-DA fluorescence and whether such an effect would be different by genotype. This was performed in cells cultured in Krebs, 20mM glucose, insulin+glucose, and menadione. Since the studies described in sections 8.1.3 and 8.1.4, suggest a possible greater antioxidant role with respect to the -866G allele, one *might* expect that antisense might be associated with increased cellular oxidative stress in GG subjects, but this might remain higher and unchanged in AA subjects. These are the results of two experiments performed in duplicate for each subject (5 AA, 5GG) after adjusting for wells with DCF-DA and no cells.

8.1.5.b Results

As shown in figure 8.3, the effect of *UCP2* antisense was to abolish the difference observed by genotype in cellular DCF-DA fluorescence, and this effect was observed with cells incubated in Krebs, 20mM glucose, insulin+glucose and menadione. In all four experiments, the common GG genotype showed an increase in cellular DCF-DA fluorescence following the addition of *UCP2* antisense. In subjects with the AA genotype, basal cellular DCF-DA fluorescence was higher compared to GG, but no significant increase was observed after the addition of antisense. The percentage changes in cellular DCF-DA fluorescence following the addition of *UCP2* antisense, by genotype

are shown in table 8.1. In the GG subjects, the greatest increase in DCF-DA fluorescence was observed in cells incubated with insulin+glucose (168% increase). As described in section 8.1.3, the greatest difference in DCF-DA fluorescence by genotype was observed with insulin+glucose, with relative protection being afforded by the GG genotype. *UCP2* antisense had an effect to make GG subjects respond in a similar way to AA subjects.

Table 8.1: Percentage change in DCF-DA fluorescence in AA and GG subjects following the addition of *UCP2* antisense

	AA	p	GG	p
Krebs	-7.5%	0.71	+44.1%	0.15
20mM glucose	-27.0%	0.13	+58.6%	0.26
Menadione	+5.0%	0.79	+49.4%	0.09
Insulin+glucose	+4.9%	0.88	+168.2%	0.02

As a control for the above experiments, the effect of scrambled *UCP2* antisense was also examined in relation to the absence and presence of *UCP2* antisense with respect to cell culture with Krebs reagent (figure 8.4). No difference was observed in DCF-DA fluorescence between scrambled *UCP2* antisense and those without *UCP2* antisense.

Figure 8.3: Cellular ROS generation by *UCP2* -866 genotype with UCP2 antisense

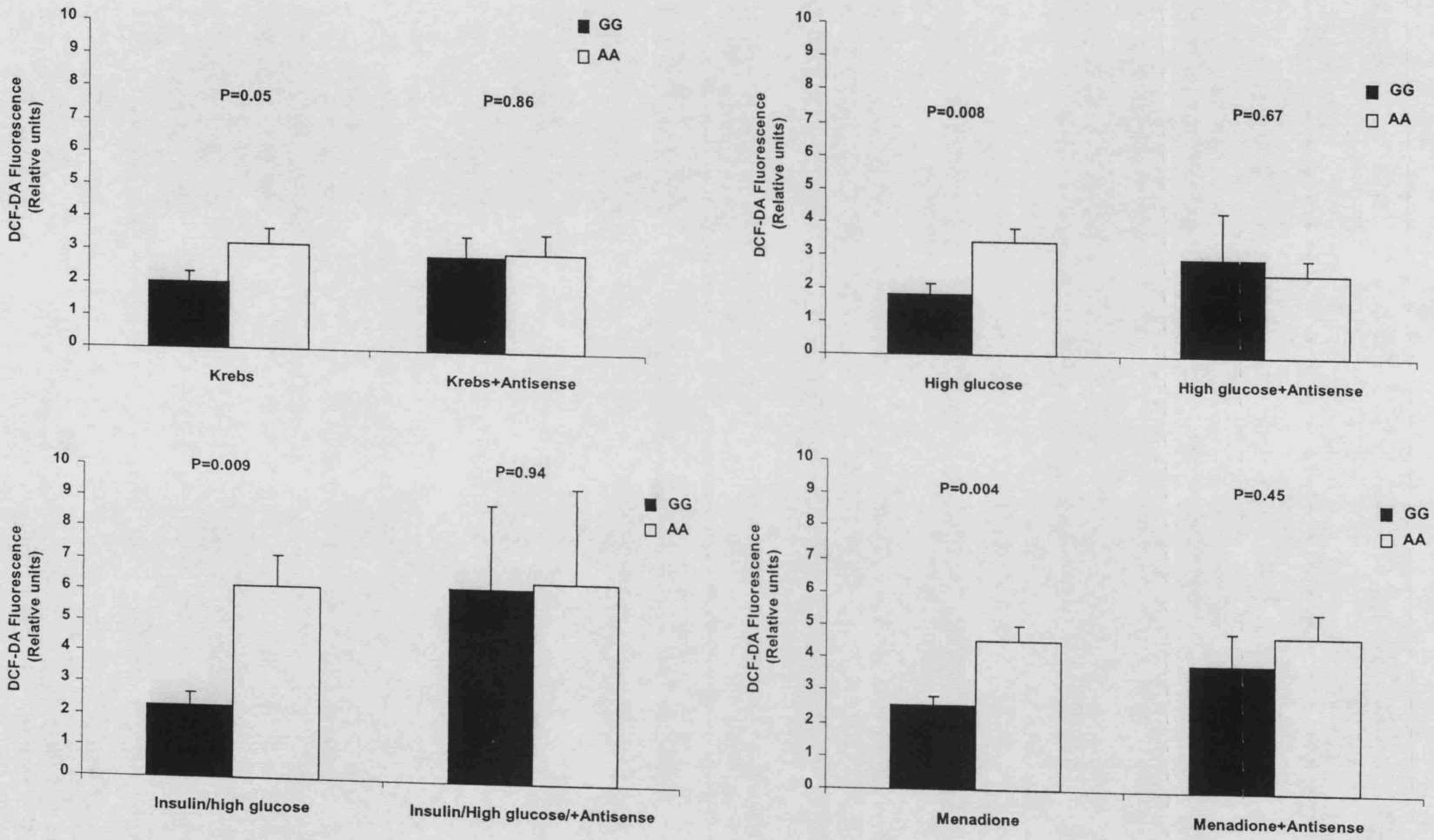
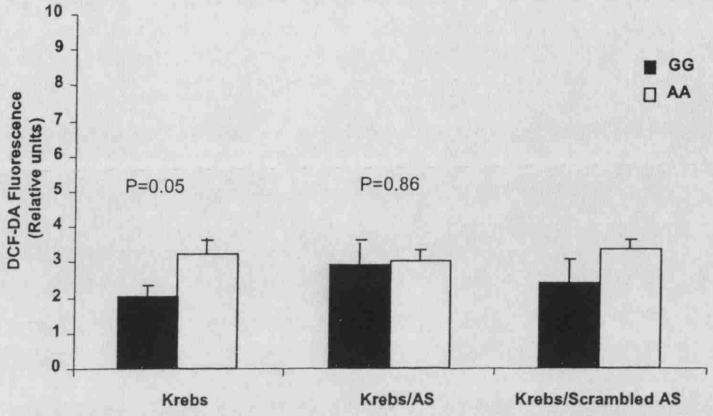


