# The use of a genetic strategy to study the role of modulation of oxidative stress by uncoupling proteins 2 and 3 in the pathogenesis of Type 2 Diabetes.

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Declaration

All the work reported in this thesis is my own, or has been carried out as part of a collaboration in which I played a major part.  All collaborative work has been acknowledged in the text [see chapter 2 (p132)].  No part of this thesis has been submitted for a degree, diploma or other qualification at any other University

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Abstract


Mitochondrial dysfunction has been implicated in the early pathogenesis of Type 2 Diabetes.  The uncoupling proteins 2 and 3 are mitochondrial proteins found in man that have been implicated in protecting mammals from the effects of over-nutrition.  Examination of the effect of genetic variation in the *UCP2-UCP3* genetic cluster has so far been inconclusive.  The aim of this thesis was to examine, using a genetic strategy, the hypothesis that the role of the uncoupling proteins 2 and 3 in the pathogenesis of Type 2 Diabetes is via modification of oxidative stress.

In a prospective study of nearly 3000 men the risk of type 2 diabetes at 10 years was increased for both the *UCP2-866AA* (1.94 [1.18-3.19]: p=0.009) and the *UCP3-55TT* (2.06 [1.06-3.99]: p=0.03) homozygotes.  This increased risk was not explained by the association with any measured conventional risk factors. Paradoxically, in a Europe-wide cross-sectional study of 598 subjects the *UCP2-866A* variant was associated with lower waist-hip ratio (GX v AA,1.00 [0.06] v 0.98 [0.07]; p=0.003), although also associated with lower insulin secretion (42.6 [24.6] v 35.6 [18.6]; p=0.03). The *UCP3* variant was not significantly associated with any metabolic trait.

The significant heritability of plasma markers of oxidative stress (TAS 0.54, TOAS 0.49) suggests anti-oxidant function is a plausible mechanism to determine Type 2 Diabetes risk.  The predictors of anti-oxidant stress in a family study were examined, as was the impact of *UCP2-UCP3* gene cluster variation. Genetic variation in the *UCP2-UCP3* was found to increase the risk of the Type 2 diabetes.  While UCP2 may modify insulin secretion directly, the mechanism of action for UCP3 is likely to involve novel risk factors for Type 2 Diabetes such as modification of mitochondrial oxidative stress.   Finally, the development of a human model is described to examine genetic influences on oxidative stress burden using a meal rich in used cooking oil.

Publications Arising from Work in this Thesis

**David R Gable**, Jeffrey W. Stephens, Jackie A. Cooper, George J. Miller, Steve E Humphries.Variation in the UCP2-UCP3 gene cluster predicts the development of type 2 diabetes in healthy middle-aged men
*Diabetes ;55:1504-11. 2006*

**David R Gable,** Jeffrey W Stephens, Sukhbir S Dhamrait, Emma Hawe, Steve E Humphries on behalf of the HIFMECH Study Group. European differences in the association between the UCP2 -866G>A common gene variant and markers of body mass and fasting plasma insulin.
*Diabetes, Obesity and Metabolism,9:130-131:2007*

**David R Gable**, Jeffrey W. Stephens, Jackie A. Cooper, George J. Miller, Steve E Humphries.  Variation in the UCP2-UCP3 gene cluster predicts the development of type 2 diabetes in healthy middle-aged men. *Atherosclerosis supplements 7:3:312:2007*

**Gable DR**, Stephens JW, Cooper JA, Miller GE, Humphries SE. The Association of UCP2-866G>A with Prospective Risk of Type 2 Diabetes is due to reduced insulin secretion from the Pancreas. *Atherosclerosis; 186:S6.2006*

**Gable D**, Stephens J, Hurel S. The 75g oral glucose load does not induce an increase in markers of oxidative stress markers in healthy volunteers. *Diabetic Medicine;23:S2:P76.2006*

**Gable DR**, Hurel SJ, Humphries SE. Adiponectin and its gene variants as Risk Factors for Insulin Resistance, The Metabolic syndrome and Cardiovascular Disease.  *Atherosclerosis 468:231-244 2006*

Jeffrey W. Stephens, **David R. Gable,** Steven J. Hurel, George J. Miller , Jackie A. Cooper, Steve E. Humphries. Increased Plasma Markers of Oxidative Stress Are Associated with Coronary Heart Disease in Males with Diabetes Mellitus and with 10-Year Risk in a Prospective Sample of Males *Clinical Chemistry;52:446-52. 2006*

**D.R. Gable**, R. Whittall, Ka Wah Li, J. Cooper, S.E. Humphries.  Identified Variants in the adiponectin gene act as markers for the metabolic syndrome in southern Europe but not the north. *Atherosclerosis supplements 7:3:368:2006*

**Gable D,** Stephens J, Humphries S, Hurel S.  +276G>T adiponectin gene SNP is associated with cardiovascular disease in patients with type 2 diabetes mellitus. *Diabetologica 48 (Suppl1) A131 349: 2005*

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**Abbreviations**

**GLUT**  Glucose transporters

**GSK-3**  Glycogen synthase kinase 3

**NEFA**  Non-Esterified Fatty Acids

**UCP-2**  Uncoupling Protein 2

**UCP-3**  Uncoupling Protein 3

**IRS-**  Insulin receptor substrate

**AMPK**  AMP-activated protein kinase

**SOCS**  Suppressor of cytokine signalling

**NF-Kβ**  Nuclear factor kappa β

**IKK**  I-Kappa-B Kinase

**IMCL**  Intra myocellular lipid

**MODY**  Maturity onset diabetes in the young

**ATP**  Adenosine Triphosphate

**ROS**  Reactive Oxygen species

**RNS**  Reactive Nitrogen species

**MDA**  Malondialdehyde

**HNE**  4-Hydroxynonenal

**LDL**  Low density Lipoprotein

**HDL**  High density Lipoprotein

**DNA**  Deoxyribonucleic acid

**TBARS**  Thiobarbituric AcidReactive Substances

**HPLC**  High Performance Liquid Chromatography

**ELISA**  Enzyme-Linked ImmunoSorbent Assay

**GCMS**  Gas chromatography-mass spectrometry

**GSH**  Glutathione

**GSSG**  Oxidised glutathione

**M2VP**  1-methyl-2-vinylpyridinium trifluoromethanesulfonate

**NADPH**  β-nicotinamide adenine dinucleotide phosphate

**FADH**  Flavin adenine dinucleotide-Hydrogen

**mRNA**  Messenger Ribonucleic Acid

**PPAR**  Peroxisome proliferator-activated receptors

**UTR**  Untranslated region

**LD**  Linkage disequilibrium

**CHAPTER ONE**

**INTRODUCTION**

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## 1.1 Type 2 Diabetes Mellitus

### 1.1.1 Definition

Diabetes Mellitus is a disorder characterised by hyperglycaemia. Diabetes is diagnosed on the basis of World Health Organisation (WHO) recommendations (Table 1.1) which also defines the pre-diabetic states of impaired glucose tolerance and Impaired fasting glucose.

**Table 1.1** The diagnosis of diabetes mellitus and pre-diabetes states from the Expert Committee on the Diagnosis and classification of diabetes mellitus (Diabetes Care: 20: 1183-1197:1997)

|  | **Glucose concentration in venous plasma (mmol/L)** |
|---|---|
| Diabetes mellitus | Fasting ≥ 7·0 or 2-h post-glucose load ≥11·1 |
| Impaired glucose tolerance | Fasting (if measured) <7·0 and<br>2-h post-glucose load ≥ 7·8 and <11·1 |
| Impaired fasting glucose | Fasting ≥ 6·1 and <7·0 and<br>2 h post-glucose load (if measured) <7·8 |

Glycaemia is regulated by a number of hormones but the key hormone is insulin. Failure to maintain normal glucose homeostasis is due to the inability of the organism to maintain normal insulin secretion from the β-cells of the pancreas or to maintain normal insulin action, a state defined as insulin resistance. Insulin resistance is present when the biological effects of insulin are less than expected for both glucose disposal in skeletal muscle and suppression of endogenous glucose production, primarily in the liver (Dineen S,1992). The WHO criteria for the classification of diabetes are based on current understanding of the aetiology of the disease. Type 1 diabetes results from a cell-mediated autoimmune attack on β cells [reviewed by Daneman D, 2006]. In summary, a genetic susceptibility to type 1 diabetes is inherited, mainly in the HLA-genotype, and the condition is triggered by an as yet unidentified, probably

infective, environmental trigger.  The abnormal activation of the T-cell-mediated immune system in susceptible individuals leads to an inflammatory response within the islets (insulitis) as well as to a humoral (B cell) response with production of antibodies to β-cell antigens.  Continuing destruction of β cells leads to progressive loss of insulin-secretory reserve and, when insulin secretion falls below a critical amount to a state of absolute insulin deficiency (Daneman D,2006).

Type 2 diabetes is by far the most common form of diabetes, it is a heterogeneous condition, diagnosed empirically by the absence of features of a secondary cause or features suggesting type 1 diabetes.

**Table 1.2.** Classification of diabetes mellitus according to aetiology (World Health Organization (1998)

| **Type 1** | β-Cell destruction, usually leading to absolute insulin deficiency | 15–20% of cases in Europe and the United States |
|---|---|---|
| **Type 2** | Some degree of insulin resistance, with relative insulin deficiency | 75–80% of cases in Europe and the United States |
| **Other** | Other types with specific causes | 5% of cases in Europe and the United States |

1.1.2 Demographics

Diabetes Mellitus is now considered as one of the main threats to human health in the 21$^{st}$ Century (Zimmet P,2000).  Changes in the human environment, behaviour and lifestyle have resulted in escalating rates of both obesity and diabetes and the number of people with diabetes is expected to double between 2000 and 2030 (Wild S,2004).  The diabetes epidemic relates mainly to type 2 diabetes which accounts for over 90% of cases globally (Zimmet P,2004) which explains the increase in the older population (Fig1.1).  Estimates suggest that

5.2% of World all cause mortality is attributable to diabetes (2.9 million deaths in 2000), which is equivalent to one in ten deaths in economically productive individuals aged 35-64 years in most developing countries.(Roglic G,2005).

**Fig. 1.1.** Global diabetes prevalence by age and sex for 2000.(Wild S,2004)
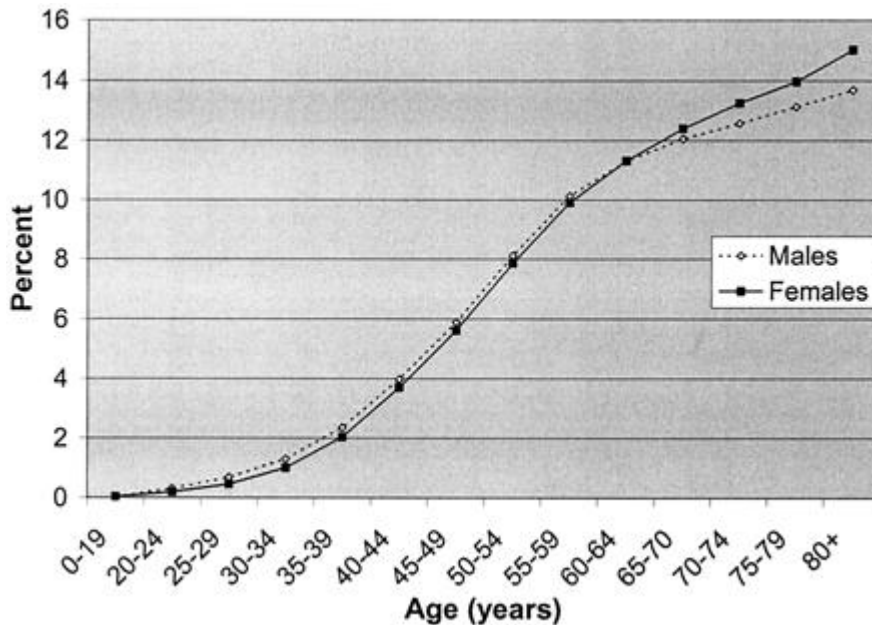


The increase in mortality is in part due to a significant increase in the risk of coronary artery disease, where men with type 2 diabetes have the same risk of myocardial infarction as men without type 2 diabetes who have already had a myocardial infarction (Fig.1.2).  This is equivalent to the increased risk of myocardial infarction associated with an increase in age of 15 years (Booth GL,2006).

The link between type 2 diabetes and cardiovascular disease is partly explained by a cluster of adverse changes to lipid profiles and blood pressure.  This cluster has been termed the metabolic syndrome. An attempt to define this syndrome was first made by the WHO in 1998, of which abnormal glucose tolerance was a key factor.  A clinical definition of metabolic syndrome was produced by the National Cholesterol Education Program- Adult treatment program III in 2001, which includes abdominal obesity, dyslipidaemia, hypertension, insulin resistance and prothrombotic and inflammatory states (Expert Panel on Detection EaToHBCiA,2001).  There is, however, much

debate as to whether current definitions identify an entity that adds any cardiovascular disease risk above the individual components in the definition or other primary risk factors not included in the definition, such as smoking and LDL cholesterol, which also tends to cluster with hypertension etc. (Alberti KGMM,2005; Kahn R,2005).

**Fig. 1.2**. Relation between age and rates of AMI by diabetes status and sex [All lines fitted according to a polynomial equation, R >0·99 for each fitted line (Booth GL,2006)]
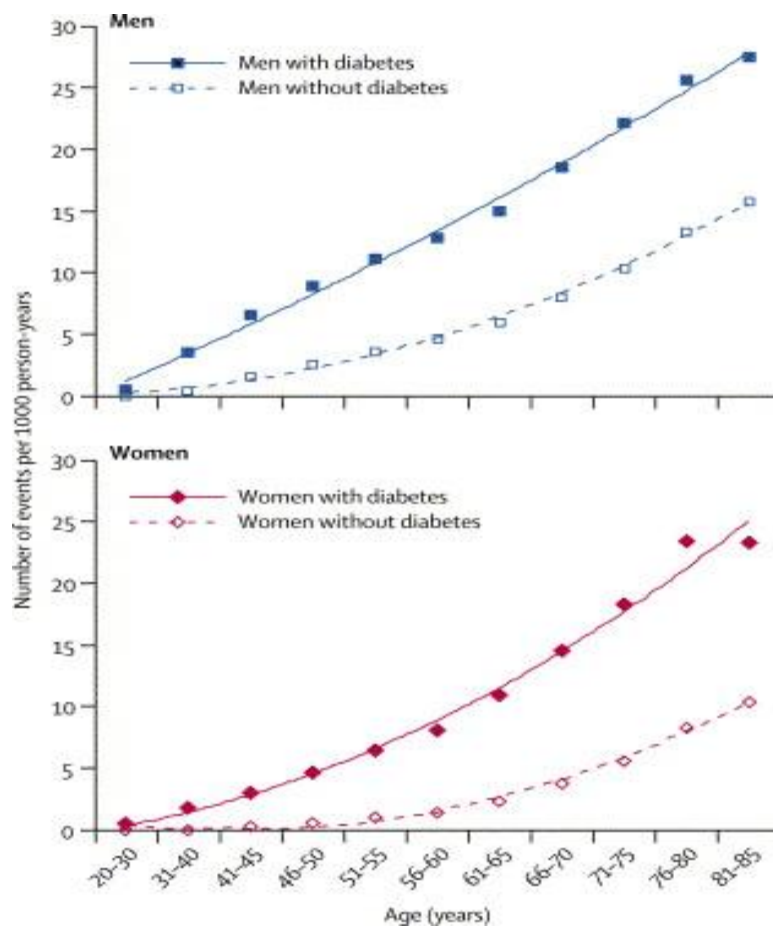
## 1.2 Pathogenesis of type 2 diabetes

The core elements involved in the pathogenesis of type 2 diabetes are impaired insulin action, known as insulin resistance, and impaired insulin secretion leading to a relative insulin deficiency. The core elements can be influenced both genetically and environmentally. Longitudinal studies suggest that a defect in insulin action precedes the development of type 2 diabetes, and the pre diabetic states, with the disease only becoming evident when β-cell failure means that insulin secretion is unable to compensate for the increased requirements (Weyer C,1999). Adiposity, especially central adiposity leads to increased release and circulating levels of non-esterified fatty acids [NEFA] which aggravates insulin resistance in liver and muscle (Boden G,1997). The excess lipid is also stored in ectopic sites, such as skeletal muscle, where it further impairs insulin action (Pan DA,1997).

**Fig. 1.3.** A summary of the pathogenesis of type 2 diabetes
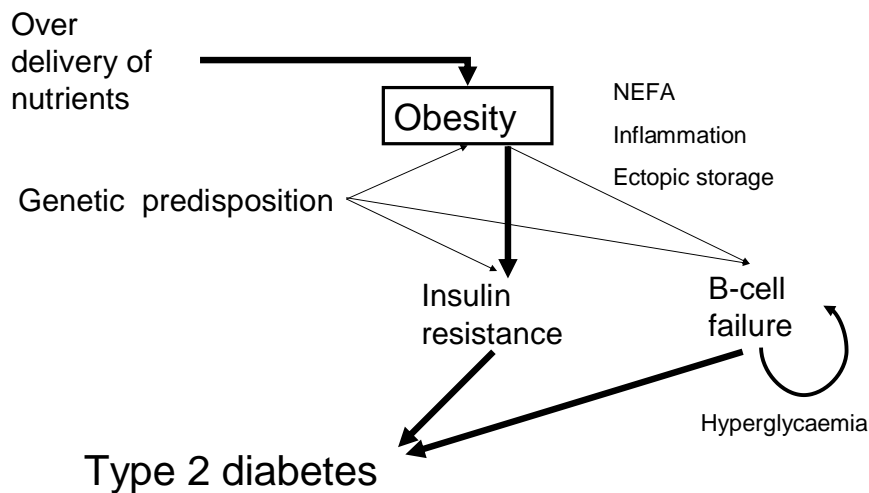
### 1.2.1 Genetic predisposition to Type 2 Diabetes

### 1.2.1a Evidence for a genetic basis for type 2 diabetes

The recent epidemic of type 2 diabetes is a clear indication of the importance of environmental factors in type 2 diabetes onset.   However, there is also an abundance of evidence concerning the presence of a genetic component.

Ethnic Differences

There is wide variation in the prevalence of type 2 diabetes by ethnic group, with very low levels in some, such as rural Chinese, to extremely high rates found in the Pima Indians in Arizona (WHO Ad Hoc Diabetes Reporting Group,1993).   However, this could be due to non-genetic cultural and environmental factors, but as the prevalence of type 2 diabetes reduces with the extent of Pima/European American interbreeding (Williams RC,2000) this is less likely.   The prevalence of type 2 diabetes also differs between ethnic groups that share similar environments, such as the United Kingdom where the prevalence of type 2 diabetes in Caucasians is 2.4% but prevalence is 3-6 fold higher in individuals of South Asian descent (Simmons D,1991).

Family Studies

A higher prevalence in family members is thought to be because of an increased number of shared genes, including those that play a role in disease predisposition shared between family members.  Such a relationship has been described a number of times, most recently in the Framingham Offspring study where risk of type 2 diabetes increased by 3.5 times with one affected parent and to 6.1 with both parents affected (Meigs J,2000).  However, there is still the possibility of these results being confounded by shared culture, environment and habits.  This can be addressed by twin studies.  Several twin studies have been carried out and although concordance rates vary they are higher in

monozygotic twins than dizygotic twins (Barroso I,2005).   The fact that concordance in monozygotic twins may be as high as 80% (Ghosh S,1996), together with the other studies, suggest that type 2 diabetes has a substantial genetic component.

1.2.1b  The role of insulin resistance

The requirement for over fifty essential nutrients in modern man to achieve maximum health and longevity means that a diverse diet has been linked to health and that this relationship developed early in hominid pre-history (Hockett B,2003).   The survival of the genes predisposing to type 2 diabetes, not only in man but in other species, such as Free-Ranging Baboons (Kemnitz JW,2002) would imply that they may have been selected for in the past.    Pre-human ancestors (2-4 million years) consumed a significant proportion of carbohydrate in their diet (Colagiuri S,2002), as a result the brain developed a specific requirement of glucose  as a source of fuel.    Brain stores of glycogen are sufficient for only a few minutes survival without blood flow, so systems had to develop to ensure a robust supply of glucose (Kitano H,2004).   These systems evolved in past environments far different to those found today and our genome remains largely adapted to our Palaeolithic existence (Johanson D,1998).  This pre-agricultural age diet overlaps little with current foods and was based mainly on meat protein (Hunter) and protein from nuts and shellfish, with little carbohydrate from fruits and vegetables (Gatherer)(Colagiuri S,2002).    To maintain blood glucose for the brain the organism would develop reduced peripheral blood glucose utilisation and increased hepatic glucose production i.e., insulin resistance (Rossetti L,1989), a state that is also a feature of periodic starvation (Newman WP,1983).   At the end of the last Ice Age the population was insulin resistant.   The advent of agriculture increased the amount of carbohydrate in the human diet (Eaton SB,1985), although it was not until the industrial revolution that highly processed carbohydrate was widely available, which, coupled with the lack of energy (activity) spent in food procurement (Eaton SB,2002), and high calorie diet, produced an environment where this adaptation started to become deleterious.   This then confers higher susceptibility to a number of chronic degenerative diseases.