Figure 8.4: DCF-DA fluorescence with *UCP2* antisense and scrambled antisense



This data, suggests that if *UCP2* transcription is abolished/reduced with antisense, then in the presence of a 'stressor', the GG genotype is associated with increase cellular oxidative burden similar to the AA genotype. Hence, this data supports the concept that UCP2 has an anti-oxidant role in GG (homozygous wild-type) subjects, but in AA subjects, UCP2 is defective. This might arise at the level of transcription, translation or in post-translational modification of the protein.

8.2 Quantitative mRNA expression and the *UCP2* -866G>A variant

8.2.1 Introduction

The work in this section describes *in vitro* studies to further explore the association between the *UCP2* -866G>A genotype in relation to *UCP2* mRNA expression. *UCP2* mRNA expression in monocytes will be studied by genotype in a basal and stimulated state.

8.2.2 Aims

The primary aim of the work described in this section was to examine *UCP2* expression in monocytes from subjects selected by homozygosity for the -866A and -866G allele, and secondly to examine any change in *UCP2* expression associated with increased cellular 'stress'.

8.2.3 Methods

This work was performed on peripheral blood monocytes obtained from the same subjects described in section 8.1. Quantification of *UCP2* expression was performed using Taqman, where *UCP2* expression was compared to β-actin. Further details are described in chapter 2.

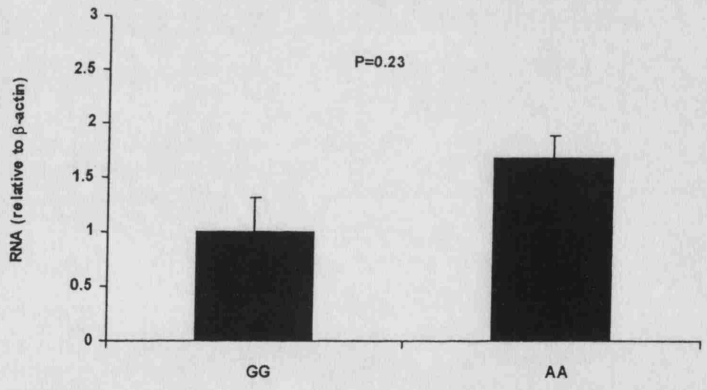
Initially, *UCP2* expression was compared by genotype in monocytes extracted and purified from whole blood, without cell culture. Subsequently, all experiments were performed on cultured monocytes by the methods described in chapter 2. In these experiments, *UCP2* expression was compared in a basal state (culture in Krebs reagent) and after culture with menadione or insulin+glucose. Experiments were performed in

duplicate and mRNA quantification performed in triplicate. In the analysis, comparisons were then made between changes in expression by *UCP2* genotype (GG v AA) in a basal state (Krebs buffer) and following stimulation (insulin+glucose or menadione). The GG cells in basal Krebs solution were set as the reference point.

8.2.4 Results

Firstly, in extracted uncultured monocytes, as demonstrated in figure 8.5, AA cells had a 1.7-fold higher expression compared to cells from GG subjects, however this was not statistically significant, p=0.23.

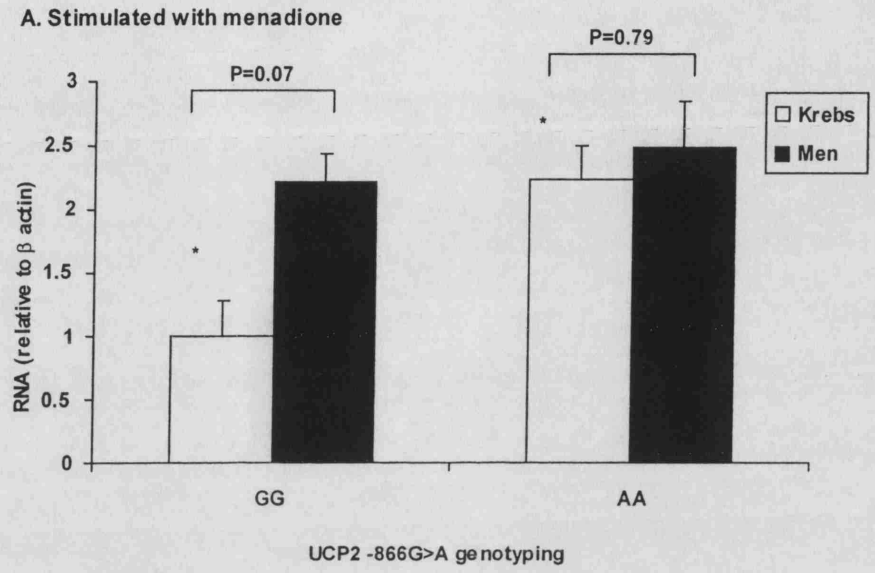
Figure 8.5: *UCP2* expression by genotype in uncultured monocytes



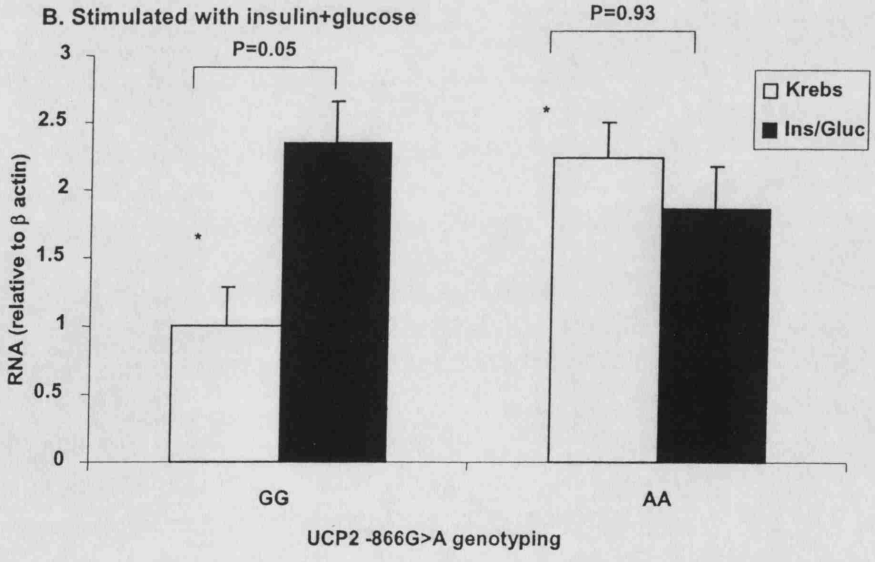
Cultured monocytes were incubated with the 'stressors':- menadione, and insulin+glucose respectively (figure 8.6). *UCP2* expression was non-statistically higher in cells from AA subjects compared to those from GG subjects (p=0.07). Following stimulation with menadione, there was a 2-fold increase in mRNA in GG subjects

(p=0.07), but no change in AA subjects. Similar results were observed following stimulation with insulin+glucose. In GG subjects there was again a 2-fold increase (p=0.05), whilst no difference was observed in AA subjects.