## 1.2.1c   Using Genetics in the study of type 2 Diabetes

Epidemiology is "the study of distribution determinants [and control] of health related states and events in populations".   The concept of "genetic epidemiology" is less well defined but regarded as the study of the genetic determinants of disease, and also the joint effect of genes and non-genetic determinants (Burton PR,2005).   The disease-exposure association of traditional epidemiology is essentially replaced with the genetic variation-exposure association.   There are a number of methods available to locate and identify causative genes.

Learning from Monogenic Disorders

A small proportion of diabetes cases are due to mutation in a single gene. There are three main groups of disorders, MODY [maturity onset diabetes of the young], neonatal diabetes and Mitochondrial diabetes.   The understanding of the genetic basis for these conditions has improved understanding of the physiology of metabolism and energy balance and the pathophysiology of polygenic diabetes (McCarthy MI, 2008).

Although 10% of cases MODY do not have a cause identified the remaining cases fall into two types  (Vaxillaire M, 2006).  Glucokinase mutations account for 14% and give rise to a non-progressive mild impairment of glucose sensing in the pancreatic β-cell (Hattersley AT, 1993).  The remaining cases are due to mutations in the genes for a number of transcription factors that are thought to play key roles in pancreatic development and function, including TCF-1,TCF-2, IDF-1, Nuerod 1 and CEL (McCarthy MI, 2008).

Neonatal diabetes presents in the first six months of life.  The genes identified all code for proteins that form an ATP dependant potassium channel [KCNJII/ABCC8].  Identification of the underlying genetic aetiology has enabled an effective and tailored treatment strategy to be developed for these` patients

using the sulponylurea group.   Mitochondrial diabetes is discussed in a following section on insulin resistance.

Unravelling the genetic variation –exposure in polygenic disease

One option is to move straight to the obvious candidate genes, however, in complex diseases such as type 2 diabetes there are a very large number of plausible candidate genes.   Linkage analysis can be used to identify broad genomic regions that might contain a disease gene, even without a plausible candidate being present in that area (Teare MD,2005).   Two genetic loci are linked if they are transmitted together from parent to offspring more often than expected under independent inheritance (c.f. Linkage disequilibrium, which refers to loci found together on haplotypes in the population at a frequency greater than expected).   Linkage analysis identifies markers that are passed down through a family, that consistently accompany the disease of interest. They have been more successfully used for monogenic conditions than complex polygenic conditions (Botstein D,2003).   To date, over 50 type 2 diabetes linkage studies  have been conducted in a variety of populations, in which some chromosomal regions have now been replicated in multiple studies (Barroso I,2005).    This is in part due to the intrinsic limitations of linkage analysis studies due to low statistical power, which can also be reduced by missing pedigree information, genetic differences between populations and genotype errors, such that the sample size to detect genetic linkage of the modest size expected in complex traits may be unobtainable (Risch N,2000). The power of linkage analysis in type 2 diabetes is also reduced by difficulty defining the phenotype, with the possibility that early onset type 2 diabetes may have different genetic determinants, and the difficulty in identifying subjects who may have latent autoimmune diabetes.   The power of the study will be diluted further by the possibility of genetic heterogeneity within families, where a different combination of genes may determine a complex trait within a family (Teare MD,2005). However, despite these difficulties, a number of regions have now been replicated (reviewed by (Florez JC,2003), and at least two important genes have been identified by this approach namely, calpain 10 (Horikawa

Y,2000) and TCF7L2 (Grant SFA,2006), neither of which were plausible candidate genes.

Association studies have more in common with traditional epidemiology, where the risk is a particular allele, genotype or haplotype of a genetic marker. Most performed so far employ a case-control design, and have been of the candidate gene type. They have also not been well replicated, because of poor matching of cases and controls, use of convenient samples, small study size, limited number of markers per gene and failure to take into account environmental interaction (Barroso I,2005) although, these can somewhat be overcome if a prospective sample is used (Humphries SE,2003). The presence of an association does not always imply causation, but may reflect an association with a nearby causal variant (i.e. in strong linkage disequilibrium with the variant studied) or be due to some underlying stratification or admixture of the population (Cordell HJ,2005). Genetic association studies have identified a number of polymorphisms reproducibly associated with type 2 diabetes, including Pro12Ala in *PPAR-gamma* (Altshuler D,2000). A review of the published candidate gene polymorphism association studies can be found in Barroso I(2005).

New techniques in genetic epidemiology

The Genome Wide Association study

The development of high performance genotyping (Fan JB, 2006) coupled with the identification of most known patterns of common variation by the HapMap consortium (The International HapMap Conssortium, 2003) have enabled researchers who have either through collaboration or design amassed more suitable sample sizes to perform genome wide association studies (McCarthy MI, 2008). This approach is not based on any plausible biological basis but aims to capture most of the common variation in the whole genome in one study. This approach .has led to a recent increase in the number of replicated gene-type 2 diabetes association studies. The following have been identified as

important genes by genome wide association studies, HHEX/IDE, SLC30A8, FTO, CDKAL1, CDKN2A and IGF2BP2 (McCarthy MI, 2008).

Mendelian Randomisation

When a robust finding from observational studies [vitamin C and CHD (Khaw KT, 2001)] is not backed up by interventional studies (Heart Protection Study Collaborative group, 2002) one of the likely causes is the presence of confounding variables.  Mendelian randomisation overcomes some of problems as the association can be tested on the basis of random genetic variation.  A genetic variant that alters a biological exposure eg CRP should increase the prevalence of the related disease as predicted by its effect on the risk factor (Smith GD, 2007).  If this is not the case then the disease-risk factor relationship observed is likely to be due to confounding factors.

1.2.2   Molecular Mechanisms in type 2 diabetes- gene-lifestyle interaction

The cardinal features of type 2 diabetes are the development of insulin resistance and the failure of insulin secretion.  Both of these develop from a combination of genetic predisposition and environmental influences.  There is continued debate about which is the initial abnormality.  Early studies suggested that insulin resistance was the strongest predictor of type 2 diabetes in Pima Indians (Lillioja S,1993) and that insulin resistance was present at least ten years before development of the disease, whilst reduction in insulin secretion occurred only a few years prior to development of type 2 diabetes (Warram JH,1990).  However, recently this data has been questioned, with evidence that β-cell dysfunction is present in normal glucose tolerant individuals predisposed to type 2 diabetes (van Haeften TW,1998), and that people with type 2 diabetes may not be more insulin resistant than appropriate obese controls (Weyer C,1999).  Certainly both are present at time of hyperglycaemia (Chiasson JL,2004), and it may be the relationship between them that is more important than each individually (see 1.2.2b).

1.2.2a The development of insulin resistance


Insulin is a peptide hormone secreted from the β-cells of the islets of Langerhans, in the pancreas. The actions of insulin include increased glucose uptake into muscle and fat, decreased hepatic glucose production, and increased protein, glycogen and lipid synthesis with concurrent reductions in breakdown and cell growth and differentiation (Saltiel AR,2001). Sensitivity to insulin action is influenced by age, genetics, exercise and fitness, diet, medication and obesity (Kahn SE.,2003). Insulin resistance is strongly associated with "over-nutrition" driving obesity, although, the over-nutrition may not be all dietary, as defects in mitochondrial fat oxidation have also been described in type 2 diabetes. The consequences of obesity and insulin resistance include impaired glucose tolerance, hypertension and dyslipidemia (low High Density Lipoprotein [HDL], high Very Low Density Lipoprotein [VLDL] and a preponderance of small dense Low Density Lipoprotein [LDL]) and can even be detected in obese children (Weiss R,2004). The metabolic changes all promote vascular damage, and cardiovascular disease and mortality and morbidity is increased in the presence of insulin resistance (Laasko M,1996).


Normal Insulin Action (Fig.1.4)


The insulin receptor consists of two α and two β subunits. The α units make up the extracellular ligand binding domain and normally inhibit the action of the intracellular tyrosine kinases domain (β subunits). When insulin binds to the receptor this inhibition of tyrosine kinase activity is reduced, and conformational changes occur in the β subunits which enhance tyrosine kinase activity (Patti MR,1998). The β-subunits activate, by phosphorylation, a cascade of insulin-receptor substrate proteins (IRS), which act as docking proteins for and activate phosphatidylinositol-3 kinase [PI(3)K] (Kahn BB,1996). The IRS cascade also

activates Mitogen-Activated Kinase [MAP-Kinase], which is responsible for some of the cell growth and proliferation effects and is not down-regulated in insulin resistance (Cusi K,2000). PI(3)K activates a number of pathways related to glucose metabolism, through activation of protein kinase B/Akt and protein kinase C (Schinner S,2005). These include transposition of GLUT-4 glucose uptake proteins to the cell membrane, and the reduction of glycogen synthase kinase 3 activity which allows glycogen synthesis to occur (Shulman GI,2000). The activation of a number of forkhead transcription factors (FkHR/FOXO) inhibits the key enzymes of glycogenolysis, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (Schmoll D,2000), whilst activation of the mTOR pathway can control the translation of a number of proteins with metabolic actions, either directly or through $P70^{rsk}$ activation (Saltiel AR,2001). There is also a second pathway of GLUT-4 activation, the Cbl/CAP pathway. On binding of insulin to the insulin receptor Casitas B-lineage lymphoma protein (Cbl) is activated, which then forms a complex with Cbl associated protein (CAP). This complex locates the G-protein TC10 in membrane rafts and activates it, which then provides a signal for GLUT-4 activation (Chiang SH,2001) .

**Fig 1.4.** Signal transduction in insulin action (Saltiel AR,2001).
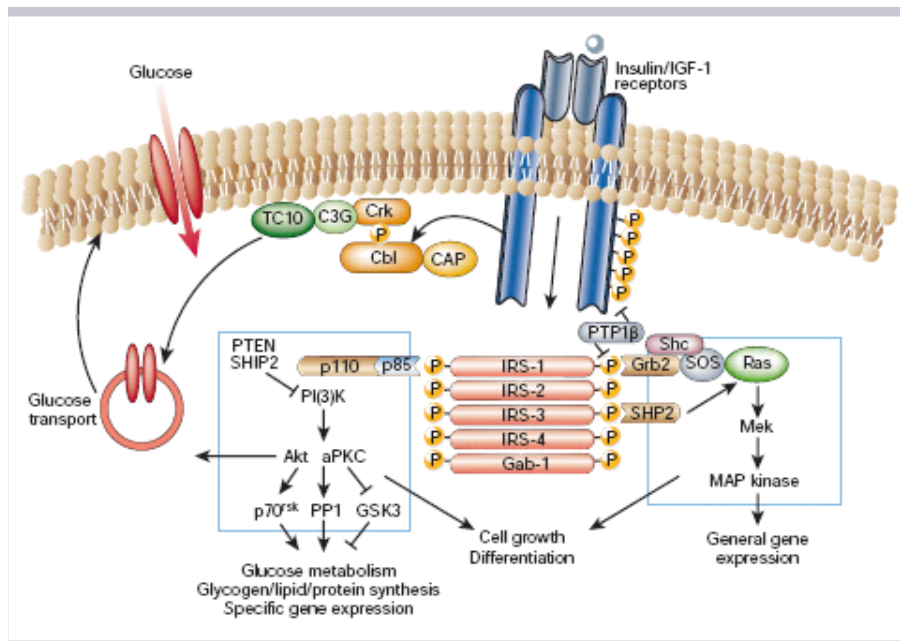


Insulin receptor pathways are terminated by protein tyrosine-phosphatase 1B which dephosphorylates the signal pathways.  The insulin signal can also be inhibited by serine/threonine phosphorylation (Zick Y,2001) by atypical Protein Kinase C isoforms or the C-Jun Pathway (White MF,2002) or by enhanced degradation of the IRS proteins as occurs in SOCS signalling (Rui L,2002).


Consequences of Obesity


Adipose tissue is an important determinant of whole body insulin sensitivity. The distribution of the excess body fat is also important, with central fat being more important in driving insulin resistance than peripheral fat (Fujimoto WY,1994).   Central fat is intra-abdominal visceral fat which tends to collect around the waist, which is itself a strong predictor of type 2 diabetes (Wahrenberg H,2005).   Visceral fat has a different phenotype, with increased lipolysis compared to subcutaneous fat (Abate N,1995).   The important role of adipose tissue in whole body insulin sensitivity is indicated by animal models. The GLUT-4 adipose tissue specific mouse is severely insulin resistant (Abel ED,2001) while the skeletal muscle tissue specific GLUT-4 knockout has mild diabetes only (Zisman A,2000).   This may be because Insulin Growth factor

signalling can compensate in skeletal muscle (Shefi-Friedman L,2001) but the adipose tissue GLUT-4 over-expression model is also more insulin sensitive despite being more obese (Shepherd PR,1993). Adipose tissue specific GLUT-4 over-expression also corrects the mild insulin resistance seen in the muscle specific knockout, without any direct effect on skeletal muscle but by increasing the number of small adipocytes (Carvalho E,2005). In man, GLUT-4 expression is reduced in adipose tissue and not skeletal muscle in those with obesity or type 2 diabetes (Carvalho E,2001).

Expanded adipose tissue can promote the development of type 2 diabetes by a number of mechanisms. These are triggered by a change in adipocyte size. Large adipocytes, that occur in obesity, undergo changes in gene expression such as reduced adiponectin, and tend to promote insulin resistance (Yang X,2004) with increased intracellular lipid in non adipose tissue. The presence of large adipocytes is also a predictor of type 2 diabetes and numbers inversely correlate with insulin sensitivity (Weyer C,2000), whilst in patients with type 2 diabetes adipogenesis is impaired, decreasing the availability of new adipose cells and increasing average cell size (Yang X,2004). Large adipocytes have diverse functional abnormalities (Le Lay S,2001) with impairment of proliferation pathways (β-catenin), insulin signalling pathways (GSK-3) and reduced adiponectin secretion (Yang X,2004).

There are a number of candidates for the signal from adipose tissue that induces insulin resistance in liver and muscle. Non-Esterified Fatty Acids [NEFA] are released in larger quantities by expanded adipose tissue (Ravussin E,2002) and are increased in the offspring of patients with type 2 diabetes even if glucose tolerance is normal (Perseghin G,1997). Circulating levels of NEFA are inversely correlated with net glucose disposal (Baldeweg SE,2000). The infusion of NEFA inhibits glucose uptake after 3-4 hours (Fig 1.4), (Boden G,1997) which with decreased glycogen synthesis (Krebs M,2001) means that there is a reduction in the effectiveness of insulin-driven pathways. NEFAs reduce PI(3)K activity (Dresner A,1999) possibly through activation of atypical PKC isoforms and Serine/Threonine phosphorylation of IRS proteins (Shulman GI,2000). Increased delivery of NEFA alters membrane composition (Borkman

M,1993) and has a direct effect on glucose transporters (Long SD,1996), which also contributes to other mechanisms driving insulin resistance, including intracellular lipid stores and inflammation.

Adipose tissue forms over 10% of total body weight, but it is now clear that adipocytes have functions other than simple storage cells (Shimada K,2004). The most significant of these appears to be the secretory capacity of the adipocyte. The adipocyte secretes a number of peptides that have been labelled adipocytokines or adipokines (Kadowaki T,2005). Adipokines identified to date seem to function as modulators of metabolism, such as Leptin and Resistin, or of inflammation, such as Tumour Necrosis Factor-α (TNF-α), Interleukin 6 [IL-6], adipsin (also known as complement factor D), acetylation-stimulating protein, visfatin (also known as B-cell colony-enhancing factor), plasminogen-activator inhibitor type 1, as well as other complement components and interleukins (Hug C,2005; Rajala MW,2003).

The most abundantly secreted adipokine is adiponectin, making up 0.01% of circulating protein, with serum concentration a thousand times greater than other hormones and $10^6$ times greater than other inflammatory cytokines (Shimada K,2004). Studies from animal models and human subjects confirm that adiponectin is an insulin-sensitising hormone, that is negatively regulated by obesity [Reviewed in Gable D, 2006a]. A significant number of the metabolic actions of adiponectin are dependent on the activation of AMP-dependent kinase (AMPK) (Tomas E,2002; Wong GW,2004; Yamauchi T,2002; Yamauchi T,2003), a fuel-sensing enzyme (Wong GW,2004). AMPK dependent fuel-sensing systems have been identified in myocytes, hepatocytes, skeletal muscle and parts of the central nervous system (Wong GW,2004). AMPK is activated when ATP is required, and one of its main stimulators is the AMP/ATP ratio. Activation increases glucose transport, glycogen accumulation and fatty acid oxidation, with the aim of increasing ATP production. Adiponectin also has anti-inflammatory actions. The increase in insulin sensitivity associated with Leptin secretion is also through AMPK activation, although the secretion of anorexigenic peptides also contributes to reduced over-nutrition (Minokoshi Y,2002).

Leptin is described as a "starvation signal" as its levels decline during fasting, but in obese individuals levels are often higher. The mechanism of this Leptin resistance has yet to be elucidated (Ahima RS,1999). TNF-α is also produced by adipose tissue and acts in an autocrine and paracrine way, increasing NEFA release (Itani SI,2002), reducing adiponectin release (Ruan H,2002) and reducing insulin signalling efficiency in adipocyts (Saltiel AR,2001).

The possibility that inflammation was important in the development of insulin resistance was first suggested when it was noted that the lipid abnormalities in type 2 diabetes were similar to those seen in the acute phase response (Blackman JD,1993). Further evidence of the link between inflammation and insulin resistance is suggested by an increase in levels of pro-inflammatory cytokines in the post prandial period (Carroll MF,2003), and the relationship of type 2 diabetes and obesity with low grade inflammation. Low grade inflammation precedes and predicts the development of insulin resistance. Insulin resistance is associated with higher levels of C-Reactive Protein (CRP), and levels of this acute phase protein, as well as others such as IL-6, orosmuoid and sialic acid are associated with risk of type 2 diabetes (Duncan BB,2003). Obesity is also associated with non-specific markers of activation of the immune system including temperature, white cell count and total γ-globulin (Tataranni PA,2005). The acute phase response is part of the innate immune system and IL-6, of which up to 30% of the circulating levels are produced by adipose tissue (Mohamed-Ali V,1997), is one of the prime stimulators of acute phase proteins (Gabay C,1999). IL-6 levels correlate with obesity and insulin resistance and predict the onset of type 2 diabetes (Bastard JP,2000). Infusion of IL-6 induces insulin resistance (Tsigos C,1997), with IL-6 having a direct effect on inactivating IRS function via SOCS (Rui L,2002). One of the key transcription factors involved in the innate immune system activation is NF-κβ, which is maintained in its inactive state by binding with Iκβ. NF-κβ is activated after Iκβ is released by the action of the kinase IKK. Lipid infusion, which is associated with increased insulin resistance activates NF-κβ (Tripathy D,2003), while blockade of IKK activity lowers blood glucose in rodents (Kim JK,2001) and in man (Hundal RS,2002). The inflammatory response in obesity may be driven by changes in adipose tissue (Fig. 1.5), as a response to being unable to

store all the energy delivered (Wellen KE,2003). Adipose tissue and the immune system are similar, with both functions performed by the fat body in *Drosophila* (Xu H,2003). There are many shared pathways between T-cells, macrophages and adipocytes, with the peroxisome proliferator-activated receptors [PPAR] transcription factors, now known to regulate a large repertoire of inflammatory genes as well as their well known metabolic function (Way JM,2001). A number of macrophage and inflammatory genes are also upregulated in obesity (Xu H,2003).

Skeletal muscle is responsible for 75% of post-prandial glucose disposal (Klip A,1990), and a defect in removal of glucose and glycogen by skeletal muscle is found in all insulin-resistant subjects (Shulman GI,1990). Early investigations demonstrated increased lipid in the skeletal muscle of subjects with type 2 diabetes (Dagenais GR,1976) and although the early studies required prior removal of all extracellular lipid and were prone to error, they suggested that intramyocellular lipid (IMCL) was a good predictor of insulin resistance (Machann J,2004). Advanced imaging techniques (Simoneau JA,1995), and more recently the use of magnetic resonance spectroscopy, has confirmed this association (Thamer C,2003). However, endurance athletes also have elevated levels of IMCL but are very insulin sensitive (Goodpasture BH,2001). Fatty acid metabolites, such as long chain acyl-CoA (Ellis BA,2000), are correlated with level of insulin resistance and diacylglycerol, and can increase serine/threonine phosphorylation of the insulin signaling cascade, possibly by activating atypical PKC isoforms or through pro-inflammatory pathways (Itani SI,2002). However this relationship can be modified by the oxidative capacity of skeletal muscle (Thamer C,2003).