Figure 8.6: *UCP2* expression by genotype in cultured monocytes



*P=0.07



*P=0.07

8.3 Discussion

This section demonstrated that *UCP2* has an anti-oxidant role *in vitro*, and this response is dependent on the -866G>A variant. Monocytes from subjects homozygous for the -866G allele had lower basal and stimulated cellular oxidative burden, compared to those homozygous for the -866A allele. This effect was observed not only in relation to increased mitochondrial oxidative burden, but also in relation to total cellular oxidative burden. Therefore, this supports an anti-oxidant role for UCP2. An interesting observation was that reducing the overall cellular oxidative burden with DPI, abolished the difference observed by genotype, thus re-enforcing the concept of a greater anti-oxidant effect of the GG compared to the AA genotype in the face of a 'stressor' stimulus. These *in vitro* observations support the *in vivo* findings of chapter 6, where increased plasma oxidative stress was associated with the -866A allele in the presence of pro-oxidant risk factors. Furthermore, 'blocking' *UCP2* transcription with *UCP2* antisense, in cells of the common-wild type genotype (GG), was associated with increased cellular oxidative burden, similar to that observed in cells from subjects homozygous for the -866A allele. This observation further supports the anti-oxidant role of the wild-type variant, as reducing *UCP2* expression results in lower anti-oxidant potential.

These findings are in line with previous studies supporting the anti-oxidant role of UCP2 in animal and *in vitro* studies. In mice chronically infected with *Toxoplasmais gonadii*, in-situ hybridisation showed increased UCP2 expression, associated with cellular infiltration of monocytes and macrophages (Arsenijevic *et al.* 2000). Furthermore, previously published, *in vitro* studies have shown that *UCP2* gene expression is induced

by both extra- and intra-mitochondrial sources of ROS (Pecqueur *et al.* 2001; Echtay *et al.* 2002). Therefore, UCP2 appears to have an important intrinsic anti-oxidant role. The antisense experiments are also in line with a previously published result, showing that selective down-regulation of *UCP2* with antisense increases endothelial cell ROS generation (Duval *et al.* 2002).

A difference in *UCP2* expression was observed by genotype. In basal culture, with Krebs reagent (containing 5mM glucose), cells from AA subjects had a greater than 2-fold increase in mRNA expression compared to GG subjects. Following stimulation with insulin+glucose or menadione, *UCP2* expression increased in GG subjects (2-fold), but not in AA subjects. In GG subjects, *UCP2* expression was therefore increased in a pro-oxidant environment, compatible with the anti-oxidant role of UCP2. In line with previous studies, in AA subjects, basal *UCP2* mRNA levels were higher than GG subjects. In adipose tissue, the -866A allele has been associated with enhanced mRNA expression *in vivo* and *in vitro*, in reporter gene constructs expressed in human adipocytes cell line (Esterbauer *et al.* 2001). This difference may be related to different transcriptional activities of the wild type and variant promoters. Computational analysis of bp -879/-839 has shown putative binding sites for several *trans* factors. Preferential binding of some of the presumptive *trans* factors to the variant or wild type promoter may confer specific advantages for either allele. The presence of binding sites for the dimmers aryl-hydrocarbon nuclear translocator (ARNT)-aryl-hydrocarbon receptor (AHR) and ARNT-hypoxia inducible factor 1a (HIF1A) may indicate a possible link between oxidative stress and toxic signals with *UCP2* expression. Furthermore, in rat β-cells, PAX6, a *trans* factor for pancreatic islet function, influenced transcription in an

allele-specific manner with increased transcription being associated with the A allele (Krempler *et al.* 2002). Unlike the experiments described in this chapter, unfortunately, these studies have not looked at difference in expression by genotype following stimulation.

The increased basal expression associated with the AA compared to the GG genotype might be due to the possibility that protein synthesis is somehow 'defective' in cells from AA subjects. 'Sensing' this, in these subjects there might be increased transcription in an attempt to maintain the homeostasis of oxidative cellular burden. Furthermore, this might explain the failure of increased expression following stimulation and the higher oxidative cellular burden observed with this genotype. This is speculative, and direct determination of UCP2 protein level would help to clarify this. An important limitation of the study, which is shared with other studies in the field, is the lack of measurements of UCP2 protein. Such measurements have been hampered by the non-availability of specific antibodies (Pecqueur *et al.* 2001; Li *et al.* 2003).

In summary, the *in vitro* studies described in this chapter, complement the results of the *in vivo* association between the *UCP2* -866G>A variant and plasma markers of oxidative stress and CHD risk described in chapter 6. These studies provide the first *in vitro* evidence for modulation of the anti-oxidant role of *UCP2* by genotype, in the face of different cellular 'stressors', in relation to the measurement of cellular oxidative burden and at the level of UCP transcription. The development of a specific monoclonal antibody to UCP2 will further clarify this.

CHAPTER NINE

GENERAL DISCUSSION AND OVERVIEW

The work presented in this thesis explores the role of total plasma oxidative stress, and more specifically LDL-oxidation, in relation to CHD, its associated risk factors, and specific variants in selected candidate genes. The majority of the work focused on a high risk group of subjects with diabetes mellitus, which is a major risk factor for CHD morbidity and mortality. In these subjects, no more than 25% of the excess CHD risk can be accounted for by traditional risk factors (Pyorala *et al.* 1987). Considerable evidence exists to support the concept that increased oxidative stress has a role in the pathogenesis of CHD at the molecular level (Cai *et al.* 2000; Harrison *et al.* 2003), but previously, no data exists to support an aetiological role at the clinical level. Oxidative stress may result in numerous adverse effects at the molecular level. One of these deleterious effects is to result in LDL-oxidation (Witztum *et al.* 1997), a specific end-point measure of oxidative damage. As described in this thesis (and supported by previous studies), global oxidative stress and LDL-oxidation are not correlated (Kopprasch *et al.* 2002; Weinbrenner *et al.* 2003). This paradoxical observation is not surprising, since, in the plasma as a whole, antioxidants and pro-oxidant molecules will continually change in response to the surrounding environment. In this respect, plasma TAOS is a useful measure, as it provides the net result of these effects.

Biochemical markers of oxidative stress

The measurement of plasma TAOS is cheap, rapid and relatively easy to perform. The high correlation with plasma F_2-isoprostane re-enforces its possible use as a cheaper and less technically-demanding alternative, which is of particular value in large epidemiological studies. Of interest, the strongest correlates with plasma TAOS were

random glucose and Tg, two of the common abnormalities associated with T2DM. In UDACS, plasma TAOS was higher in subjects with CHD, and a threshold effect was observed by quartiles of plasma TAOS, with the lowest quartile having a significantly higher proportion of subjects with CHD. No previous studies have looked at prospective risk associated with baseline measures of plasma oxidative stress. In the prospective NPHSII sample, lower plasma TAOS was associated with a significant increase in CHD cases. Again a threshold effect was observed as in UDACS, with subjects in the lowest quartile having a doubling in CHD risk, which was independent of 'traditional' risk factors. This risk is substantial and equates to that of cigarette smokers or subjects with diabetes. Measuring plasma TAOS in another prospective sample is required to replicate this observation. To this end, measurement is currently being performed in samples from WOSCOPS, which will also allow more accurate assessment of the influence of statin therapy on plasma oxidative stress. Replication and confirmation of this observation in other studies might suggest the usefulness of measuring plasma TAOS, as a way to identify subjects at high risk of CHD.

In UDACS, plasma Ox-LDL:LDL was also significantly higher in male subjects with CHD. This is a measure of the proportion of detectable Ox-LDL per mmol LDL. Again, a threshold effect was observed, with a higher prevalence of CHD in subjects in the upper quartile of Ox-LDL:LDL. As described, this effect was observed regardless of statin therapy. Of interest, absolute Ox-LDL, like total cholesterol and LDL, appeared to be influenced by statin therapy, and levels did not differ by CHD status (presumably due to increased statin use in CHD subjects). These observations suggest even though statins

lower plasma levels of both LDL and absolute Ox-LDL, the proportion of detectable Ox-LDL per mmol LDL might remain unchanged. Further prospective analysis is required in this area, as there is strong and consistent evidence to support the role of statins in reducing CHD morbidity and mortality, by both LDL lowering effects and other lipid-independent effects. Depending on these results, the measurement of Ox-LDL:LDL might be considered as an aid in assessing CHD risk.

Genetic variation and oxidative stress

Variation in three candidate genes were studied in relation to plasma total oxidative stress and LDL-oxidation. Previously, numerous gene variants implicated in oxidative stress, have been studied in relation to CHD risk (Forsberg *et al.* 2001). However, surprisingly, few have looked at the effects of gene variants in relation to intermediate biochemical phenotypes. The three candidate genes were selected in part because of previously published associations with CHD (e.g. *APOE* and *GST*) and also because each plays a very different role in cellular physiology. ApoE is an important plasma lipoprotein, GST is a cytoplasmic antioxidant protein and UCP2 an uncoupler of proton transport within the mitochondrial membrane. Importantly, the effects of these variants were studied in a sample of subjects with diabetes, which is 'typically' associated with increased oxidative stress and CHD. Analysis in such a 'stressed' sample of subjects might therefore reveal important differences which would not be apparent in a healthy 'unstressed' group of subjects.

With respect to the common variants in *APOE*, the ε4 allele was associated with increased susceptibility to LDL-oxidation by smoking. This effect was also dependent on LDL size, such that, ε4 smokers with sd-LDL had higher LDL-oxidation. This finding provides a possible explanation for the increased CHD risk observed with the ε4 allele in previously published prospective studies, where the effect of the ε4 allele appeared to be independent of 'traditional' risk factors (Humphries *et al.* 2001). Previously, *in vitro* at the protein level, ε2 has been shown to possess antioxidant activity, which is thought to be independent of lipid parameters. In UDACS, smokers with the ε2 allele had the highest plasma TAOS. Therefore, in the face of an environmental stimulus, this might reflect a stimulus-induced antioxidant effect in ε2 carriers (perhaps by increasing apoE levels).