**Fig 1.5.** Inflammation in obese adipose issue



Obese adipose tissue is characterized by inflammation and progressive infiltration by macrophages as obesity develops. Changes in adipocyte and fat pad size lead to physical changes in the surrounding area and modifications of the paracrine function of the adipocyte. For example, in obesity, adipocytes begin to secrete TNF-α, which can stimulate preadipocytes to produce monocyte chemoattractant protein-1 (MCP-1). Increased secretion of leptin (and/or decreased production of adiponectin) by adipocytes may also contribute to macrophage accumulation by stimulating transport of macrophages to adipose tissue and promoting adhesion of macrophages to endothelial cells, respectively. Whatever the initial stimulus to recruit macrophages into adipose tissue, once these cells are present and active, they, along with adipocytes and other cell types, can perpetuate a vicious cycle of macrophage recruitment, production of inflammatory cytokines, and impairment of adipocyte function. (Wellen KE,2003).

Mitochondria and insulin resistance

Oxidative capacity is determined by mitochondrial function and there is evidence of mitochondrial dysfunction in insulin resistance and type 2 diabetes. Mitochondria are also an important source of oxidative stress [see 1.3.4].

Mitochondria have their own genome, and mutations in the mitochondrial genome are associated with diabetes. The mitochondrial genome is maternally inherited, consists of 16.6kB and codes for 13 proteins that form part of the electron transport chain. Mutations have been associated with neurological disease and diabetes, and for the tRNA and rRNA involved in ribosomal protein synthesis within mitochondria (Wallace DC, 1999). The most common causes

of mitochondrial diabetes are the 3243A>G variant in the Leu,URR gene, a DNA encoded tRNA that is associated with low BMI, lipomas and hepatic fat infiltration suggesting the underlying problem is aberrant fat storage. The diabetes tends to occur around age 40 and is associated with significant β-cell damage. A second gene [LARS2] has also been implicated in mitochondrial diabetes (Maassen JA, 2007).

In mouse models there is reduced biogenesis of mitochondria (Choo HJ,2006) disease, while mitochondria in subjects with type 2 diabetes are smaller and have reduced activity of the electron transport chain (Kelley DE,2002). In subjects with type 2 diabetes or insulin resistance, gene expression profiling shows reduced electron transport chain genes, especially in visceral fat (Dahlman I,2006), and also reduced copies of oxidative phosphorylation protein encoded in mitochondrial DNA (Bogacka I,2005a). PPAR-γ co-activator 1 (PGC-1α) is a transcription factor that drives mitochondrial biogenesis (Wu Z,1999) and has a role in gluconeogenesis and fatty acid oxidation (Bogacka I,2005b). PGC-1α expression is reduced in type 2 diabetes (Mootha VK,2003) and also in insulin resistant offspring of subjects with type 2 diabetes (Patti ME,2003). The deterioration in mitochondria function that occurs with age leads to reduced oxidative phosphorylation in the elderly which correlates with the increase in insulin resistance seen with age (Petersen KF,2003) and the reduced utilization of fatty acids in those with insulin resistance (Kelley DE,2000). Total fasting fat oxidation is correlated with insulin sensitivity (Wahrenberg H,2005). Insulin sensitivity is also correlated with post-prandial thermogenesis (Robinson S,1992). Women predisposed to obesity have lower 24hr fat oxidation compared to controls (Raben A,1994), whilst in obese women relative post prandial fat oxidation is reduced (Blaak EE,2006). The mechanism linking fat oxidation and insulin resistance is unclear. ICML is important, as blocking fatty acid uptake molecules, such as CD36 (Hajri T,2002) and Fatty acid transporter-1, (Kim JK,2004) protects from insulin resistance. In insulin-sensitive individuals, insulin increases ATP synthesis and Respiratory Quotient, which does not occur in insulin-resistant states (Brehm A,2006). Fat oxidation only adapts slowly to intake (Raben A,1994) and, combined with a diminished

capacity to use fat as a fuel, it may be that mitochondrial dysfunction potentiates the problems of over-nutrition and abnormal adipose tissue biology.


1.2.2b The development of pancreatic failure

Although insulin secretion in type 2 diabetes is abnormal in response to a number of stimuli, including intravenous and oral glucose, arginine, β-agonists (isoproteronol), and sulphonylureas (Kahn SE,2003), it was not thought that poor insulin secretion contributed to the early aetiology of type 2 diabetes. Initial investigations demonstrating that subjects with type 2 diabetes continue to secrete insulin (Yalow RS,1960), and this taken with the evidence that insulin resistance appeared to be the first factor to appear, suggested that abnormal insulin secretion was a late event in the pathogenesis of type 2 diabetes. However, these studies did not take into account that insulin secretion is modified by insulin resistance (Kahn SE,1993) and recent data suggests that β-cell dysfunction occurs early, and is important in the pathogenesis of type 2 diabetes as does data from the understanding of MODY [discussed below]. Recently, genome wide association studies have also suggested an important role for insulin secretion as, to date, most of the replicated identified associated genetic variants seem to have more of a role in modulating pancreatic function rather than insulin resistance.


Normal Insulin secretion (Fig 1.6)

Insulin secretion occurs as the result of uptake of glucose into the β-cell by GLUT-2, a low affinity, high capacity glucose transporter. Glucose is "trapped" in the cell by the action of glucokinase converting the glucose to glucose-6-phosphate. Further transformation to pyruvate via the tricarboxylic acid cycle and the mitochondrial electron transport chain to ATP follows. Alteration of the intracellular ADP:ATP ratio leads to closure of ATP-sensitive potassium channels. These channels include in their structure the sulphonylurea receptors (SUR1/SUR2) and the subunits Kir6.1, Kir6.2 which

have been implicated in the aetiology of neonatal diabetes and are closed in the presence of glucose. This leads to a change in membrane electrical energy potential and opening of voltage-gated calcium channels (MacDonald PE,2005).

**Fig 1.6.** Normal Insulin secretion
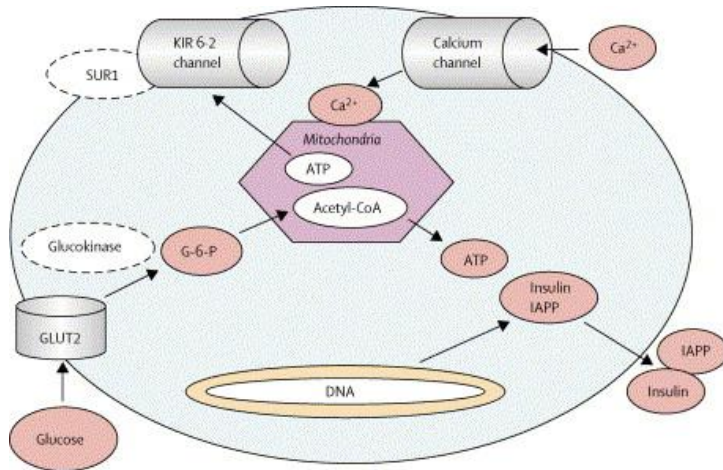


IAPP=islet amyloid polypeptide. G-6-P=glucose-6-phosphate. CoA=coenzyme A. GLUT2=glucose transporter 2.(MacDonald PE,2005).

Initially, there is release of docked and readily available vesicles and then vesicle mobilization is stimulated. Therefore, insulin release is biphasic (Del Prato S,2002) with an early peak (acute phase) followed by a more sustained plateau or second phase (Fig 1.7).

**Fig. 1.7.** Representation of the Biphasic insulin secretion
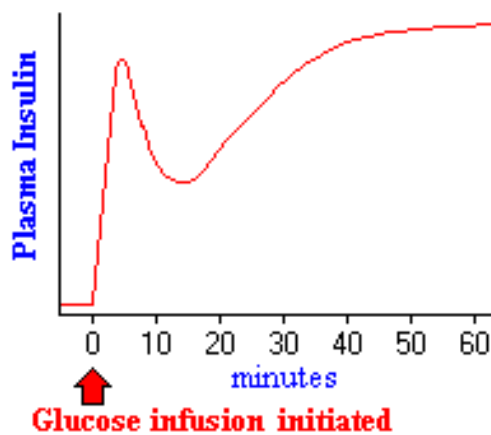
β-cell function in subjects at high risk of diabetes

As described earlier, the presence of insulin resistance in those at risk of type 2 diabetes will modify insulin secretion, which is often used as a marker of β-cell function.  This relationship is shown in Fig 1.8.

**Fig 1.8.** Relationship between insulin sensitivity and β-cell function in 93 healthy individuals  (Kahn SE,1993).
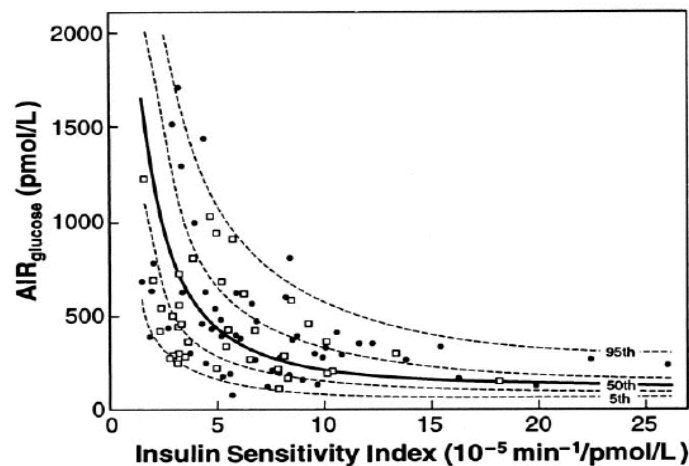


$AIR_{glucose}$ = acute insulin response to glucose.  Insulin sensitivity Index is derived from minimal model analysis of frequently sampled intravenous glucose tolerance testing.  5th,25th,50th,75th percentiles shown.

The development of insulin resistance must be associated with an increase in insulin secretion for normal glucose homeostasis to be maintained, a move to the right along the same centile.   When groups at increased risk of type 2 diabetes are examined (Fig.1.9) they are indeed found to be insulin resistant but in addition β-cell dysfunction is also present as indicated by insulin secretion falling into the lower centiles (Kahn SE,1996).

**Fig. 1.9.** Relationship between insulin sensitivity and β-cell function in groups at high risk of type 2 diabetes
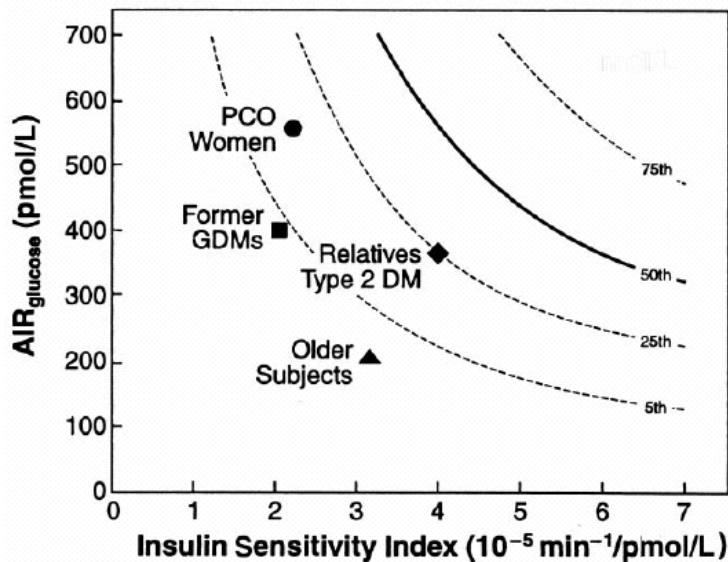


PCO= women with polycystic ovary syndrome
GDM= Gestational Diabetes Mellitus   (Kahn SE,1996)

The first defect in β-cell function that occurs, even in those with normal glucose tolerance, is the loss of the acute phase response (Del Prato S,2002), even though the relationship between second phase and insulin sensitivity is maintained.  During the development of worsening glucose tolerance the acute phase insulin release progressively worsens (Weiss R,2005).   There is also evidence of abnormal insulin processing with increased release of pro-insulin, again even in those at high risk of type 2 diabetes but with normal glucose tolerance (Saad MF,1990).  The proportion of pro-insulin released is a predictor of future development of type 2 diabetes (Kahn SE,1995).   Insulin is co-secreted with a 37 amino acid polypeptide, islet amyloid polypeptide (Cooper GJS,1987) which is also found to be secreted in lower amounts in type 2 diabetes (Kahn SE,2003).

Mechanisms of β-cell dysfunction

The investigation of monogenic Maturity Onset Diabetes Mellitus of the Young [MODY] identified the causal genes as a number of transcription factors (Barroso I,2005). Mutations in transcription factors have a different clinical phenotype of progressive beta-cell failure resulting in increasing hyperglycaemia and requirement for treatment (www.projects.ex.ac.uk/diabetesgenes/index). Knockout mouse models generally have a reduced β-cell mass which can be compensated for in early life, but normal glucose levels cannot be maintained (Hattersley AT,2004). In autopsy series, β-cell mass is reduced by 20-50%, however, this occurs many years after diagnosis and is consistent with the finding of increased apoptotic events in subjects with type 2 diabetes (Butler AE,2003). The study of the pancreas in individuals at high risk of diabetes is difficult, but differences in insulin secretion suggest the problem is not simply reduced β-cell mass. In dogs with 65% of their pancreas removed, acute phase insulin secretion is maintained, in part, by increasing the sensitivity of the existing β-cell mass to glucose (Ward WK,1988). A similar compensation occurs early in Type1 diabetes as β-cells are destroyed by the autoimmune process (Johnston C,1987), but this compensation is not seen in type 2 diabetes suggesting other processes are also underway.

There is a possibility that the increased circulating NEFA can also directly affect β-cell function. NEFA increase insulin secretion for the first 24 hours but after this inhibit β-cell function, and long term exposure is associated with β-cell dysfunction, as indicated by reduced insulin release as well as reduced pro-insulin processing (Zhou YP,1996). NEFA are converted to long chain acyl-CoA in the cell, which can counteract ATP-induced closure of potassium channels (Bränström R,2004). They can also modulate intracellullar calcium through the orphan G-protein coupled receptor GPR40 (Itoh Y,2003) and induce β-cell apoptosis through increased nitric oxide production (McLean N,1955). However, circulating fatty acids are essential for glucose-stimulated insulin secretion after prolonged fasting (Dobbins RL,1998), and long chain acyl CoA

facilitates the fusion of secretory granules to the cell membrane (Deeney JT,2000). The factors modulating the complex relationship between circulating fatty acids and insulin secretion are not fully understood. The composition of free fatty acids can have different effects, with polyunsaturated fatty acids stimulating insulin secretion and short chain and saturated fatty acids inhibiting secretion (Haber EP,2002). Glucose-stimulated insulin secretion depends on glucose oxidation, and there is a reciprocal relationship between glucose and fatty acid oxidation (Zhou YP,1996). NEFA reduce glucose uptake and oxidation, which is more pronounced in insulin resistant states (Zhou YP,1995), and feeding rats with a high fat diet reduces the rate of glucose oxidation (Carpinelli AR,1992). Complex changes in the ratio of fat to glucose oxidation may explain the effects of NEFA on insulin secretion.

The β-cell dysfunction progresses as glucose tolerance worsens, and the most severe insulin deficiency is seen after 10 years of type 2 diabetes (Wallace TM,2002). Glucose toxicity contributes to this failure, probably through a mechanism involving oxidative stress (see 1.3), increasing cytokine production (Maechler P,1999) and accelerating apoptosis (Butler AE,2003).

Autopsy of subjects with type 2 diabetes reveals another potential mechanism of β-cell dysfunction. Up to 90%, of subjects with type 2 diabetes have deposits of amyloid in the pancreas (Knowles NG,2002). The protein is based on the islet amyloid polypeptide, which is co-secreted with insulin and could accumulate with increased insulin demand. These aggregates are toxic to islets in culture and increase β-cell apoptosis (Lorenzo A,1994). The degree of amyloid replacement predicts the need for insulin therapy (Kahn SE,1998). Overexpression of islet amyloid protein in animal models fed a high fat diet leads to higher glucose levels, (Verchere CB,1996) which suggests that islet amyloid has a role in type 2 diabetes. However, the fact there is no increase in amyloid in impaired glucose tolerance states (Butler AE,2003) and that it is not present in all subjects with type 2 diabetes, means that its role has yet to be defined. It may be a response to the underlying processes of β-cell dysfunction rather than driving it.

**1.3 Type 2 Diabetes and Oxidative Stress**

Redox reactions include all chemical processes in which atoms have their oxidation number (the charge that it would bear if all the ligands were removed along with the electron pairs that were shared with the central atom) changed. Oxidation is defined as an increase in oxidation number, and reduction as a decrease in oxidation number. In most cases oxidation occurs when a molecule transfers electrons to another molecule, but dehydrogenation is also an alternative method of oxidation. Substances that have the ability to oxidize (remove electrons from) other substances are known as oxidizing agents, oxidants or oxidizers, and are themselves reduced in the reaction. In biological systems normal metabolism results in the generation of a number of oxidants known as reactive oxygen species [ROS] if based on oxygen, or reactive nitrogen species [RNS] if nitrogen based, although they are often described together as ROS. These are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. To protect the structure of the organism from oxidation a number of cellular defences have developed. Oxidative stress occurs when cellular anti-oxidant defences are inadequate to inactivate generated ROS, and they are free to react with other cellular components (Stocker R,2004).

1.3.1 Sources and effects of oxidative stress

Oxidative stress can result from exogenous processes such as radiation, pollution, smoking (Ghiselli A,2000) and toxins. ROS generation is thought to be responsible for the cardiac side effects of anthracylines (Keizer HG,1990) and the hepatic toxicity of some antibiotics (Orrenius S,1985) or from endogenous metabolism. ROS are continuously generated by the organism as a by-product of oxidative metabolism (Gate L,1999) described in more detail later (1.3.3c). The six main ROS are superoxide [$O^{2-}$], hydrogen peroxide [$H_2O_2$], peroxyl radical ROO$^-$, Hydoxyl radical [$OH^-$], singlet oxygen [$O_2$] and peroxynitrite [$NO^-$] (Huang D,2005). Cellular damage from ROS has been implicated in a number of pathological conditions including atheroma (Heinecke

JW,2003), Alzhiemer's disease (Pratico D,2004), Parkinson's disease (Giasson BI,2000), Rheumatoid arthritis (Baskol G,2005), inflammatory bowel disease (Kruidenier L,2002), cancer (Brown NS,2001) and may even be responsible for the deterioration in function associated with ageing (Finkel T,2000). ROS could be involved in pathology either through direct oxidative damage to biomolecules or through the cellular response such as apoptosis (Harrison DG,1997).

Lipids are oxidised by ROS to aldehydes, of which the two most studied are malondialdehyde (MDA) and 4-hydroxyl-2-nonenal (HNE). Oxidised lipid changes the biological properties of cell membranes, inactivating membrane-bound receptors and enzymes, and reducing cell junction communication (Dalle-Donne I,2006). In endothelial cells, oxidation of lipid increases expression of cell adhesion molecules, activates matrix metallproteinases and induces proliferation of underlying vascular smooth muscle (Suzuki YJ,1997). These induce thrombosis and endothelial dysfunction (Calingasan NY,1998), all changes associated with atheroma. A key part in the pathological process of atheroma involves oxidation of circulating LDL particles (Ceconi C,2003). These changes in function can lead to cell death, with evidence that MDA is cytotoxic (Uchida K,2003) and HNE can induce apoptosis (Esterbauer H,1991).

Oxidation of protein can alter its structure and function, and for example cataract is a consequence of photo-oxidation of the lens proteins (Taylor A,1993). Modification of proteins can generate new antigens and an immune response (Rosen A,1997), and inhibit enzyme function (Dalle-Donne I,2006) which can cause secondary damage if repair mechanisms are affected (Wiseman H,1996). The oxidised protein is often poorly degraded, and can form aggregates within the cell that can cause metabolic dysfunction and initiate apoptotic processes (Dean RT,1997).

DNA modification will have a broad range of effects on cellular function. Modified DNA contributes to risk of cancer (Block G,2006) and may contribute to ageing and reduced lifespan (Hart RW,1974) as urinary excretion of damaged DNA correlates with lifespan across a broad range of mammals (Foksinski M,2004).

## 1.3.2 Anti-oxidant systems

Anti-oxidants are substances that oppose oxidation or inhibit reactions promoted by oxygen or peroxides. Living organisms have developed a complex anti-oxidant network to protect themselves from the damaging effects of ROS (Prior RL,1999). There are essentially two main groups, the enzymatic anti-oxidants and the non-enzymatic scavengers that break down radical chain reactions and are in the process sacrificed and need to be regenerated (see Fig.1.10) (Huang D,2005). The most significant circulating anti-oxidant is probably uric acid which is metabolised to allantoin (Grootveld M,1987). Anti-oxidants can also be classified by their lipid solubility, with the lipophilic carotenoids important in the protection of lipoproteins, while vitamin C is much more hydrophilic (Gate L,1999) and may have different roles.

**Fig 1.10.** Biological anti-oxidant systems

•Enzymatic
- Superoxide Dismutase
- Catalase
- Glutathione Peroxidase

•Non Enzymatic
- Autooxidation factors [Se, Coenzyme Q10]
- Oxidative enzyme inhibitors [Aspirin]
- Transition Metal chelators [EDTA]
- Radical Scavengers [Vitamin C & E]
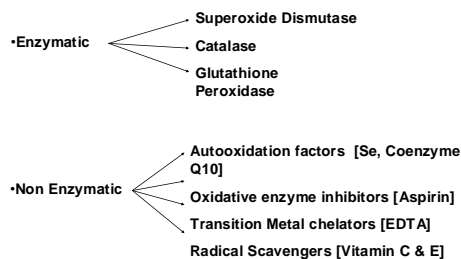
(Huang D,2005)

The function of intra-cellular anti-oxidants is supported by the enzymatic anti-oxidants superoxide dismutase, which detoxifies superoxide, and catalase, which breaks down hydrogen peroxide. Anti-oxidant defences also depend on the action of glutathione, a low molecular weight thiol made from glutamate, cysteine, and glycine. Glutathione acts as a scavenger of ROS, and is oxidised to glutathione disulfide which protects the rest of the cell. The glutathione is replenished by the action of glutathione reductase on glutathione disulfide (Wu G,2004). Anti-oxidants that can be obtained from the diet include vitamin C and E (Ames BN,1995) and the carotenoids eg lycopene in tomatoes (Stahl W,1992). Long-term anti-oxidant supplementation intervention trials have so far

been disappointing in the prevention of cancer or heart disease (2002; Beta Carotene Cancer Prevention Study Group The Alpha-Tocopherol,1994; Hennekens CH,1996; Omenn GS,1996) despite epidemiological evidence that high vitamin C and E intake protects against degenerative disease (Enstrom JE,1992; Rimm EB,1993). This may in part be due to the fact that life long exposure or genetic influences are more important and overwhelm the effect of short time (relative to total lifespan) supplementation. Single nucleotide polymorphisms in catalase and superoxide dismutase genes have been associated with differences in oxidative stress pathophysiology (Kohno S,1999; Liao F,1994; Sandstrom J,1992). When a measure of total anti-oxidant status (see 1.3 for explanation) was examined in a family study, 37.8% of the variance was thought to be genetic. The proportion increased when other factors such as smoking were taken into consideration so that in smokers 42% of the variation was genetic in nature whilst in non-smokers it was only 26%. This study also found that diet had little effect on total anti-oxidant status (Wang XL,2001).


1.3.3 Measuring the Oxidative Stress Burden


The measurement of ROS is possible, but requires electron spin resonance or chemi-luminescence. The first method is non-invasive but is expensive and as ROS are unstable, do not accumulate and are unsuitable for routine use (Dalle-Donne I,2006). Hydrogen peroxide is stable in body fluids and can be measured in urine (Varma SD,1990) and breath (Jobsis RQ,2001), but there are insufficient data at present to support its widespread use. The accuracy of the electron spin resonance method can be improved with the use of ROS "traps" such as 1,1,3-trimethylisinordole-N-oxide (Bottle SE,2003), but their safety in man has yet to be proved and they may also only "trap" a small proportion of ROS produced so their validity is also not yet certain (Halliwell B,2004). The "trap" concept can also be applied to molecules for which an assay already exists, this includes using salicylate which is non-enzymatically modified to 2-3 dihydroxybenzoate (Ingelman-Sundberg M,1991) or endogenous uric acid excreted as allantoin (Grootveld M,1987). These will suffer from the same

problems as the other "trap" methods, in so far as it is not possible to determine the percentage of generated ROS that is "trapped".

Due to the difficulties of measuring ROS directly, oxidative stress burden is most commonly assessed through biomarkers.   A biomarker is used to assess the generation of ROS indirectly.   To reflect ROS generation accurately a biomarker of oxidative stress must be

- stable, as further oxidation products are often generated during sample handling  (Firuzi O,2006).
- must accumulate to detectable concentrations.
- be non-invasive and represent specific oxidative pathways [ROS generation].
- must correlate with disease severity (Dalle-Donne I,2006).

The biomarkers of oxidative stress fall into three groups.   These are either the measurement of the oxidation of macromolecules, anti-oxidant concentration or the capacity of body fluids or tissue to interfere with *in vitro* redox reactions.

1.3.3a Oxidation of Macromolecules

Malondialdehyde [MDA] is one of the most stable of the lipid peroxidation products. It can be detected in plasma by the Thiobarbituric acid reactive substances assay [TBARS] (Buege JA,1978).  This is relatively non-specific and has now been replaced with a method using high performance liquid chromatography [HPLC] (Wong SH,1987).   However, MDA is only one of a number of products, and can be absorbed through the gut (Nelson GJ,1993). The oxidative modification of fatty acids to pentane and ethane gives products which can be detected in breath, but this method is problematic because of the difficulty in preventing contamination by airbourne sources (Knutson MD,2000). The most accurate method of measuring lipid peroxidation products is the assay of $F_2$-isoprostanes.   These are derived from the non-enzymatic oxidation of arachidonic acid (Morrow JD,1990).   They can be measured in plasma using

gas chromatography-mass spectroscopy [GCMS]. This method has high sensitivity and specificity and is not affected by passage across the gut (Blaak EE,2006). However, $F_2$ isoprostanes are only a minor end product (Lawson JA,1999) and this method is very labour intensive and requires expensive equipment (Milne GL,2005). An immunoassay is now available (Morrow JD,2005) but there is limited information available regarding accuracy (Dalle-Donne I,2006), and GCMS remains the most common method used.

The most useful assay of modified proteins is also a mass spectroscopy assay of nitro-chloro-orthtyrosine, however, this can be generated in sample handling (Halliwell B,2004). An attempt to design simpler methods has not so far led to the development of an improved technique. Antibodies to tyrosine derivatives have not been specific enough (Duncan MW,2003) and also suffer from the same problems of generation during sample handling. A calorimetric assay using dinitrophenylhydrazine has been developed to detect the more stable carbonylated proteins that result from oxidation of side chains (Levine RL,1990). These are stable, even when stored frozen (Stadtman ER,2003) but the assay is not specific and is confounded by glycated proteins and protein oxidation that occurs during cooking (Halliwell B,2004).

A huge range of base and sugar modification products are generated during DNA oxidation (Dizdaroglu M,2002). The most widely measured is 8-oxo,7,8-dihydro-2-deoxyguanasine [8-OHdG] which can be measured by HPLC or Enzyme Linked Immunosorbent Assay [ELISA] (Cooke MS,2000). The assay is limited by low availability of tissue, as DNA is intracellular, and leucocytes are often used as a surrogate. When 8-OHdG is measured in the urine as a marker it can be confounded by variation in the efficiency of the DNA repair mechanisms (Halliwell B,2002).

### 1.3.3b Anti-oxidant concentrations

The concentration of a number of anti-oxidants is decreased by ROS attack, and therefore, may function as a marker of oxidative stress (Rossi R,2006). Vitamin C, carotenoids and glutathione/glutathione disulfide [GSH/GSSG] can all be measured by HPLC (Craft NE,1992; Margolis SA,1996; Paolisso G,1992). These methods are time consuming, expensive, yield little information about combined effectiveness, and do not account for those that are undiscovered or difficult to measure (Maxwell SR,2006). The measurement of lipid-soluble anti-oxidants in plasma could also be misleading, and it has been reported that the risk of myocardial infarction correlates with adipose tissue levels of carotenoids but not plasma levels (Kohlmeier L,1997; Su LC,1998). The measurement of anti-oxidants is further confounded by degradation during sample handling (Rossi R,2002). GSH/GSSG can be stabilised by the addition of Methyl-2-vinyl-pyridium trifluoromethane sulfonate [M2VP] to the sample when it is taken. The development of a colorimetric assay which is as accurate as the HPLC method (Floreani M,1997) has helped to allow the simple measurement of the GSH:GSSG ratio as a marker of intracellular redox status (Schafer FQ,2001).

### 1.3.3c  Total anti-oxidant capacity assays

The anti-oxidant function of plasma is a result of the interaction  between many different compounds, and often co-operation provides greater protection than each one alone,  as they regenerate and recycle each other (Packer JE,1979). Total anti-oxidant capacity assays [TAC] test all the multifunctional anti-oxidants present in the sample (Frankel EN,2000). Although, this concept is attractive there are a number of disadvantages to the methods developed. The redox reactions do not occur in a biological system, and the chain propagation that follows lipid peroxidation *in vivo* does not occur (Antunes F,1999). The assays will all detect the anti-oxidant effect of uric acid, but this can be increased as a consequence of conditions where oxidative stress is higher, such as renal failure (MacKinnon KL,1999). These assays are also only able to detect

scavenger anti-oxidants (Huang D,2005), and can only look at either the hydrophilic or hydrophobic phase at one time. *In vitro* TAC depends on the distribution and interaction of anti-oxidants in the two phases (Huang SW,1996). The results also need to be interpreted with caution, as increased anti-oxidant levels may reflect an adaptive response to increased ROS generation, and low levels may just reflect low background ROS generation eg in low calorie feeding (Prior RL,1999). There are many versions of this assay but they can be divided into two main groups. Firstly, where plasma is used to interfere with an oxidation reaction, this requires an oxidant and a marker that undergoes oxidation. Secondly, to drive a reducing reaction, which requires an oxidized marker that undergoes a reduction.

Total Radical Trapping Anti-oxidant Parameter [TRAP]

Method:        Inhibition of oxidation

Oxidant:       2,2'-azobis(2-amidinopropane) [AAPH]

Marker:        $O_2$ production (oxygen electrode) (Wayner DD,1985)

               Luminol (Alho H,1999)

               Fluoroscein (Valkonen M,1997)

Result:        Lag phase

The result is calculated on the basis of the delay in the reaction seen as the anti-oxidants in the serum are consumed, and this is compared to the lag phase seen with the addition of a standard amount of the anti-oxidant Trolox (Fig 1.11). The initial method was modified because of difficulties in maintaining the oxygen electrode (Ghiselli A,2000).

**Fig. 1.11** The use of lag phase to calculate TRAP result
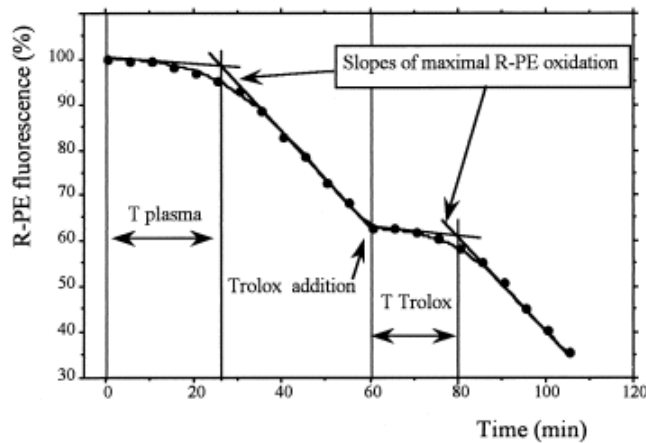


The kinetics of oxidation reaction in the presence of plasma after the addition of AAPH and trolox. The result is calculated by comparing the two lag phases obtained.

The method is more time-consuming than others because of the method of calculating the result, and the method is also susceptible to error if the plasma is diluted, as this leads to significantly different values (DeLange RJ,1989).

Oxygen Radical Absorbance Capacity [ORAC]

Method:        Inhibition of oxidation

Oxidant:       AAPH

Marker:        Phycoerythin (Cao G,1998)

                    Fluoroscein (Naguib YM,2000)

Result:        Area under the curve method

This method is similar to the TRAP method but the result is calculated differently. The oxidation reaction is allowed to proceed to completion and the result is calculated from the area under the curve in comparison to trolox standards (Prior RL,1999). This method will not be able to detect slowly-reacting anti-oxidants such as plasma proteins, which may react with ROS generated at a rate slower than the marker (DeLange RJ,1989). This will change the slope of the oxidation curve and can alter the area under the curve (Cao G,1993).

Trolox Equivalent Anti-oxidant Capacity [TEAC]

| Method: | Inhibition of oxidation | Reduction |
|---|---|---|
| Oxidant: | Ferrylmyoglobin* | persulphate used to generate |
| | Hydrogen peroxide/ | preformed ABTS$^+$ |
| | Horseradish peroxidase^ | |
| Marker: | ABTS$^+$ Generation (Green) | ABTS Generation (Clear) |
| Result: | Endpoint | Endpoint (Re R,1999) |

* Commercialised by Randox Laboratories and known as Total anti-oxidant status [TAS] (Miller NJ,1993).

^ Known as Total anti-oxidant status [TAOS] (Sampson MJ,2002). This combination has also been used in a chemilumenescence assay with luminol as the marker (Maxwell SR,2006)

ABTS= 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

ABTS forms a clear solution, that when oxidised to ABTS$^+$, undergoes a colour change which can be detected in a plate reader. The assay is quick because of the use of an endpoint method to calculate the results. The assay has also undergone significant commercial development, which enables it to be run at high speed on multiple samples on a number of automatic multi-channel analysis machines.

Ferric ion Reducing Anti-oxidant Parameter [FRAP]

| Method: | Reduction |
|---|---|
| Oxidant: | Fe$^{3+}$ |
| Marker: | Fe$^{2+}$ generation |
| Result: | Endpoint (Benzie IF,1996) |

The main disadvantage of this assay is that it is confounded by elevations in serum bilirubin (Huang D,2005), and, as with other reduction methods the anti-oxidants present may not be able to reduce Fe$^{3+}$ with the same efficiency that they would achieve with ROS, especially as *in vivo* transition metal pro-oxidant

states tend to be limited by chelators not scavenging anti-oxidants (Prior RL,1999). A similar assay has been developed using copper instead of iron, total anti-oxidant potential (Zaporozhets OA,2004) or using the Folin-Ciocalteu regent which is misleading known as total phenol assay (Singleton VL,1999).

Crocin Bleaching Assay

Method:         Inhibition of oxidation
Oxidant:        AAPH
Marker:         Bleaching of crocin
Result:         rate of bleaching

Crocin is a natural pigment extracted from saffron and is subject to significant lot-to-lot variability. The reaction rate of some anti-oxidants is very similar to blank controls, so the assay is prone to inaccuracy, and as the colour of crocin is very similar to some of the samples in which it is used to measure, this assay has found only limited applications (Huang D,2005).

1.3.3d  Which Biomarker of oxidative stress?

No biomarker has yet fulfilled all the criteria set out in 1.3.3 (Dalle-Donne I,2006). This explains why the evidence for oxidative stress being involved in disease in humans *in vivo* is not definitive (Halliwell B,2004). The poor correlation between the different methods is an indication of the high level of confounding that occurs when attempts are made to estimate oxidative stress burden. Prior to assay, different biomarkers will appear under different time courses, so that at any one time, after an oxidative stress insult, products of lipid oxidation and DNA oxidation will correlate poorly (England T,2000). Variation in sample handling is also a source of error, as anti-oxidants are unstable and degrade when exposed to air and light (Ghiselli A,2000), while any clotting that occurs during sampling will lead to the release of ROS from platelets (Leo R,1997). DNA oxidation assays are more variable if performed on isolated DNA than on urine, suggesting that the additional handling introduces error, to the extent that there is currently no agreement on basal

levels (Collins AR,2004). When considering the TAC assays, they measure the chemical activity of the plasma anti-oxidants under very specific conditions. The affinity of different anti-oxidants with the radicals generated will differ between assays, so that the anti-oxidant activity of vitamin C is low in TRAP [AAPH] but high in TAS [Ferrylmyoglobin] (Schlesier K,2002). When different methods have been compared the correlation has been poor. When FRAP was compared to markers of lipid and protein oxidation, although correlation was described, it was found to be weak (r<0.5) (Firuzi O,2006).

Three studies have compared TAC methods, no correlation was found between ORAC and TEAC, or FRAP and TEAC in one, with a weak correlation between ORAC and FRAP(r=0.35,p=0.019) only. When the proportion of the TAC due to individual anti-oxidants was calculated the proportions were significantly different, indicating that the assays have different affinities for the variety of anti-oxidants found in plasma (Fig1.12) (Cao G,1998). A similar weak correlation was found when a luminol method was used comparing ABTS and AAPH (r=0.37,p=0.03) (Waring WS,2003).

**Fig 1.12** The difference in the proportion of TAC due to individual anti-oxidants in different TAC methods (Cao G,1998)
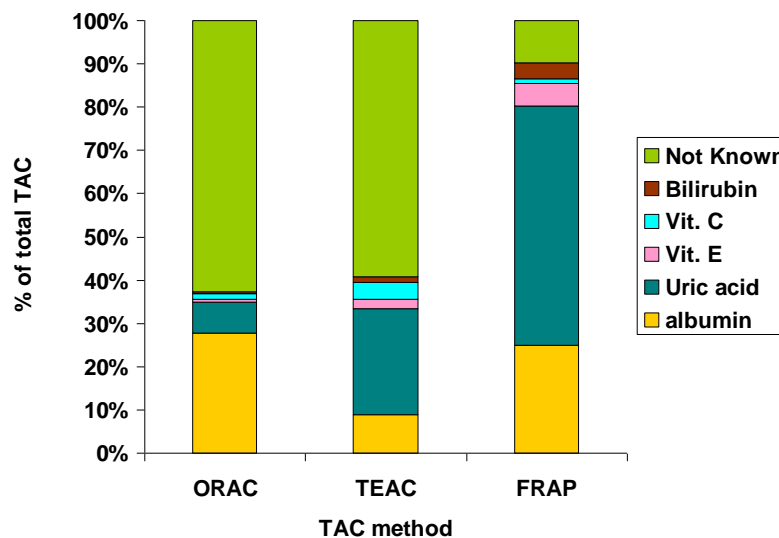
When the anti-oxidant capacity of ten drinks was compared using five different methods the absolute values were different, although the drinks that ranked higher tended to be the same regardless of the method used (table 1.3) (Schlesier K,2002). This has led to the suggestion that investigators should report two replicate biomarkers when reporting associations with oxidative stress burden (Block G,2006; Schlesier K,2002). This would overcome the two main confounding issues, firstly, the variability of the baseline between studies reflecting sampling artefact, and secondly, the variability in the substrate/ROS interaction, where different ROS have different affinity for different substrates (Mayne ST,2003).

**Table 1.3** The ranking by anti-oxidant capacity of different drinks using five different TAC methods. See paper for details of methods used. (Schlesier K,2002)

|                      | TEAC | DPPH | DMPD | FRAP | PCL |
|----------------------|------|------|------|------|-----|
| Blackcurrant juice   | 1    | 1    | 1    | 2    | 1   |
| Blackcurrant Nectar  | 2    | 2    | 2    | 1    | 6   |
| Green Assam Tea      | 3    | 6    | 6    | 4    | 5   |
| Black Assam Tea      | 4    | 4    | 4    | 7    | 3   |
| Green Darjeeling Tea | 5    | 3    | 3    | 6    | 4   |
| Black Darjeeling Tea | 6    | 5    | 5    | 5    | 2   |
| Apple Juice 1        | 7    | 8    | 8    | 3    | 7   |
| Apple Juice 2        | 8    | 7    | 7    | 8    | 10  |
| Tomato Juice 1       | 9    | 10   | 10   | 10   | 9   |
| Tomato Juice 2       | 10   | 9    | 9    | 9    | 8   |

1.3.4 Role of oxidative stress in type 2 diabetes

There are a number of steps in the natural history of type 2 diabetes where oxidative stress may play a role. These include the development of insulin resistance, β-cell failure and end organ damage secondary to hyperglycaemia. States that predispose to the development of type 2 diabetes appear to be

associated with increased oxidative stress. ROS production increases with fat accumulation in humans, with higher levels of both TBARS (Furukawa S,2004) and isoprostanes (Keaney JF,2003) in obese subjects compared to controls. Reduced anti-oxidant expression has also been demonstrated in obese mice compared to controls (Furukawa S,2004). Oxidative stress is also increased in the metabolic syndrome, with 3.7 times greater levels of isoprostanes and a 41% reduction in the anti-oxidant activity of HDL in a AAPH system compared to controls (Hansel B,2004). Diabetogenic cytokines such as TNFα are also associated with higher levels of oxidative stress (Qi C,2000). If ROS are important in the development of type 2 diabetes then they should be associated with over-nutrition. Post-prandial increases in lipid and carbohydrate levels increase oxidative stress (Tsai WC,2004), in part, driven by increased rates of non-enzymatic glycation (Dandona P,1996) and glucose auto-oxidation (Mullarkey CJ,1990) seen after a meal, but also by the presence, in the circulation, of oxidized lipids, from a processed diet (Blaak EE,2006). This may be more important in western societies where a significant part of the day is spent in the post-prandial state (Sies H,2005).