Variation in the *GSTT1* gene variant was associated with difference in LDL size, Ox-LDL and Ox-LDL:LDL. Furthermore, there was appeared to be an interaction (albeit of borderline significance) between the *GSTT1-1* variant with smoking to increase LDL-oxidation. Plasma CRP was also higher in *GSTT1-1* subjects compared to *GSTT1-0* subjects, except in *GSTT1-0* smokers, where the levels were similar. No previous data has been published looking at the association of plasma markers of oxidative stress with this variant. The association of this gene variant with LDL-size may be a reflection of the genotype difference in LDL-oxidation.

Much of the thesis focused on the *UCP2* -866G>A variant. Apriori, UCP2 expression by virtue of its uncoupling role, would be expected to influence mitochondrial ROS

generation in an antioxidant manner. UCP2 is induced by increased oxidative stress (Echtay *et al.* 2002). The -866A allele was associated with increased plasma oxidative stress (lower TAOS and higher F_2-isoprostane) in men with CHD (a state associated with high oxidative stress/and associated risk factors). The same effect was observed in men free from CHD, with previous exposure to cigarette smoke. This data suggests that the rare A allele, is 'less effective' compared to the G allele, in uncoupling the proton gradient and allowing the proton entry into the mitochondria and subsequent neutralisation of superoxide. This association did not translate into CHD risk within UDACS, as described in chapter 6. However, in the prospective NPHSII sample, ten year prospective CHD risk was doubled in AA subjects compared to G allele carriers, and this effect was independent of other classical risk factors. The presence of other risk factors (diabetes, hypertension, obesity) was associated with a further substantial increase in risk in AA subjects. Therefore, analysis of this gene variant in these two datasets provides a possible mechanism for increased CHD risk in terms of a biochemical intermediate which is relevant to our understanding of UCP2. Strong LD exists between variants within this gene. Analysis of the *UCP2* 45bp D/I variant, did not provide any robust association with plasma markers of oxidative stress. In line with previous studies (Esterbauer *et al.* 2001), haplotype analysis with the -866G>A variant, suggested that the association of the latter was the result of a single locus.

The gene association data was substantiated by *in vitro* studies in monocytes, selected by homozygosity for the *UCP2* -866G>A variant, which demonstrated increased cellular ROS associated with the A allele. Of note, the greatest difference by genotype was

observed in cell culture with insulin and glucose, which would increase electron flow through the electron transport chain and subsequently increase ROS generation. Supporting a potential antioxidant role for UCP2, reducing the overall oxidative burden on the cell with a NADPH oxidase inhibitor, reduced the genotype difference in cellular ROS. Furthermore, 'blocking' UCP2 transcription with an antisense oligonucleotide, resulted in an increase in cellular ROS in subjects of the wild-type genotype (GG), to a level observed in those with the AA genotype. In line with previous studies (Esterbauer *et al.* 2001; Krempler *et al.* 2002), the AA genotype was associated with greater than 2-fold increase in mRNA expression. However following incubation with a 'stressor', expression increased in GG cells, but no change was observed in AA cells. A possible explanation for this would be that protein synthesis was 'defective' in AA cells, and 'sensing' this, the cellular response might be increased transcription (and hence increased mRNA) to maintain cellular homeostasis. As described, further work at the protein level will clarify this, when a specific antibody has successfully been developed.

In conclusion, the data obtained in this thesis has identified an association of plasma markers of oxidative stress with CHD and associated risk factors in diabetes mellitus. It has also identified the effects of selected gene variants, and their interaction with environmental factors, on plasma levels of oxidative stress, which suggest that increased oxidative stress is important in the development of CHD. Increased plasma oxidative stress (and lower plasma TAOS) could be a *cause* or *consequence* of CHD. However, this effect can partly be distinguished by the use of gene association studies (as a genetic tool). This may be observed by the fact that subjects homozygous for the *UCP2* -866A

allele had increased plasma markers of oxidative stress and were also at risk a higher prospective CHD risk. This observation strongly suggests that increased oxidative stress is *causal* and not simply a *consequence* of CHD (i.e. genotype predisposes to increased oxidative stress and subsequently CHD risk). This is consistent with the concept of Mendelian Randomisation (Little *et al.* 2003). This method may prove useful in identifying other candidate genes which may influence plasma biochemical intermediate phenotypes of oxidative stress, and their effect on CHD risk. Using this approach, key molecules transcribed from important candidate genes might be identified which may offer therapeutic options to reduce oxidative stress and CHD risk (e.g. mitochondrial uncouplers). This is compelling, as to date, several antioxidants studied in clinical practice have shown no effects on CHD risk (Kritharides *et al.* 2002; Tornwall *et al.* 2004). This work provides novel insight and fundamental understanding of the role of oxidative stress in CHD, at a population and a cellular level, in terms of biochemistry and genetics.

CHAPTER TEN

STUDY LIMITATIONS, WEAKNESSES AND FUTURE WORK

CONTENTS OF CHAPTER TEN

10.1 Study limitations and weaknesses

Although every effort was made to address limitations and weaknesses within the study, it was unavoidable that some would arise. These are discussed below.