This increase in ROS may act as a sensor of the nutrient environment (Nemoto S,2000). This is because increased nutrient delivery will increase mitochondrial activity, and ROS generation is an obligatory consequence of aerobic metabolism (Bloch-Damti A,2005). Mitochondria are the principle source of ROS in cells (Zhou YP,1995), and the primary factor determining the rate of production is the redox state of the electron transport chain (Skulachev VP,1996). Glycolysis and the citric acid cycle generate NADPH and FADH, which donate electrons to the electron transport chain. As electrons are moved along the four complexes, hydrogen ions are pumped out of the mitochondria and an electrochemical gradient is established (Nishikawa T,2000a). This gradient is dissipated through the ATP synthase complex, converting ADP to ATP for use as an energy source in the cell (Fig 1.13).

**Fig.1.13** A representation of the electron transport chain for the production of energy in the form of ATP from NADPH and FADH generated during oxidation



(http://www.biologycorner.com/resources/electrontransport.jpg)
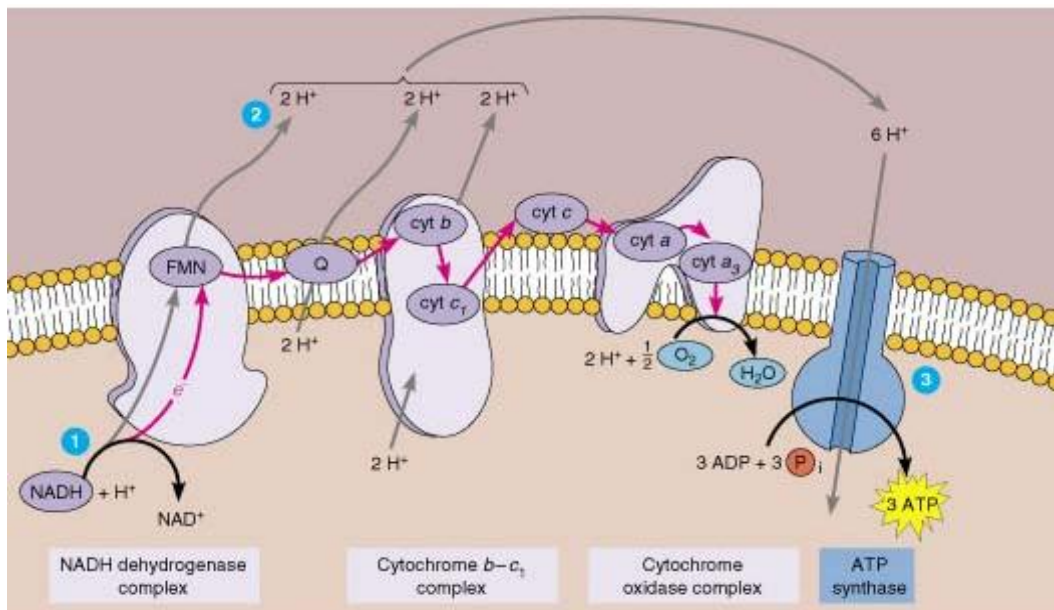
The amplitude of this gradient regulates chain activity and increased nutrient delivery will increase this gradient (Maddux BA,2001), which reduces activity and leads to the partial reduction of oxygen and the generation of ROS, whilst electrons are "held up" in the chain (Nishikawa T,2000b). Reducing the membrane potential, such as for ATP production, reduces ROS generation (Fridlyand LE,2006).

Mitochondrial DNA is in close proximity to the electron transport chain, and it is possible that mitochondrial DNA damage is the cause of the small dysfunctional mitochondria found in type 2 diabetes (Maassen JA,2004). Significant oxidative stress leads to programmed death of mitochondria, reducing oxidative capacity further (Watson RT,2004). Reducing the delivery of electrons to the electron transport chain would reduce oxidative stress. The best way to achieve this is to prevent nutrient entrance into cells i.e. insulin resistance. It may be that insulin resistance is a protective mechanism when large amounts of ROS are being generated by the electron transport chain (Fridlyand LE,2006).

The generation of ROS has a number of deleterious effects on insulin secretion and sensitivity. A transient increase in ROS enhances insulin signalling (Keaney JF,2003), possibly because insulin signalling itself may require ROS as a second messenger (Kaneto H,1999). A burst of ROS is triggered with a variety of ligands including Platelet Derived Growth Factor (Sundaresan M,1995), Epidermal Growth Factor (Bae YS,1997) and Angiotensin II (Griendling KK,2000) and ROS is becoming increasingly recognised as an important intracellular signal (Goldstein BJ,2005). However, chronic oxidative stress is damaging to insulin action (Furukawa S,2004) and may precede the development of type 2 diabetes (Salonen JT,1995). Hydrogen peroxide impairs insulin signalling in 3T3-L1 adipocytes, increases serine/threonine phosphorylation of IRS (Potashnik R,2003), reduces PI3 kinase and Akt activation (Furukawa S,2004; Tirosh A,1999) and GLUT 4 translocation (Pessler D,2001; Rudich A,1998). Hydrogen peroxide can also alter gene expression, reducing the expression of the insulin sensitisers adiponectin and leptin (Kamigaki M,2006)

ROS also stimulates intracellular processes that will lead to secondary increases in insulin resistance. These stimulate a number of transcription factors such as Mitogen Activated Protein Kinase, p38 kinase, protein kinase C isoforms and c-Jun kinases (Kaneto H,2005) and, hence, activate inflammatory pathways which regulate gene expression, promoting insulin resistance as described earlier. Exposure to oxidative stress therefore promotes insulin resistance by, firstly, impairment of insulin signalling and secondly, activation of cellular stress kinases (Pessler D,2001).

The important role of oxidative stress is further supported by the effect of anti-oxidants on insulin sensitivity. In either cell (Benhamou PY,1998) or animal (Kubisch HM,1997) models of diabetes, genetic modifications of the oxidative stress load maintain insulin sensitivity, and the anti-oxidant α-lipoic acid improves glucose utilisation in cell culture (Maddux BA,2001) and when given orally (Konrad T,1999). Insulin sensitivity is also improved by the administration of the anti-oxidants vitamin C, E, N-Actylcysteine and Glutathione (Fridlyand LE,2006; Hildebrandt W,2004).

B-cell function and insulin secretion are also similarly affected by ROS. ROS lowers glucose-induced insulin secretion as early as 30 minutes after exposure (Maechler P,1999), and in keeping with the functional abnormalities described in type 2 diabetes, hydrogen peroxide can suppress first phase insulin secretion (Sakai K,2003), while glucokinase, a crucial part of the glucose sensor, can be inactivated by ROS (Lenzen S,1988). The pancreas is particularly vulnerable to oxidative stress as it has the lowest intrinsic anti-oxidant capacity (Grankvist K,1981), and does not activate anti-oxidant enzymes during times of cellular stress (Maechler P,1999). B-cells are also susceptible to overfeeding as they are not dependant on insulin for glucose uptake (Ceriello A,2004). Activation of stress signalling pathways is associated with reduced insulin gene expression and cell apoptosis (Kaneto H,2005), which may explain why the pancreas is unable to compensate for increased insulin resistance and type 2 diabetes develops (Pick A,1998). These defects in β-cell function can also be reversed by anti-oxidant strategies, such as over-expression models of glutathione (Tran PO,2004) or C-Jun inhibitors (Duval A,2000) or by the administration of N-acetylcysteine (Tanaka Y,2002).

Oxidative stress may also play a role in the progression of β-cell dysfunction, glucose intolerance and development of the complications of type 2 diabetes (Maritim AC,2003). Development of hyperglycaemia in rats is associated with more severe defects in insulin secretion and sensitivity (Tanaka Y,1999). The effect on β-cells is known as glucose toxicity (Robertson RP,2006). In cultured human islet cells high levels of glucose switch on expression of apoptosis genes Bad, Bid and Bik, while reducing expression of the anti-apoptosis gene Bcl-x1 (Federici M,2001). This may explain the failure of β-cell mass to expand in order to counteract the effect of early glucose intolerance (Pick A,1998).

Hyperglycaemia also appears to increase the oxidative stress burden. TRAP is lower (higher oxidative stress) in both type 1 (Tsai EC,1994) and type 2 diabetes (Ceriello A,1997). Markers of oxidative stress, including salicylate hydroxylation (Ghiselli A,1992), $F_2$-isprostanes (Gopaul NK,1995) and oxidised DNA (Rehman A,1999), are consistently elevated in type 2 diabetes, and this

seems to be related to blood glucose levels as $F_2$-isprostane levels fall with improvements in diabetes control (Davi G,1999). Acute rises in plasma glucose (after a glucose tolerance test) have been demonstrated to be associated with increased $F_2$-isoprostanes (Sampson MJ,2002), reduced anti-oxidant vitamins, uric acid and TRAP in subjects with type 2 diabetes (Ceriello A,1998), although similar findings in healthy volunteers were not replicated in a separate study measuring anti-oxidant vitamins, FRAP and allantoin (Ma SW,2005). This may be because a higher blood glucose is needed as during a clamp study a serum glucose of 15mmol/l increased nitrotyrosine levels in healthy volunteers (Marfella R,2001). The role of oxidative stress in glucose toxicity is further supported by the reduction of severity of insulin resistance and β-cell dysfunction by the use of anti-oxidants in animal models, using the zucker diabetic rat (Tanaka Y,1999)and *db/db* mouse (Kaneto H,1999).

The potential mechanisms by which high glucose levels could increase ROS generation are shown in Fig 1.14. Glucose can generate ROS through non-enzymatic pathways, including the generation of ketoaldehydes (Maritim AC,2003) and the formation of Advanced Glycation End products [AGE]. Glycation of proteins produces a Schiff base, which undergoes rearrangement to form amadori products, which after further auto-oxidation of glucose form AGE. AGE can bind to specific receptors, triggering stress signalling through NF-κβ activation, as well as generating ROS (Yan SD,1994). The upregulation of alternative metabolic pathways also increases ROS generation and stress signalling, and is responsible for the development of a number of complications of type 2 diabetes (Brownlee M,2001).

The sorbitol pathway leads to the accumulation of fructose, which consumes intracellular anti-oxidants and promotes stress signalling (Brownlee M,2001; Evans JL,2002). The hexosamine pathway is also upregulated, and the end product of this pathway, UDP-*N*-acetylglucosamine, is the substrate for the glycosylation of important intracellular factors including transcription factors, particularily those involved in stress signalling (Rolo AP,2006).

**Fig1.14** The pathways leading to the generation of ROS during hyperglycemia.

GLUCOSE

Glucose-6-P

Fructose-1:6-bis-P

Sorbitol Metabolism (4)

Glucosamine

Glyceraldehyde-3-P

Hexosamine Metabolism (5)

Dihydroxyacetone

Enediol

1:3 bis-P-glycerate

Methylglyoxal

Glycerol-3-P

α-Ketoaldehyde (1)

Pyruvate

Glycation (3)

DAG

AcCoA    Oxaloacetate

PKC Activation (2)

Oxidative Phosphorylation (6)

ROS
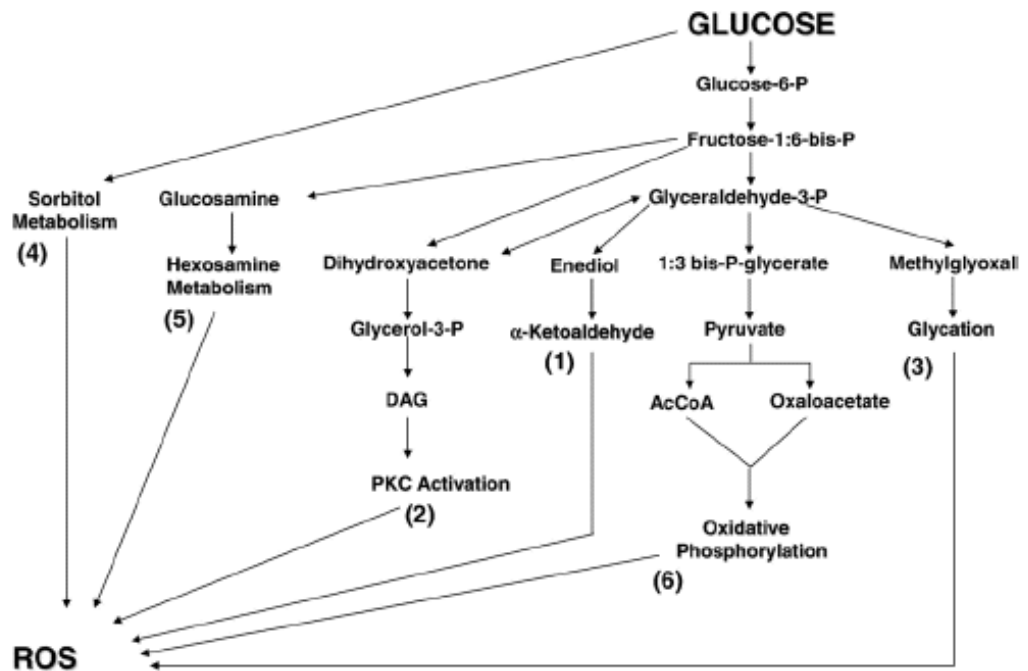
Six biochemical pathways along which glucose metabolism can form reactive oxygen species (ROS). Under physiological conditions, glucose primarily undergoes glycolysis and oxidative phosphorylation (6). Under pathological conditions of hyperglycemia, excessive glucose levels can overwhelm the glycolytic process and inhibit glyceraldehyde catabolism, which causes glucose, fructose 1:6-bis-P, and glyceraldehyde 3-P to be shunted to other pathways: (1) enolization and α-ketoaldehyde formation, (2) PKC activation, (3) dicarbonyl formation and glycation, (4) sorbitol metabolism, and (5) hexosamine metabolism (Robertson RP,2006).

In hyperglycaemia, increased synthesis of diacylglycerol [DAG] activates up to 9 isoforms of PKC, which can also activate stress signalling and lead to the generation of ROS (Brownlee M,2001). The deleterious effects of these pathways are not all dependent on ROS, but oxidative stress from hyperglycemia-induced overproduction of superoxide by mitochondria is the trigger that drives each of these pathways (Rolo AP,2006) (Fig1.15).

**Fig 1.15.** The stimulation of pathological pathways by hyperglycemia-induced ROS generation
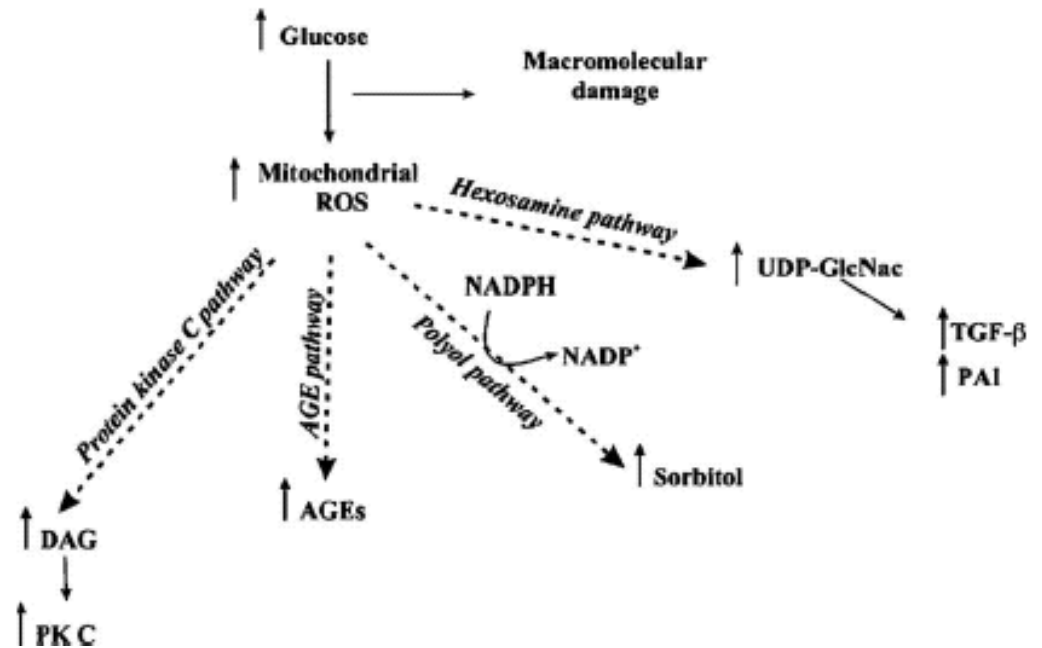


(___, direct role; -----, indirect role). GlcNac, O-linked N-acetylglucosamine; PAI, plasminogen activator inhibitor; TGF-β, transforming growth factor-β. (Rolo AP,2006)

The effect of NEFA on the induction of insulin resistance and β-cell function may also in part be modified by generation of ROS. In healthy volunteers infusion of NEFA increases levels of MDA, reduces intracellular GSH (Paolisso G,1996) and activates NF-κβ (Evans JL,2003). The long chain fatty acid sodium palmitate increases ROS generation in isolated rat β-cells, in parallel with decreases in glucose-stimulated insulin secretion, indicating ROS may also play a part in NEFA-induced β-cell dysfunction.

Therefore, despite significant difficulties in the assessment of oxidative stress there is considerable evidence that the development of insulin resistance and β-cell dysfunction are driven by oxidative stress-dependent mechanisms and that once established, glucose tolerance is perpetuated by increased generation of ROS. The mitochondria, and more specifically the rate of mitochondrial oxidation and the mitochondrial membrane, appear to be key components of this process.

## 1.4 Mitochondrial Uncoupling Proteins

The uncoupling proteins 2 and 3 (UCP2/UCP3) are part of a mitochondrial carrier superfamily that are membrane proteins of around 300 amino acids with six transmembrane domains (Bross O,2000).   Their description as uncoupling proteins is based on their homology to uncoupling protein 1 (UCP1), the first of the family to be described.

The generation of a proton gradient across the mitochondrial membrane during fuel substrate oxidation is described earlier in this chapter.  However, isolated mitochondria can continue to respire in the absence of ATP (Brand MD,1994). This is due to leak of the protons back across the membrane rather than through ATP synthase.  The stored energy is released as heat, and fuel substrate metabolism is said to be "uncoupled" from ATP production (Fig. 1.16). Proton leaks are a feature of all living cells (Schrauwen P,2002a) but the mechanism of these leaks was not previously understood.

**Fig 1.16** The dissipation of the mitochondrial proton gradient to provide ATP synthesis (coupled) or with energy wasting (uncoupled) (Schrauwen P,2002a)
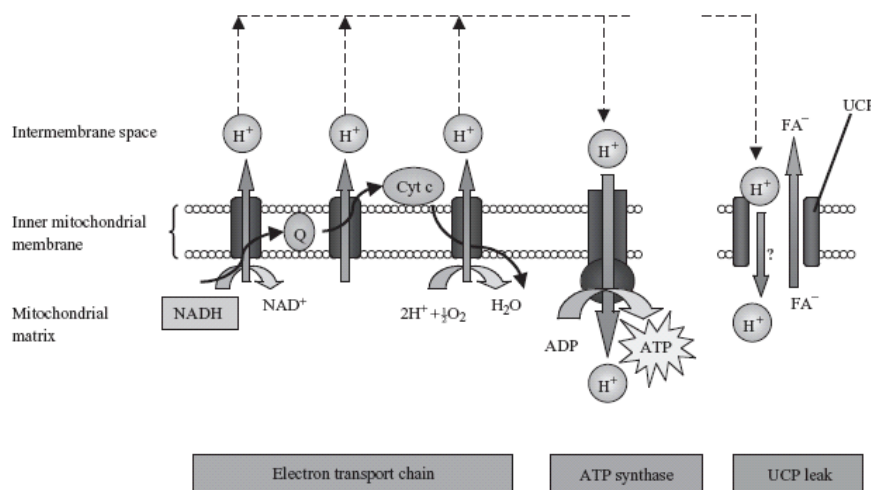


In rodents, body temperature is maintained by uncoupling (Nicholls DG,1999), and is mediated primarily by brown adipose tissue (BAT) (Rothwell NJ,1979).

This led to the identification of a uniquely expressed protein, UCP 1 (Lin CS,1980) which was responsible for the thermogenic proton leak. UCP 1 expression increases with cold stress (Silva JE,1997), and the UCP1 knockout mice were more likely to develop hypothermia in cold conditions than wild-type mice (Enerback S,1997).

However, uncoupling was not unique to BAT (Rolfe DF,1996) with proton leaks responsible for 26-50% of resting energy expenditure (Rolfe DF,1996). This led to the identification of UCP2 and UCP3, which show 55% and 57% homology with UCP1 respectively and 71% homology with each other (Vidal-Puig A,1997). These two UCPs are both located in a gene cluster on chromosome 11q13, within 8kb of each other, and share similar genomic structures (Pecqueur C,1999). Two central nervous system UCPs have also been identified, known as UCP4 (Mao W,1999) and UCP5 or Brain Mitochondrial carrier protein-1 (Sanchis D,1998), although the homology of these two proteins with UCP1 is low (<40%) (Dalgaard LT,2001a). UCP-like proteins have also been identified in plants and birds, indicating that they are an ancient, well-conserved family likely to be important physiologically (Rousset S,2004).


1.4.1. Physiology of Uncoupling Protein 2

UCP 2 was discovered in 1997 (Fleury C,1997; Gimeno RE,1997). The protein is expressed widely in spleen, lung, stomach, skeletal muscle, pancreas, heart, liver, kidney and macrophages (Fleury C,1997; Gimeno RE,1997; Millet L,1997; Pecqueur C,2001),with high homology among species e.g. 95% between rat and human (Matsuda J,1997). The ability of UCP1 to dissipate the proton gradient without the generation of ATP (Nicholls DG,1984) identifies its function as an uncoupler of metabolism, although the exact mechanism of proton transfer is still unclear (Esteves TC,2005). The similarity in structure suggests that UCP2 should also function as an uncoupling protein. A number of experimental systems suggest UCP2 does possess uncoupling properties, including *E. coli* inclusion bodies (Jaburek M,1999) or liposomes (Echtay KS,2001), overexpression in yeast systems (Fleury C,1997; Gimeno RE,1997;

Rial E,1999), and cell culture systems [Hela cells (Mills EH,2002), INSE-1 cells (Hong Y,2001), Human thymus (Krauss S,2002), *Ob/Ob* mice hepatocytes (Chavin KD,1999)and Zucker diabetic rat islet cells (Cortez-Pinto H,1999)]. However, there is not a close link between tissue proton leak and UCP2 levels (Dalgaard LT,2001a), and some of these systems such as liver cells and yeast mitochondria can exhibit a proton leak in the absence of UCPs (Porter RK,1995; Stuart JA,1999a). The yeast system also has other potential confounding features that may apply to the other systems. When UCP2 is expressed in yeast it is misfolded and only poorly incorporated into the membrane. Replication at low levels of expression did not demonstrate a proton leak, and it is thought that the earlier results could be due to an over-expression artefact (Stuart JA,2001). The structure of UCP2 also lacks a histidine pair thought to be essential for UCP1 function (Masaki T,1997). Therefore, the evidence that UCP2 functions as an uncoupler of mitochondrial metabolism is not conclusive. There is no change in proton conductance in the UCP2 mouse under basal conditions, although this does change under certain conditions such as activation by ROS or free fatty acids (Esteves TC,2005). These results suggest that UCP2 has a novel function that may be dependent on conventional uncoupling, or may act through a non-uncoupling mechanism.

1.4.1a UCP2 and Metabolism

Despite there being little BAT in adult humans (Garruti G,1992) there is no evidence to suggest that UCP2 has a role in thermogenesis. The UCP1 knockout mouse remains cold-sensitive despite increased expression of UCP2 (Enerback S,1997), while the UCP2 knockout mouse is not cold sensitive (Arsenijevic D,2003). The expression of UCP2 in ectothermic fish, which remain the temperature of the surrounding water, also suggests that UCP2 has no role in the regulation of body temperature (Stuart JA,1999b).

The ability to waste energy derived from substrate utilisation would make UCP2 an attractive molecule for a role in the adaptive response to over-nutrition. Resting metabolic rate is dependent on thyroid and steroid hormone levels, sympathetic nervous system activity and ratio of fat to fat-free mass (Toubro

S,1996). Energy expenditure over 24hrs is in part genetic, with a heritability of between 0.26-0.70 (Dalgaard LT,2001a), and lower expenditure is more predictive of future weight gain than increased nutrient intake (Roberts SB,1988) with only very small differences increasing the risk of obesity significantly (Leibel RL,1997). UCP2 expression is increased in obese mouse models, the *Ob/Ob* and *db/db* mouse (Chavin KD,1999; Gimeno RE,1997), and the obesity prone C57b/6 mouse fails to upregulate UCP2 when fed a high fat diet (Fleury C,1997). In man, abdominal muscle tissue mRNA was 28% lower in obese subjects compared to those without obesity (Nordfors L,1998) and muscle mRNA levels correlated with percentage body fat and BMI (Bao S,1998). Low expression was also found in intraperitoneal fat tissue taken at surgery, which persisted even when the subjects lost weight (Oberkofler H,1998). However, most of this evidence is circumstantial, and UCP2 expression could be following other obesity-induced metabolic changes. The UCP2 knockout mouse does not gain weight on a high fat diet (Arsenijevic D,2003), UCP2 levels do not change after weight loss in man (Vidal-Puig A,1999) and groups with lower UCP2 expression, such as first degree relatives of those with type 2 diabetes, do not have lower energy expenditure. This suggests either that UCP2 does not have a major role in energy balance, or that there is significant overlap in energy control mechanisms (Erlanson-Albertsson C,2003). The data, so far, does not support a critical role for UCP2 in energy balance. This is confirmed by the upregulation of UCP2 by fasting, a state of reduced energy expenditure and reduction in body temperature.

The upregulation of UCP2 by fasting has been consistently demonstrated in rats (Boss O,1997a; Cadenas S,1999; Memon RA,2000; Samec S,1998a; Samec S,1998b; Teshima Y,2003; Xiao H,2004), mice (Kersten S,1999; Samec S,1999a) and humans (Millet L,1997). Interestingly, these studies demonstrated a reduction in mRNA expression with re-feeding, although not if re-fed with a high fat diet (Samec S,1999a; Teshima Y,2003; Xiao H,2004). The fasting state and the re-fed high fat diet state are both associated with elevations in free fatty acids, and abolishing this increase with intervention prevents the induction of UCP2 expression (Dulloo AG,2001). The observation that free fatty acids uncouple mitochondrial metabolism (Challoner DR,1966)

raises the possibility that this is due to UCP2, which has a function in fuel substrate switching or protection of the high rate of oxidation associated with fatty acid delivery. The changes seen with fasting were more pronounced in muscle that is usually glucose dependent (fast twitch-gastrocnemius) than muscle groups with high rates of basal fat oxidation (slow twitch-soleus) (Samec S,1998a). Free fatty acids appear to be key regulators of UCP2 mRNA expression. The higher fatty acid levels associated with a high fat diet (Aubert J,1997; Chan CB,2001; Fleury C,1997; Gong DW,1999; Joseph JW,2002; Matsuda J,1997; Surwit RS,1998; Tsuboyama-Kasaoka N,1999), chemically induced diabetes (Hidaka S,2000; Kageyama H,1998) and acute exercise (Thompson MP,2004) all increase UCP2 mRNA levels in a number of rodent models and tissues, including in hepatocytes which do not normally express UCP2 (Cortez-Pinto H,1999). The transition from glucose to free fatty acid metabolism in cardiomyocytes at birth is associated with a five times increase in levels of UCP2 mRNA in rats (Van Dere Lee KAJM,2000). Interventions such as fibrates (Tsuboyama-Kasaoka N,1999) and nicotinic acid (Samec S,1998a), which lower free fatty acid levels, also lower UCP2 mRNA in rodents. In humans, lipid infusion increased mRNA expression in subcutaneous fat (Nisoli E,2000) although no change was found in skeletal muscle in another study (Khalfallah Y,2000). Cell culture experiments confirm these studies with palmitate, α-bromopalmitate, oleic acid and linoleic acid all increasing UCP2 mRNA in a variety of cell lines, including rat cardiomyocytes and hepatocytes, 3T3-L1, INS-1, H9c2 and L6-myotubules (Armstrong MB,2001; Aubert J,1997; Camirand A,1998; Lameloise N,2001; Medvedev AV,2002; Reilly JM,2000; Van Dere Lee KAJM,2000; Viguerie-Bascands N,1999). In rat islet cells the increase in mRNA expression paralleled the increase in fat oxidation and was inhibited by high glucose levels, which lowers fat oxidation (Medvedev AV,2002).

A role in fatty acid metabolism is also suggested by the regulation of the gene by the key metabolic transcription factors of the PPAR group (Masaki T,1997), as well as other regulators that modify fatty acid metabolism, including thyroid hormones (Nagase I,2001), catecholamines (Masaki T,1997) and the sterol regulatory element binding protein-1 (Medvedev AV,2002). The result of this is that changes in UCP2 mRNA parallel changes in other genes in fat oxidation

such as carnitine palmitoyltransferase, acylcoA dehydrogenase and long chain CoA dehydrogenase (Samec S,2001).

Overexpression of uncoupling proteins in mice induced resistance to weight gain, even on a high fat diet with higher oxygen metabolism (Li B,2000). The widespread expression would make UCP2 an attractive candidate for energy balance control. The expression of UCP2 suggests an important role in fatty acid metabolism but does not support the hypothesis that this role is in energy balance. The ability of fatty acids to uncouple is probably not dependent on UCP2 (Hirabara SM,2006), and may just be a reflection of the fatty acid content of cell membranes (Porter RK,2001). Therefore, despite a large body of evidence that UCP2 is an important component of fatty acid metabolism its physiological role is unclear. From an evolutionary standpoint the development of a mechanism to waste energy, when food seeking and energy conservation is given high priority, is surprising (Erlanson-Albertsson C,2003). The earlier sections of this chapter describe an important difference between glucose and fat oxidation, documenting the higher levels of ROS production by the electron transport chain during fat metabolism. Non-thermogenic uncoupling does, however, reduce ROS generation (Vidal-Puig AJ,2000). The possibility that UCP2 is a modulator of ROS production is examined in more detail in 1.4.1c, but if this is the case then the involvement in fatty acid metabolism may be purely a feature of the higher levels of ROS generated therein.

1.4.1b UCP2 and Islet Cell Function

The dependence of insulin secretion on ATP production makes it likely that expression of UCP2 in the pancreas will have a modifying effect on β-cell function. A small study described 80% higher skeletal muscle UCP2 mRNA levels in type 2 diabetes, although the small number of subjects in the study meant that this difference was not statistically significant (p=0.09)(Bao S,1998). However, UCP2 mRNA levels in skeletal muscle correlate with fasting blood glucose in subjects with obesity/type 2 diabetes (Bao S,1998). The UCP2 knockout mouse has increased β-cell ATP levels, and higher glucose-stimulated

insulin secretion, leading to 18% lower blood glucose levels (Zhang C-Y,2001), as well as higher β-cell mass and insulin content even on a high fat diet (Joseph JW,2002). If UCP2 is knocked out in the *Ob/Ob* mouse, then first phase insulin secretion is restored and higher insulin levels and lower blood glucose levels are observed, with no changes to insulin sensitivity (Zhang C-Y,2001) Although one early dissenting report suggested the opposite (Wang MY,1999), UCP2 over-expression models have now shown consistently lower insulin secretion in rat islet cells (Chan CB,1999), human insulinoma cells (Hong Y,2001) and mouse and human islet cells. The defect in insulin secretion is isolated to glucose, with no difference in response to direct stimulators of ATP-dependent K channels (Chan CB,2001) .

Studies on the UCP2 knockout mouse have suggested UCP2 may be an important modulator of insulin secretion. The reduction of β-cell function, and increase in UCP2 mRNA, seen with free fatty acids, may in part explain their diabetogenic effect. The exposure of the UCP2 knockout mouse to palmitate does not cause the lower glucose-stimulated insulin secretion that is normally seen in response to free fatty acids. Isolated islet cells have higher ATP levels, and the mitochondrial membrane is hyperpolarized faster and to a greater extent (Joseph JW,2004). Superoxide can also have a negative effect on β-cell function, which is absent in the UCP2 knockout mouse (Krauss S,2003).

Thus UCP2 appears to be a critical modulator of insulin secretion, but once again it is not clear if that is the primary function of the protein, or if it is simply a side effect of UCP2 being activated by fatty acid-derived ROS. This means that potentially enhanced UCP2 function would be associated with protection from obesity but increased risk of type 2 diabetes, an "Adiposity Angel and Diabetes Devil" (O'Rahilly S,2001). This effect also raises the possibility that UCP2 could be a therapeutic target for the treatment of type 2 diabetes. In mice, a pharmacological inhibitor of UCP2-determined proton leak did reverse obesity-induced β-cell dysfunction through higher levels of ATP production, although it is not clear how long this effect persists (Zhang CY,2006).

1.4.1c  UCP2 and oxidative stress.

In most cells the majority of ROS is generated by the electron transport chain, so that a protein that can modulate mitochondrial function would be a good candidate for being part of the cellular defense against ROS (Skulachev VP,1996).   Mitochondrial production of superoxide is inversely correlated with the amount of uncoupling (Gimeno RE,1997), and mitochondrial ROS generation can be modulated by UCP2 activation (Negre-Salvayre A,1997), whilst inactivation using an antisense RNA method increases ROS generation in a mouse endothelial cell line (Duval C,2002).  The immune system is also a high producer of ROS, and oxidative stress. The regulation of UCP2 with immune function further suggests that it may have a role in the modulation of oxidative stress.  UCP2 mRNA expression is activated by superoxide (Echtay KS,2001; Echtay KS,2002a), hydroxynonenal (Echtay KS,2003), AAPH (Murphy MP,2003), lipopolysaccharide [LPS] (Faggioni R,1998; Viguerie-Bascands N,1999) and TNF α (Cortez-Pinto H,1998; Viguerie-Bascands N,1999) and levels become high in macrophages as they differentiate into functioning phagocytes (Nishio K,2005).   The induction of UCP2 expression by LPS can be blocked by the administration of the anti-oxidant n-acetylcysteine, indicating the role of ROS generation in UCP2 expression regulation (Alves-Guerra MC,2003). UCP2 expression is also increased in pathological situations where ROS is thought to be important, such as ischaemia (de Bilbao F,2004), ischaemic pre-conditioning (McLeod CJ,2005), irradiation (Voehringer DW,2000), left ventricular dilatation and failure (Guo P,2005) and inflammatory conditions (Rousset S,2004).  The findings of increased ROS in UCP2 knockout models (Bai Y,2005; Joseph JW,2004), and decreased ROS in over-expression models (Kizaki T,2003), confirms the finding that ROS is a modulator of UCP2 function, and that changes to UCP2 function can modulate ROS production.

The first study to demonstrate that this may be of physiological importance was a study on the immune function of the UCP2 knockout mouse.  Pathogen killing is dependent on the generation of superoxide by   macrophages (Babior

BM,2000). The UCP2 knockout mouse had a higher bactericidal activity against Salmonella typhimurium, and was able to clear Toxoplasma gondii infection, which remains a chronic infection in wildtype mice (Arsenijevic D,2003). The activation of the immune system by LPS is also higher with higher levels of inflammatory cytokines, NF-κβ activation and hydrogen peroxide generation during bacterial killing compared to wildtype mice (Bai Y,2005). The role of UCP2 modulation of ROS has also been studied in a number of models of neuroprotection. UCP2 over-expression reduces the toxicity of 1,2,3,6-methyl-phenyl-tetrahydropyridine [a toxin used to induce an animal model of Parkinsons disease] on isolated neurones (Conti B,2005), and injury size in stroke and traumatic brain injury models (Mattiasson G,2003). In a mouse model of Multiple Sclerosis the knock out of *UCP2* led to higher disease scores and increased T-cell activity and cytokine production (Vogler S,2006). The response to toxins that induce ROS-dependent disease is also higher in UCP2 knockout mice, including tumour development to azooxymethane (Derdak Z,2006) and cell damage to mendione in hepatocytes (Collins P,2005), while hepatic regeneration after injury is also delayed as ROS interferes with the cell cycle (Horimoto M,2004) .

ROS are thought to play an important part in the generation of cardiovascular disease. LDL oxidation is one of the first steps of atheroma. The LDL-receptor knockout mouse is a model of cardiovascular disease, and when the *UCP2* gene was also knocked out in this mouse the plaque area was increased by 42% compared to the LDL-receptor knock out "wildtype" (Blanc J,2003). The plaques in the UCP2/LDL receptor knockout mouse were strongly positive for nitrotyrosine staining [a marker of peroxynitrite generation form superoxide and, thus, oxidative damage] while the UCP2 wildtype/LDL receptor knockout mouse nitrotyrosine staining was barely detectable (Blanc J,2003). In human vascular smooth muscle cells overexpression of UCP2 lowered the toxic effects of glucose and angiotensin II (Park JY,2005) .

Cell death pathways including apoptosis can be stimulated by ROS or mitochondrial dysfunction. The early steps often include changes to mitochondrial membrane potential, mitochondrial calcium entry and ROS

generation. UCP2 can protect mitochondrial function by modulating membrane potential. Cell survival after exposure to hydrogen peroxide is higher with UCP2 over-expression in β-cell (Li LX,2001), cardiomyocytes (Teshima Y,2003; Zackova M,2003) and endocrine cell models (Diano S,2003). In human aortic endothelial cells UCP2 over expression inhibited all the steps of mitochondrial dysfunction and cell death that occurred with administration of lysophophatidylcholine [LPC]. This effect appears to be physiologically relevant as LPC induces impaired endothelial function, an effect not seen in isolated rat aorta infected with an UCP2 over-expression vector (Lee KU,2005).

Summary

The function of UCP2 is still not fully understood. The protein may be a conventional uncoupler, but there is no evidence of a role in thermogenesis or obesity/energy balance. Expression studies indicate a potential role in fatty acid metabolism, or protection from the redox pressure on the electron transport chain from the higher ATP production associated with fatty acid metabolism. The modulation of ATP production means that UCP2 clearly modulates insulin secretion, although it is not clear if this is a primary function. The immune system is a large generator of ROS and patterns of expression, such as in the liver where it is located in the phagocytic Kuppfer cells only (Larrouy D,1997), suggests that protection from immune system ROS is a potential function, while a modulator of immune function or even inflammation-induced pyrexia (Faggioni R,1998) are potential functions. The possibility that the mRNA expression observed in other tissues is due to the presence of entrapped circulating or tissue immune cells has yet to be examined. However, expression studies need to be interpreted with caution, as UCP2 mRNA undergoes significant translational modification and regulation, and a number of tissues may have mRNA identified in them but not UCP2 protein (Pecqueur C,2001). The absence of a specific antibody further impedes the understanding of the role of UCP2.

## 1.4.2  Physiology of Uncoupling Protein 3

UCP3 was also identified by cDNA library screening (Boss O,1997b; Vidal-Puig A,1997) and mapped to chromosome 11q13 in man, adjacent to UCP2 (Solanes G,1997).   UCP3 mRNA was identified in two forms, UCP3L and UCP3S, the second form lacking the sixth transmembrane domain and the nucleotide binding site (Boss O,1997b).   However, levels of the two forms are highly correlated, indicating regulation by a common mechanism (Bao S,1998).   UCP3 also shows high homology between species, mouse UCP3 is 88% identical and rat 86% to humans, and both also express a UCP3S mRNA also lacking a transmembrane domain (Solanes G,1997) and a purine binding site (Bouillaud F,1994).    The tissue distribution of UCP2 and UCP3 are very different, with UCP3 being located predominantly in skeletal muscle, with weak signals seen in the heart, thyroid and bone marrow, and in rodents, in brown adipose tissue (Boss O,1997b; Vidal-Puig A,1997).

## 1.4.2a UCP3 thermogenesis and uncoupling

The tissue-specific distribution in skeletal muscle makes UCP3 an attractive candidate for a role in energy balance in man.  Skeletal muscle is responsible for roughly 80% of resting energy expenditure (Ravussin E,1992) and 40% of adrenaline-induced thermogenesis (Simonsen L,1993).  Skeletal muscle metabolism is also uncoupled, with up to 50% of resting metabolic rate due to proton leaks (Rolfe DF,1996).   UCP3 uncouples metabolism in yeast over-expression systems (Gong DW,1997; Hagen T,1999; Hinz W,1999; Zhang CY,1999), insulinoma cells (Hong Y,2001), MCF7 cells (Stock MJ,1999), 293T cells (Mao W,1999), E. Coli (Echtay KS,2001; Jaburek M,1999), C2C12 myoblasts (Boss O,1998a) and L6 myocytes (Guerini D,2002).  However, most of these systems are susceptible to overexpression artefact, and the proton leak is not regulated by purine nucleotides in a physiological manner (Schrauwen P,2006a).  Like UCP2, UCP3 lacks the histidine pair thought to be required for conventional uncoupling activity (Masaki T,1997).