10.1.1 Recruitment of samples

Analysis in UDACS focused on Caucasian subjects with diabetes. The number of subjects from other ethnic origins was small and hence these were excluded from further analysis (as described in chapter 2). This is being addressed in another study by Dr Helen Ireland, in the Ealing Diabetes Study where the focus is on subjects with a South Indian origin. These samples would become available for further work and replication of the findings within UDACS.

As described throughout the thesis, subjects within UDACS were taking a mixture of oral hypoglycaemic agents, insulin, antihypertensive agents and lipid lowering therapy. Although I have stratified for some of these therapies, it was not possible to adjust for all of these therapies in the analysis, as this would considerably reduce power. Furthermore, these therapies *might* lead to confounding or masking of genotype effects (e.g. on TAOS). Therefore, the results must be interpreted with this in mind. The effect that statin therapy *might* potentially have on Ox-LDL is described in chapter 3, page 157. Of note, no differences were observed by treatments with ACE inhibitors and aspirin on the biochemical measures of oxidative stress (as described in chapter 3). Furthermore, no difference was observed in the prevalence of the various therapies by genotype for the variants described in the thesis.

Within UDACS, subjects with type 1 and type 2 diabetes mellitus were analysed together, i.e. the sample was not stratified by type of diabetes. It is possible that the aetiology of CHD risk phenotypes may be different in type 1 and type 2 diabetes, and combining the data in this way may lead to confounding. In any future study or in any extension of the current sample this would need to be addressed rigorously by recruiting enough of both groups of subjects to be statistically robust.

10.1.2 Biochemical measures

Since I was the sole recruiter of UDACS and there was a limited time interval for sample collection, not all samples collected were non-fasting. This *might* therefore cause a problem in interpreting lipid measurements and their impact on CHD risk. This is particularly important with respect to plasma triglyceride levels and less so for LDL. The only results likely to be influenced by this are the LDL particle size measurements described in the thesis. Furthermore, Ox-LDL *might* be influenced by the non-fasting state, however there is no strong evidence to suggest this. In a 'true clinic population' this might also provide a more accurate representation of a clinic-based sample.

Within UDACS, plasma proteins and uric acid concentration was not measured, and thus plasma TAOS was not adjusted for these measures. As described in chapter 1, previous studies have suggested that plasma TAOS *might* be influenced by these plasma components (Erel 2004). These factors would be unlikely to introduce a systematic bias to

the genetic results, but they may add "noise" to the data, and reduce the power to pick up the expected modest genotype effects on risk traits.

With regard to the NPHSII data, plasma for the measurement of TAOS was only available on 310 samples from the NPHSII sample. As described in chapter 3, this plasma had been stored as a batch for 10 years at -80°C and, perhaps not surprisingly, the mean plasma TAOS levels were lower in these samples compared to those of UDACS. This *might* therefore represent degradation in plasma antioxidants over the period of storage. Nevertheless, all NPHSII samples were stored under identical conditions so this could not be a confounder.

In the NPHSII sample, plasma HDL was not measured at recruitment. This would be a useful measure to study, since HDL has been shown to possess antioxidant and anti-inflammatory properties and the relationship between HDL and TAOS would be relevant to examine.

10.1.3 Genotyping

All genotyping was performed using MADGE. This was performed using 96-well array plates. Within the sample, there were four duplicate DNA samples within the cohort. These were used as controls to ensure that the PCR was working efficiently. A weakness arises in that more duplicate DNA samples should have been placed in the individual PCR plates. A duplicate rate of 5% would be re-assuring with respect to the accuracy of genotyping. Nevertheless, any genotype which was unclear underwent repeat PCR and analysis, and

genotypes were assessed by two observers. In other samples when 'hidden' duplicates were included and MADGE analysis used, reported error rates were <0.5% (Cook *et al* 2001). Any genotyping errors would be unlikely to add any systematic bias to the data, but would reduce the overall power of the study to find a statistically significant result.

10.1.4 UCP2 antisense studies

Even though the UCP2 antisense studies provided interesting results, there is a specific weakness. To clearly demonstrate that UCP2 antisense prevented or reduced transcription, the UCP2 mRNA should be quantified to ensure that mRNA quantity did actually decrease with antisense. This was not performed as there was difficulty in obtaining blood on repeat venesection of the recruited subjects in this study. To ensure that the same subjects were enrolled for each experiment, every attempt was made to ensure that the same subjects underwent venesection on each occasion. However, due to migration of subjects only a limited number of experiments could be performed. UCP2 protein was not assessed in relation to UCP2 antisense, since as described in chapter 8, no reliable antibody has been developed. This *might* be addressed in the future.