Evidence from whole organisms is also unclear. Mice *UCP-3* over-expression models have smaller adipose tissue depots, are resistant to obesity and do demonstrate increased energy expenditure, fat oxidation, oxygen consumption and a decline in mitochondrial membrane potential (Clapham JC,2000), but the proton leak is again not regulated in a physiological manner, indicating that this may be an artefact (Cadenas S,2002). The UCP3 knockout mouse is not obese and has normal energy expenditure even though the mitochondria are more coupled. There appears to be no effect on bodyweight or temperature regulation phenotype (Cline GW,2001; Gong DW,2000; Vidal-Puig AJ,2000). Temperature maintenance in hibernating squirrels is not dependent on UCP3 even at temperatures as low as -10C (Barger JL,2006). In Pima Indians mRNA levels are associated with 24hr energy expenditure and adjusted metabolic rates and inversely correlated with BMI and percentage body fat (Schrauwen P,1999). Women who successfully lost weight were more uncoupled and had higher UCP3 mRNA levels than women who were unable to lose weight in a simple trial of dietary advice (Harper ME,2002). When UCP3 protein is measured directly no such relationship with BMI (Schrauwen P,2001a), sleeping metabolic rate or 24 hour energy expenditure (Schrauwen P,2002a) could be detected. In rodents, although free fatty acids uncouple mitochondrial metabolism, this does not occur in conjunction with changes in UCP3 mRNA levels (Hirabara SM,2006). Acute exposure to cold does upregulate UCP3, mRNA which later falls on chronic exposure (Lin B,1998), but again this does not correspond to the time course of non-shivering heat production (Schrauwen P,2002a), while in humans mild cold exposure has no effect on UCP3 mRNA or protein levels (Schrauwen P,2002b). Although *UCP3* expression is regulated by thyroid hormone, and in pathological thyroid states changes in energy metabolism may be in part due to altered UCP3 levels (Hesselink MK,2005), there is no evidence that the primary role of UCP3 is mitochondrial uncoupling and regulation of energy expenditure. This data, combined with similar evidence reviewed later, shows that UCP3 is regulated by skeletal muscle fatty acid tissue delivery regardless of body temperature or energy balance, which makes it unlikely that the primary function of UCP3 is the regulation of either of these parameters.

1.4.2b UCP3 and fatty acid metabolism

The paradoxical increase in UCP3 mRNA with fasting again led to a number of studies that suggest that UCP3 has an important role in fatty acid metabolism. UCP3 mRNA levels correlate with free fatty acids (Boss O,1998a; Vidal H,1999) and in most cases, physiological or pharmacological increases in free fatty acids and their metabolism increase UCP3 mRNA levels or protein.  This includes in the rat, fasting (Boss O,1998c; Cadenas S,1999; Gong DW,1997; Samec S,1999b; Samec S,1998b; Weigle DS,1998), sepsis (Sun X,2003), acute exercise (Cortright RN,1999), high fat feeding and lipid infusion (Weigle DS,1998) in skeletal muscle; fasting (Gong DW,1997; Samec S,1998b; Sivitz WI,1999), high fat feeding  (Matsuda J,1997) in brown and white adipose tissue and fasting in the heart (Hidaka S,1999).  In mice, fasting, high fat feeding , and acute exercise have similar effects in both adipose tissue and skeletal muscle (Gong DW,1999; Hwang CS,1999; Schrauwen P,2003; Tsuboyama-Kasaoka N,1998; Tsuboyama-Kasaoka N,1999).  At the beginning of suckling, levels of free fatty acids rise over a couple of weeks in mice, and this is accompanied by a rise in UCP3 mRNA over the exact same time course (Brun S,1999).  In humans, UCP3 is upregulated by fasting (Millet L,1997; Vidal H,1999), lipid infusion (Khalfallah Y,2000; Nisoli E,2000), acute exercise (Noland RC,2003; Schrauwen P,2002a) and by a high fat diet (Hesselink MKC,2003).  The switch to fat metabolism in the failing heart is also associated with higher UCP3 mRNA levels (Murray AJ,2004).  The effect of fatty acids persists at thermoneutrality (Boss O,1998a) and is more pronounced in glycolytic muscle fibres (Samec S,1998a).  It does not occur if levels of free fatty acids are suppressed (Samec S,1998a) or if fatty acid oxidation is suppressed (Schrauwen P,2002a).  The reversal of these changes, such as by refeeding, also reverses the effect on UCP3.  Cell systems also demonstrate increased UCP3 mRNA levels, with free fatty acids in C2C12 cells (Cabrero A,2000; Hwang CS,1999), human myocytes (Sbraccia P,2002), L6 myocytes (Costello A,2003; Nagase I,1999; Son C,2001) and rat myocytes (Hoeks J,2003).

Fasting is not accompanied by a change in proton conductance (Cadenas S,1999), and changes in proton gradient with any of these stimuli follow a

different time course to the changes in UCP3 (Bevilacqua L,2005), so it is likely that the role of UCP3 is related to fatty acid oxidation rather than energy balance or conventional uncoupling.    A number of studies support this hypothesis;  during lipid infusion in humans the lipid oxidation rates correlate with UCP3 mRNA and free fatty acid levels (Khalfallah Y,2000), and the regulation of *UCP3* expression occurs in parallel to other fatty acid oxidation genes (Samec S,2001).   The PPAR family are also important regulators of *UCP3* expression   (Cabrero A,2000; Nagase I,1999; Son C,2001) and both PPAR α (Silvestri E,2006) and γ (Brunmair B,2004; Hwang CS,1999) agonists increase UCP3 mRNA, while levels are lower in the PPARγ knockout mouse (Murray AJ,2005).   However there are a number of situations when fatty acid metabolism is not associated with an increase in UCP3 mRNA or protein levels. UCP3 mRNA levels are higher in muscle groups with a higher proportion of fast twitch glycolytic fibres and lower fat oxidation (Schrauwen P,2001b).   Exercise training (Boss O,1998b; Russell AP,2003; Schrauwen P,2005; Tsuboyama-Kasaoka N,1998) and weight reduction (Esterbauer H,1999; Schrauwen P.,2000; Vidal-Puig A,1999), which increase fat oxidation capacity, are also associated with lower levels of UCP3 mRNA or protein.   The pharmacological inhibition of fatty acid oxidation is also associated with an increase in UCP3 mRNA (Cabrero A,2001; Samec S,1999b).   In all these conditions UCP3 levels do not appear to correlate with the rate of fat oxidation, however, the muscles with low oxidative capacity tend to accumulate excess free fatty acids (Schrauwen P,2004), and it appears that *UCP3* expression may be regulated by intracellular  fatty acid surplus, which has led to the hypothesis that UCP3 functions in mitochondrial fatty acid transport.

### 1.4.2c UCP3 and fatty acid transport

Although thought to be the classical uncoupling protein the actual mechanism of mitochondrial uncoupling by UCP1 is unclear and it is possible that rather than directly transporting protons the protein transports fatty acid anions (Schrauwen P,2002a).   The function of UCP3 is dependent on the availability of free fatty acids, and the mRNA is specifically upregulated by the oversupply of long chain but not medium chain fatty acids (Schrauwen P,2003).   There is an important

difference in the handling of these two types of fatty acid by mitochondria. Fatty acids are taken up into the mitochondrial matrix by CPT1, with carnitine as a co-factor, where they are converted to fatty acylCoA. The inner mitochondrial membrane is impermeable to fatty acylCoA, so fatty acids are transported by conversion to fatty acyl carnitine by CPT1 and then back to fatty acylCoA in the inner mitochondrial matrix by CPT2. This is then available for oxidation (Schrauwen P,2006a). Fatty acids can accumulate within the matrix by either removal of the acylCoA by thioesterases, releasing the CoA for immediate oxidation in the citric acid cycle, or by passive transport of neutral fatty acids, when fatty acid supply overwhelms the ability to incorporate them into fatty acid acylCoA. Within the inner mitochondrial matrix medium chain fatty acids can be readily oxidised without modification, whilst this is not the case for long chain fatty acids. These are deprotonated into fatty acid anions (Hamilton JA,1999), which become trapped within the mitochondria, as the membrane is impermeable to fatty acid anions (Ho JK,2002). The accumulation of fatty acid anions near the respiratory chain are prone to oxidation by lipid peroxides, which are highly reactive (Yagi K,1987), and will have the capacity to further damage important metabolic enzymes as well as mitochondrial DNA (Goglia F,2003). The impairment of mitochondrial function has been linked to insulin resistance and ageing as described earlier.

Thus there are a number of potential mechanisms by which UCP3 may protect the mitochondria from fatty acid anion and lipid peroxide accumulation (see Fig 1.18) and there are a number of studies supporting this as the primary function of UCP3. Medium chain fatty acids are less susceptible to oxidative damage, and, as discussed above, are not linked to regulation of *UCP3* expression. In mouse models UCP3 mRNA is regulated in parallel with mRNA levels of thioesterases (Moore GBT,2001). UCP3 over-expression increases fatty acid oxidation, CPT1 levels, and reduces lipid accumulation in skeletal muscle (Bezaire V,2005), without any effect on glucose oxidation or convention uncoupling (MacLellan JD,2005). Fat oxidation is reduced in the UCP3 knockout mouse (Bezaire V,2001), which also demonstrates higher levels of ROS production (Vidal-Puig AJ,2000), intramuscular lipid peroxides (Hoeks J,2006) and oxidative damage to protein lipids and DNA (Brand MD,2002;

Echtay KS,2003).  Aconitase function, inactivated by superoxide, is reduced both by purine inhibition of UCP3 function and in the UCP3 knockout mouse (Talbot DA,2005).  The effects in the knockout mouse are influenced by diet, with a decrease in lipid peroxide formation on a high fat diet, because of a decrease in intracellular fat storage by a mechanism as yet unknown (Echtay KS,2003).  Therefore, these results must be interpreted with caution, in case they are confounded by a compensatory mechanism.

**Fig 1.17** Hypothesized mechanisms by which UCP3 may protect from fatty acid accumulation and peroxidation (MacLellan JD,2005)
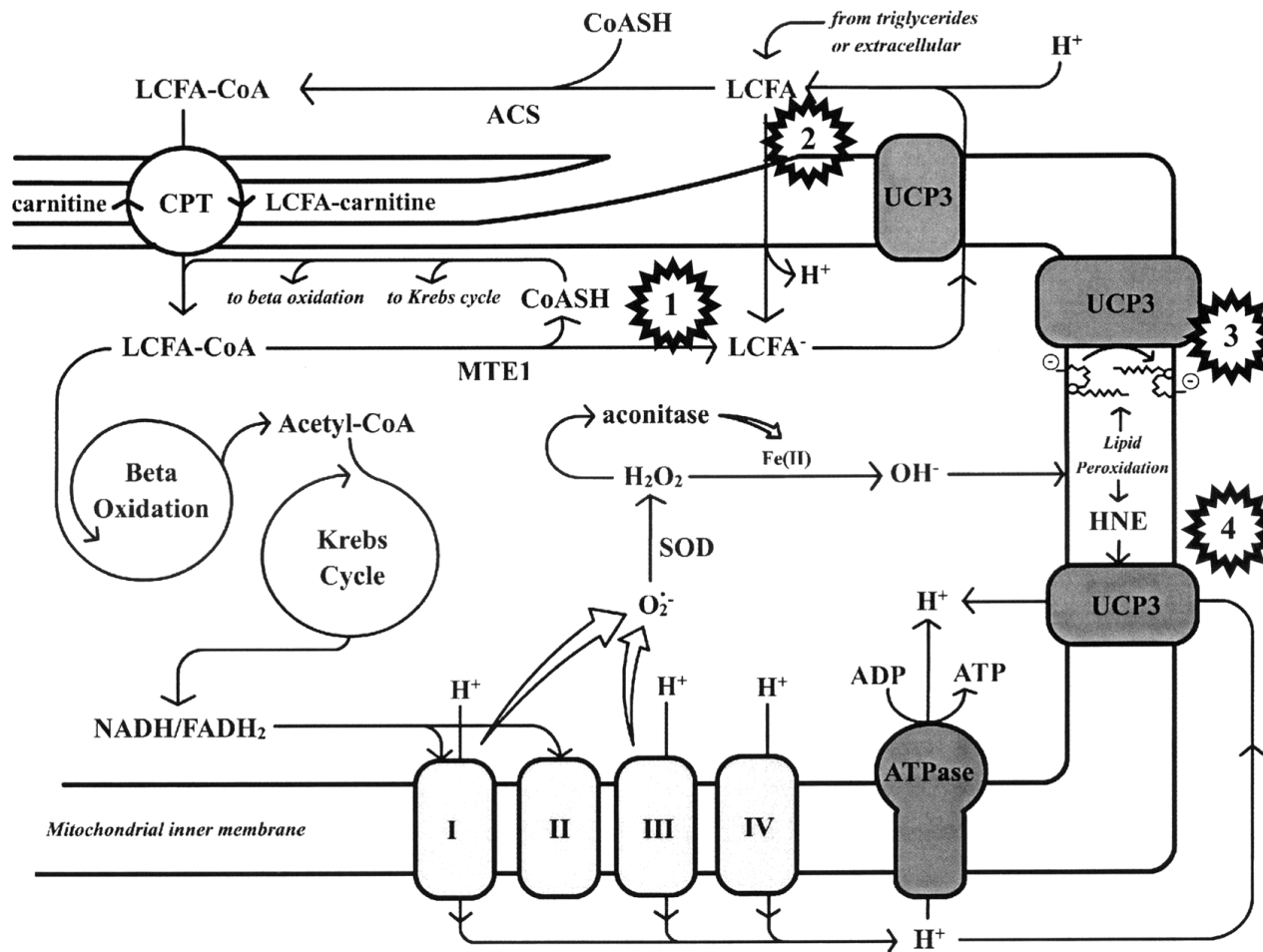


1)UCP3 removes long-chain fatty acids (LCFA) produced by MTE1; the latter liberates mitochondrial matrix CoASH, a rate-limiting coenzyme for ß-oxidation and the Krebs cycle. Long-chain fatty acids are exported from the matrix by UCP3 for reactivation by acyl-CoA synthase in the intermembrane space. Thus, MTE1 and UCP3 are proposed to function in tandem to facilitate fatty acid oxidation (Himms-Hagen J,1990).

2) UCP3 removes excess long-chain fatty acids that have entered the mitochondrial matrix independently of the CPT system. This would serve to remove potentially damaging fatty acid anions from the matrix (Schrauwen P,2003).

3) UCP3 translocates lipid peroxide anions, generated from the interaction of matrix ROS with unsaturated fatty acids of the inner leaflet, from the inner to the outer leaflets of the mitochondrial inner membrane (Goglia FERN,2003). This would remove fatty acid peroxides and prevent damage of mitochondrial DNA, aconitase, and other matrix components.

4) 4-hydroxynonenal (HNE), a lipid by-product of mitochondrial superoxide (SOD) production, is proposed to activate a UCP3-mediated proton leak (Echtay KS,2003). It is proposed that this decreases mitochondrial membrane potential and hence decreases ROS production.

If the function of UCP3 is to control lipid accumulation and peroxidation then *UCP3* expression should be induced by ROS or oxidative damage. Muscle contraction releases ROS and increases UCP3 mRNA, which is blocked by the administration of anti-oxidants (Silveira LR,2006), as does ROS induced by hypoxia in C2C12 cells (Flandin P,2005) and in peri-infarct cardiomyocytes (Almsherqi ZA,2006). The increase in mitochondrial proton conductance seen with the administration of hydrogen peroxide is dependent on free fatty acids and is not seen in the UCP3 knockout mouse, indicating the role of UCP3 in protection from ROS (Echtay KS,2002b). Interestingly, superoxide does not appear to regulate *UCP3* expression directly (Mozo J,2006) but 4-hydroxy-2-nonenol one of the major products of lipid peroxidation does have this function (Echtay KS,2003). Where higher UCP3 mRNA levels have been attributed to higher ROS generation, such as the models above, it may be the higher generation of mitochondrial peroxides by the generated ROS that increases UCP3 mRNA levels. However, ROS generation *per se* does not seem to be the main stimulus of *UCP3* expression. Metabolic changes in ischaemic pre-conditioning are only partly dependent on UCP3 (McLeod CJ,2005) and although UCP3 levels increase after administration of LPS this is thought to be due to fatty acid changes rather than higher levels of ROS (Yu XX,2000). UCP3 function will, as an added feature of its suggested function, lower the mitochondrial membrane potential and reduce metabolic ROS generation, so protecting the cells where it is found (Vincent AM,2004).

In summary, the putative function of UCP3 is, firstly, the protection of mitochondria from fatty acid anion accumulation and peroxidation. Secondly, it also has a role in the protection of mitochondrial function against further oxidative damage from these lipid oxidation products.

1.4.2d UCP3 and type 2 diabetes

The possibility that insulin resistance is a disorder of fat oxidation or mitochondrial function, as discussed earlier, suggests that UCP3 may have an important role in protection from the development of insulin resistance, and that aberrant UCP3 function might be associated with type 2 diabetes. UCP3

mRNA levels are correlated with glucose tolerance (Samec S,1999b). Studies of UCP3 mRNA in subjects with type 2 diabetes have had differing results, with studies showing higher (Bao S,1998; Vidal H,1999) and lower levels (Krook A,1998) associated with disease. Lower levels were also reported in rats with diabetes (Vincent AM,2004). The level in subjects at risk of type 2 diabetes is also variable, with no difference reported in those with first degree relatives (Pedersen SB,2005), but lower levels seen in subjects with impaired glucose tolerance which were corrected by treatment with the insulin sensitizer rosiglitazone (Schrauwen P,2006b). UCP3 protein was 50% lower in subjects with type 2 diabetes (Schrauwen P,2001a), and *UCP3* gene expression is not upregulated in fasting subjects with type 2 diabetes (Vidal H,1999). Experimental models also support a role for aberrant UCP3 function in insulin resistance. UCP3 over-expression in mice is associated with improved glucose tolerance (Clapham JC,2000; Vincent AM,2004; Wang S,2003), while over expression in L6 myocytes increases GLUT4 levels, glucose uptake and insulin signalling (Huppertz C,2001; Hwang CS,1999). The increase in UCP3 mRNA levels after acute exercise is mirrored by an increase in GLUT4 mRNA (Tsuboyama-Kasaoka N,1998) leading to increased glucose uptake. The UCP3 knockout mouse is not diabetic (Brand MD,2002), but this may be because other compensatory mechanisms are limiting the delivery of free fatty acids to mitochondria (Schrauwen P,2006a). Lipid peroxidation and mitochondrial dysfunction are features of type 2 diabetes, and, through its effects on lipid oxidation, UCP3 appears to play a protective role against insulin resistance.

## 1.5    Variation in the uncoupling Protein Genes 2 and 3

The genes for UCP2 and UCP3 are, as described earlier, located on chromosome 11q13 within 8Kb of each other (Pecqueur C,1999). *UCP2* consists of eight exons while *UCP3* consists of seven. The gene structures are represented in Fig 1.17. The region including these two genes or their equivalent has been associated, by linkage analysis, with obesity and diabetes in the mouse (Seldin MF,1994; Taylor BA,1996; Warden CH,1995). In man 11q13 has been linked to resting metabolic rate and percentage fat mass (Bouchard C,1997), type 1 (Hashimoto L,1994) and type 2 diabetes (Ghosh

S,1996; Watanabe RM,2000), all in caucasian samples.  However, replication has been poor, with a number of studies failing to find linkage with obesity or type 2 diabetes in Caucasians or Mexican Americans (Comuzzie AG,2000; Elbein SC,1997; Hager J,1998; Lee JH,1999).  However, this does suggest that this area of the genome may be important in determining risk of obesity and diabetes.