10.1.5 *UCP2* -866G>A power in UDACS

As described in chapter 6, no association was observed between the *UCP2* -866G>A variant and CHD risk within UDACS. One possibility is that UDACS simply failed to have adequate power to detect this. From NPHSII, the odds ratio (OR) for CHD associated with the AA genotype was 2.08. To achieve 80% power, with an α=0.05, if there were 609 controls, we would require 67 cases within UDACS, to achieve an OR of 2.08. To achieve

an OR of 1.70, with a power of 80%, and α=0.05, we would require 143 cases with 609 controls. Therefore, it does appear that UDACS (with 154 CHD cases) was adequately powered. The inability to detect an association with CHD in UDACS *might* therefore be related to the reasons described in the discussion in chapter 6, describing problems associated with determining risk in case-control studies. One of these problems is that case-control studies are prone to intrinsic bias, for example due to altered rates of disease progression and subsequent progression of secondary phenotypes, or genotypes associations with death or treatment. An example of this would be if the *UCP2* -866AA genotype was associated with increased CHD risk, then these subjects might have a higher mortality at a younger age in association with the clinical and biochemical phenotypes associated with diabetes. Furthermore, this *might* be 'masked' by the liberal use of pharmacological treatments known to alter the progression of CHD, such as statins, ACE inhibitors and aspirin.

10.1.6 Analysis and multiple comparisons

As described in chapter 2, no adjustment was made for multiplicity of testing and significance was considered when P <0.05. This *might* be considered as a weakness in the statistical analysis.

Correction for multiple comparisons was not applied to the results, because the study design was predominantly 'hypothesis testing'. It is recognised that adjusting statistical significance for the number of tests that have been performed on study data (the Bonferroni method) may create more problems than it solves (Perneger 1998; Rothman 1990). The

Bonferroni method is concerned with the general null hypothesis (that all null hypotheses are true simultaneously), which is rarely of interest or use to researchers exploring novel hypotheses in medical research. Furthermore, Bonferroni adjustments do not guarantee a 'prudent' interpretation of results (Perneger 1998). The likelihood of type II errors is also increased, so that truly important differences are deemed non-significant. In the thesis I have described what tests of significance have been performed and the reason why. The focus of analysis truly was on the biochemical measures of plasma oxidative stress (Ox-LDL, Ox-LDL:LDL and TAOS).

10.2 Future studies

10.2.1 Other studies within UDACS

Further work is currently being performed on the UDACS cohort to study the associations of other gene variants implicated in lipid oxidation and oxidative stress. Interest has focused on those within the *APOA4* gene (Professor Talmud) and the platelet-activating factor acetylhydrolase gene (Professor Talmud). These results will be available in the near future. Plasma interleukin-6 has also been measured on the UDACS cohort to allow further investigation of pro-inflammatory gene variants in subjects with diabetes. This work will also allow further analysis in respect to gene-environment and gene-gene interaction.

10.2.2 Prospective recruitment of a sample of subjects with diabetes

My next objective is to recruit a cohort of subjects with impaired glucose tolerance and to follow these subjects prospectively for the development of diabetes and CHD. This would allow a 'cleaner' sample to be collected at recruitment (i.e. medication free). I would also

plan to recruit a cohort of subjects where there is a first degree relative affected with type 2 diabetes and to follow these subjects prospectively. These studies will not only provide a sample for biochemical and genetic follow-up prospectively in relation to the development of diabetes, but also CHD. This will allow a prospective analysis of biochemical markers of oxidative stress and inflammation, and gene variants in relation to the above outcomes. This will provide a cohort to replicate the findings within UDACS and NPHSII (in relation to the *UCP2* -866G>A variant). Furthermore, the sample will be specific to type 2 diabetes.

10.2.3 WOSCOPS and plasma TAOS

Plasma and DNA samples are being made available from WOSCOPS to study the effect of statin therapy on plasma TAOS. The association of the *UCP2* -866G>A variant with plasma TAOS will also be studied, as will the interaction between this variant and statin therapy. This will provide a robust study group to replicate the findings from UDACS and NPHSII in relation to the *UCP2* -866G>A variant.

10.2.4 Further work relating to the *UCP2* -866G>A variant

I have been successful in co-writing a grant for further work relating to the *UCP2* -866G>A variant. The aim of this study is to look at the association of this variant in relation to endothelial function and plasma markers of oxidative stress in response to a glucose load. The work will be undertaken in subjects with normglycaemia, impaired glucose tolerance and type 2 diabetes, selected by *UCP2* -866G>A genotype. This will explore the possible influence of this gene variant on endothelial function and oxidative

stress *in vivo*, and to explore the effect of hyperglycaemia and genotype on the outcome measures.

Further cell culture studies are also planned to study the effects of ACE inhibitors and statins on cellular markers of oxidative stress, in monocytes recruited from subjects by genotype. The aim is to perform these studies in subjects from the above groups, by the methods described in chapter 8. Both statins and ACE inhibitors have been implicated to reduce oxidative stress and inflammation. These studies will build and further our understanding in this area.

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