**Fig 1.18.** The structure of the *UCP2* and *UCP3* gene on chromosome 11
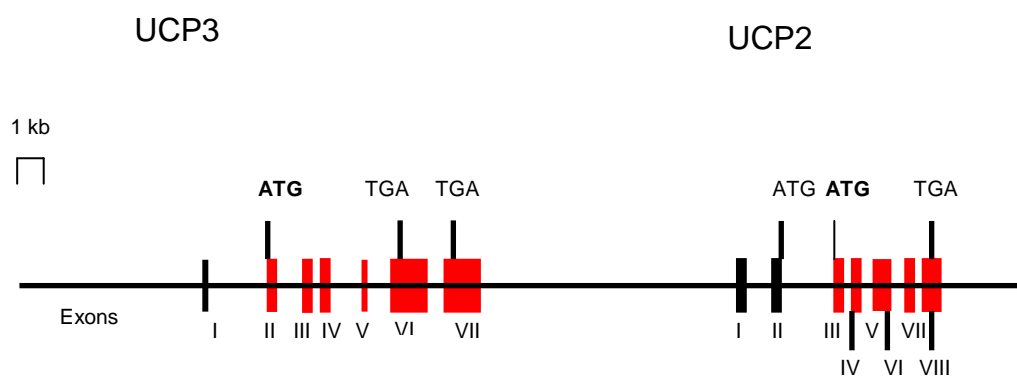


1.5.1 Uncoupling Protein 2

The human *UCP2* gene consists of 8 exons, of which exons 1 and 2 are non-coding (Pecqueur *et al.* 2001).  Exon 2 contains a short open reading fragment that is, like the protein amino acid sequence, highly conserved among species (Jastroch M,2004), and seems to act as an inhibitor of translation although the functional significance of this is unclear at present (Pecqueur C,2001).  The transcription site is preceded by a region that contains a strong *cis*-acting positive regulatory element (-141 to -65) which may underlie the ubiquitous expression of *UCP2.*  In keeping with the changes in expression described, several consensus sequences exist for transcription control elements, such as C/EBP-$\beta$, CREB-1 (cAMP response binding protein 1), two PPAR$\gamma$ responsive elements, two TREs (thyroid hormone response elements), and NF$\kappa$-B.

The first *UCP2* variants to be identified were in exon 4, a +164C>T variant which results in an amino acid change of alanine to valine (A55V) and a 45bp

insertion/ deletion in exon 8 (3'UTR). Although the +164C>T variant was not associated with obesity or diabetes markers in a Danish population (Urhammer SA,1997), in Pima Indians these two variants were associated with either sleeping metabolic rate (+164C>T) or 24 hour energy expenditure (45bp I/D). The 3'UTR variant had no effect on mRNA levels in this study (Walder K,1998). The 3'UTR was associated with BMI in South Indian women living in the UK but not in a Caucasian sample, and was not associated with type 2 diabetes in either a family study or a case control study (Cassell PC,1999). Thus early studies suggested that *UCP2* was not important in the susceptibility to either obesity or type 2 diabetes.

Interest in *UCP2* as a candidate gene for type 2 diabetes and obesity was renewed by the discovery of five new variants, -2723T>A, -1957G>A,-866G>A, -371G>C and a 13bp insertion deletion in exon 1. Esterbauer *et al* showed two of these were associated with mRNA levels in intraperitoneal fat tissue, the -2723T>A and -866G>A. The *UCP2* promoter consists of two main blocks, with most of the activity in the first 1200bp with a second inhibitory block from -1200 to -1600bp (Dalgaard LT,2003). The -866G>A variant is at a junction between a negative and positive cis-acting DNA region, and within a region that contains binding sites for hypoxia, aromatic hydrocarbons, inflammatory cytokines and the important β-cell transcription factor PAX-6. This variant is in 100% LD with the -2723T>A promoter variant, strong LD (97%) with the A55V and moderate (75%) LD with the 3'UTR. The -866G>A variant has been reported to account for 71% of the variation in mRNA transcript ratio of the 3'UTR variant, regardless of the nucleotide at -2723 indicating that the -866G>A may be more important in the LD block. The higher activity of the variant promoter, confirmed in expression studies in Paz-6 cells (derived from brown fat cells), was associated with a lower risk of obesity in two separate case-control studies in Caucasians from the Salzburg area of Austria. In the second cohort the population attributable fraction associated with this variant for obesity was 14.7% (Esterbauer H,2001). In a similar case-control study from Austria the -866A was associated with risk of type 2 diabetes. The over activity of the -866A promoter was confirmed in INSE-1 cells (a β-cell line) when stimulated with PAX-6. Interestingly, the same stimulus was associated with *lower* activity from

the -866A promoter in COS-7, cells suggesting that regulation of *UCP2* is tissue specific (Krempler F,2002). This was confirmed using electromobility shift assays which showed that different complex patterns formed with THP-1 (macrophages) and HUVEC (endothelium) cells compared to INSE-1 and Paz-6 cells, indicating differences in transcription factor binding. Baseline mRNA levels in THP-1 and HUVECS are higher for the wildtype promoter and these differences in activity appeared to be in response to an aryl hydrocarbon receptor/ nuclear translocator [AhR/ARNT], and Hypoxia inducible factor-1α/ARNT complex binding (Oberkofler H,2005). These transcription factors are involved in cell stress responses to either environmental pollution (Park H,1999) or hypoxia (Semenza GL,2001), and could explain why the -866A variant is associated with higher $F_2$-isoprostanes, lower TOAS, higher prospective risk of coronary artery disease in Caucasian men (Dhamrait SS,2004) and more extensive carotid atheroma in women (Oberkofler H,2005), all of which are associated with increased oxidative stress, and would be expected if UCP2 function (i.e. mRNA and protein levels) were lower in carriers of -866A.

Overall, cross-sectional studies have been consistent in demonstrating associations between *UCP2-866G>A* and markers of obesity, oxidative stress-related disease and type 2 diabetes. In other ethnic groups the relationship has been less consistent, with the -866G associated with type 2 diabetes in a mixed race sample from the USA (Wang H,2004), and the -866AA genotype associated with higher waist hip ratio and risk of metabolic syndrome in Chinese and South Asians (Shen H,2006). This indicates that the effect may be race-specific or that *UCP2* variation has only a modest effect, confounded by environmental differences, such as diet, between ethnic groups. The gene association studies for *UCP2* are summarized in table 1.4.

**Table 1.4a** The associations of the A55V variant in the *UCP2* gene and obesity and diabetes markers.

| Phenotype | Population | Assoc^n | Risk Allele | Reference |
|---|---|---|---|---|
| **Obesity** | | | | |
| Sleeping MR | Pima Indians | Y | AV↑ | (Walder K,1998) |
| Exercise Eff↑ | Denmark | Y | V | (Buemann B,2001) |
| 24hrEE ↓ | Denmark | Y | V | (Astrup A,1999) |
| ↓Fat Oxid | Denmark | Y | V | (Astrup A,1999) |
| Weight loss ↓ | USA –overfed* | Y | V | (Ukkola O,2001) |
| Morbid Obesity | Taiwan | Y | V | (Chen HH, 2007) |
| Weight Loss post Surgery | Taiwan | Y | V | (Chen HH, 2007) |
| Obesity | Taiwan | Y | V | (Wang TN, 2007) |
| Weight Loss | Korea | Y | A | (Yoon, Y,2007) |
| 24hr EE | Pima Indians | N | | (Walder K,1998) |
| Juvenile cc | Denmark | N | | (Urhammer SA,1997) |
| BMI | Utah | N | | (Wang H,2004) |
| Obesity cc | Italy | N | | (Mancini FP,2003) |
| Obesity cc | Japan | N | | (Kubota T,1998) |
| Juvenile cc | USA-mixed | N | | (Yanovski JA,2000) |
| Resting MR | USA-baseline/overfed* | N | | (Ukkola O,2001) |
| Weight gain | USA-baseline/overfed* | N | | (Ukkola O,2001) |
| BMI,%Fat | Korea | N | | (Yoon, Y,2007) |
| **Diabetes** | | | | |
| Type 2 | Pima Indians | Y | V | (Walder K,1998) |
| Type 2 | Utah | Y | V | (Wang H,2004) |
| Type 2 | USA-Mixed [obese] | Y | V | (Yu X,2005) |
| Type 2 | Pima Indians | N | | (Walder K,1998) |
| Type 2 | Denmark | N | | (Urhammer SA,1997) |
| Glucose/Insulin | Italy | N | | (Mancini FP,2003) |
| Type 2 | Pima Indians | N | | (Kovacs P,2005) |
| Type 2 | Japan | N | | (Kubota T,1998) |
| **Other** | | | | |
| Multiple Sclerosis | Basque | Y | A | (Otaegui D,2007) |
| HDL Cholesterol | Korea | Y | V | (Cha MH, 2007) |
| Plasma Leptin | Scotland | Y | V | (Rance KA,2007) |

**Table 1.4b** The associations of the 45bp 3UTR insertion/deletion [I/D] in the *UCP2* gene with mRNA levels and obesity and diabetes markers.

| Phenotype | Population | Associ^n | Risk Allele | Reference |
|---|---|---|---|---|
| **mRNA** | | | | |
| Half life | Paz-6 cells | Y | D | (Esterbauer H,2001) |
| Levels | Muscle biopsy | N | | (Walder K,1998) |
| **Obesity** | | | | |
| Sleeping MR | Pima Indians | Y | I/D ↑ | (Walder K,1998) |
| 24hr EE | Pima Indians | Y | D | (Walder K,1998) |
| BMI | South Indian [F] | Y | D | (Cassell PC,1999) |
| BMI | Utah | Y | D | (Wang H,2004) |
| 24hr EE | Pima Indians | Y | D↓ | (Kovacs P,2005) |
| Obesity | Germany | Y | D | (Evans D,2001) |
| Juvenile Obesity | USA-Mixed | Y | D | (Yanovski JA,2000) |
| Fat Mass [Dialysis] | Sweden | Y | D | (Wang X, 2006) |
| Obesity | Spain | Y | D | (Ochoa MC,2007) |
| Weight gain | USA-baseline/overfed* | N | | (Ukkola O,2001) |
| Obesity cc | Denmark | N | | (Dalgaard LT,1999) |
| BMI | England | N | | (Cassell PC,1999) |
| 24hr EE | Pima Indians | N | | (Kovacs P,2005) |
| 10 yr wt gain | Denmark | N | | (Berentzen T,2005) |
| Adiposity [Dexa] | Scotland | N | | (Rance KA,2007) |
| Plasma Leptin | Scotland | N | | (Rance KA,2007) |
| Weight Loss | Korea | N | | (Yoon, Y,2007) |
| BMI,%Fat | Korea | N | | (Yoon, Y,2007) |
| **Diabetes** | | | | |
| Type 2 | Pima Indians | Y | D | (Walder K,1998) |
| Type 2 | Utah | Y | D | (Wang H,2004) |
| Insulin Resistance | Spain | Y | D | (Ochoa MC,2007) |
| Type 2 | Pima Indians | N | | (Walder K,1998) |
| Type 2 | South Indians | N | | (Cassell PC,1999) |
| Type 2 | Pima Indians | N | | (Kovacs P,2005) |

**Table 1.4c** The association the -866 variant in the *UCP2* gene with mRNA levels and obesity and diabetes markers.

| Phenotype | Population | Assoc^n | Risk | Reference |
|---|---|---|---|---|
| **mRNA** | | | | |
| (Low activity) | | | | |
| Promoter activity | Paz-6 cells | Y | G | (Esterbauer H,2001) |
| Promoter activity | INSE-1 cells | Y | G | (Krempler F,2002) |
| Promoter activity | COS-7 cells | Y | A | (Krempler F,2002) |
| Promoter activity | THP/HUVEC cells | Y | A | (Oberkofler H,2005) |
| Levels | SC Fat (mixed) | Y | A | (Wang H,2004) |
| Promoter Activity | INSE-1 cells | Y | G | (Sasahara M,2004) |
| Levels | Blood (cauc) | Y | G | (Vogler S,2005) |
| Promoter activity | Jukhat (T-cell) U9377 (Mphage) | Y | G | (Vogler S,2005) |
| **Obesity** | | | | |
| BMI | Austria | Y | G | (Esterbauer H,2001) |
| Fat:Glc OX | Europe | Y | G | (Le Fur S,2004) |
| 24hr EE | Pima Indians | Y | G | (Kovacs P,2005) |
| WHR | Indian/Chinese | Y | A | (Shen H,2006) |
| Weight Loss | Korea | Y | A | (Yoon, Y,2007) |
| Obesity | Spain | Y | G | (Ochoa MC,2007) |
| BMI,%Fat | Korea | N | | (Yoon, Y,2007) |
| BMI,%FAT | Denmark | N | | (Dalgaard LT,2003) |
| Juvenile onset | Europe | N | | (Le Fur S,2004) |
| Obesity cc | Japan T2 | N | | (Sasahara M,2004) |
| BMI | France T2 | N | | (Reis AF,2004) |
| BMI | Japan | N | | (Ji Q,2004) |
| Morbid Obesity | Taiwan | N | | (Chen HH, 2007) |
| Weight Loss post Surgery | Taiwan | N | | (Chen HH, 2007) |
| Obesity | Taiwan | N | | (Wang TN, 2007) |
| Body Adiposity [Dexa] | Scotland | N | | (Rance KA,2007) |
| **Diabetes** | | | | |
| Type 2 | Austria | Y | A | (Krempler F,2002) |
| GSIS | Italy | Y | A | (Sesti G,2003) |
| Type 2 | Utah | Y | G | (Wang H,2004) |
| Type 2 | Italy [F] | Y | A | (D'Adamo M,2004) |
| Insulin therapy | Japan | Y | A | (Sasahara M,2004) |
| Type 2 | Italy | Y | G | (Bulotta A,2005) |
| Glucose/Insulin | Italy | N | | (Mancini FP,2003) |
| NEFA/Insulin Relationship | Hungary | Y | Lost in A | (Bokor S, 2007) |
| Type 2 Diabetes | North India | Y | G | (Rai E, 2007) |
| Insulin Resistance | Spain | Y | G | (Ochoa MC,2007) |
| Glucose/Insulin | Denmark | N | | (Dalgaard LT,2003) |
| Glucose/Insulin | France | N | | (Reis AF,2004) |
| HOMA-R | Japan | N | | (Ji Q,2004) |
| Type 2 | Pima Indians | N | | (Kovacs P,2005) |

**Oxidative Stress**

| | | | | |
|---|---|---|---|---|
| Multiple Sclerosis | Germany | Y | G | (Vogler S,2005) |
| TOAS | England | Y | A | (Dhamrait SS,2004) |
| CAD | England | Y | A | (Dhamrait SS,2004) |
| Carotid Atheroma | Austria [F] | Y | A | (Oberkofler H,2005) |
| Neuropathy | Japan Type 2 | Y | A | (Yamasaki H,2006) |
| Neuropathy | USA- Europe/Type | Y | G | (Rudofsky G,2006) |
| TOAS [Smokers] | England | Y | A | (Stephens J, 2007) |

**Other**

| | | | | |
|---|---|---|---|---|
| Triglycerides | France T2 | Y | A | (Reis AF,2004) |
| Hypertension | Japan | Y | A | (Ji Q,2004) |
| Triglycerides | Indian/Chinese | Y | A | (Shen H,2006) |
| Long Chain FA | Hungary | Y | A | (Bokor S, 2007) |
| HDL Cholesterol | Iran | Y | A | (Akrami SM, 2007) |
| HDL Cholesterol | Korea | Y | A | (Cha MH, 2007) |
| Plasma Leptin | Scotland | N | | (Rance KA,2007) |

1.5.2 Uncoupling Protein 3

The UCP3 gene has 7 exons and the presence of binding sites for muscle dependent transcription factors CCAC and E box, Mefz and MyoD explains how *UCP3* expression is targeted to skeletal muscle cells (Acin A,1999). The promoter has several other binding sites, and is regulated by other important metabolic signals including retinoic acid (Solanes G,2000), PPAR, thyroid hormone (Acin A,1999), leptin, steroids and β3 (Gong DW,1997) and β2 receptors (Masaki T,1997). The activity of the metabolic response elements in most cases is also dependent on activation of muscle-dependent transcription factors ensuring metabolic response is also targeted to skeletal muscle cells (Solanes G,2000)

Sequencing and gene association studies involving *UCP3* have been less successful in identifying important variants with consistent effects. The discovery of significant mutations in obese African Americans with type 2 diabetes suggested that *UCP3* may be important. One of these sites, a mutation at the splice site of exon 6, was associated with a significant 50% reduction in fat oxidation (Argyropoulos G,1998).

A number of variants have been identified throughout the gene (see table 1.5) but only one of these has been associated with differences in mRNA levels. The -55C>T was originally located near a TATA box (6bp) although a recent report (Acin A,1999) places the variant in the '5UTR near (4bp) a PPAR responsive element, which also suggests it could modify regulation of *UCP3* expression. The variant allele was associated with higher levels of mRNA but not with obesity (Schrauwen P,1999). This variant is also near a DR1 retinoic acid response element (6bp) and an area responsible for MyoD activity (61bp) (Solanes G,2000), so may affect the action of other important factors as well.

Phenotype association studies have been very inconsistent, and often in conflict with the association with mRNA levels. The T allele was associated with higher BMI or WHR, consistent with decreased function, in French and German Caucasians, South Asian Indian parent-offspring trios, South Asian Indians and the British Diabetic Association Warren 2 trios collection (Cassell PG,2000; Halsall DJ,2001; Herrmann SM,2003; Otabe S,2000). However, one study showed a lower BMI in TT subjects in a United Kingdom sample (Halsall DJ,2001), and increased function is also suggested with the association of protection from diabetic neuropathy (Rudofsky G,2006). In these studies association with T2DM was examined only once and a relationship not found. The variant allele was found to be protective against T2DM in two French cohorts, suggesting increased function, although also associated with an atherogenic lipid profile (Meirhaeghe A,2000). These studies are summarized in table 1.6. The results in both tables are not well replicated. The frequency of a number of the variants is different across ethnic groups (Kimm SY,2002; Liu YJ,2005), so differences could be seen depending on the origin of the sample. The effect of variation in *UCP3* on obesity is likely to be small, estimated at 2-3% of total variation in one study (Liu YJ,2005), which means that not all the studies will be adequately powered to detect a difference. There is strong LD between the variants in both genes (Walder K,1998) and some signal from a functional variant is likely to be detected in studies of several "non-functional" variants across the cluster.

**Table 1.5** A summary of association studies for variants within *UCP3* excluding the -55C>T polymorphism. [The variants are described as the appear most commonly in the literature.]

| Variant | Phenotype | population | Risk | Reference |
|---|---|---|---|---|
| **Promoter** | | | | |
| -155C>T | Obesity | France | No | (Otabe S,2000) |
| -439Del A | Obesity | France | No | " |
| **Exon 1** | | | | |
| +5 G>A | Obesity | France | No | (Otabe S,2000) |
| **Exon 2** | | | | |
| Val9Met | Obesity | France | No | (Otabe S,1999) |
| | Growth/O2 use | Yeast | No | (Hagen T,1999) |
| **Intron 2** | | | | |
| +521G>C | BMI/Weight loss | Korea | No | (Cha MH,2006) |
| **Exon 3** | | | | |
| Arg70Trp | Growth/O2 use | Yeast | Variant ↓ | (Brown AM,1999) |
| Ala83Ala | Obesity/DM | France | No | (Otabe S,1999) |
| Gly84Ser | Juvenile Obesity | Denmark | No | (Urhammer SA,1998) |
| Tyr99Tyr | Obesity/DM | France | CC ↑BMI | (Otabe S,1999) |
| | Juvenile Obesity | Denmark | No | (Urhammer SA,1998) |
| | Weight Loss | Canada [F] | No | (Harper ME,2002) |
| | Resting EE | USA-mixed | No | (Kimm SY,2002) |
| | BMI | USA- Cauc | No | (Liu YJ,2005) |
| | Met R./Obesity | Pima | No | (Walder K,1998) |
| | TSH r TRH | USA | ↓ CC | (Ukkola O,2001) |
| | Weight loss after overfeeding | USA | ↓ CC | (Ukkola O,2001) |
| | RQ Baseline + after overfeeding | USA | ↓ CC | (Ukkola O,2001) |
| | BMI/Weight loss | Korea | No | (Cha MH,2006) |
| | BMI/Weight loss | Korea | No | (Yoon Y, 2007) |
| Val109Ile | Resting EE | USA-mixed | No | (Cha MH,2006) |
| | Growth/O2 use | Yeast | No | (Brown AM,1999; Hagen T,1999) |
| | Obesity | Tiawan | No | (Wang TN, 2007) |
| **Intron 3** | | | | |
| -46A>T | Juvenile Obesity | Denmark | No | (Urhammer SA,1998) |
| -47A>G | Juvenile Obesity | Denmark | No | " |
| | BMI/Weight loss | Korea | No | (Cha MH,2006) |
| -96C>T | Juvenile Obesity | Denmark | No | " |
| -143A>G | Juvenile Obesity | Denmark | No | " |
| **Intron 4** | | | | |
| +36C>T | Obesity/DM | France | TT↑DM | (Otabe S,1999) |
| +1811C>T | BMI/Weight loss | Korea | No | (Cha MH,2006) |

| Variant | Phenotype | population | Risk | Reference |
|---|---|---|---|---|
| **Exon 5** | | | | |
| Tyr210Tyr | Obesity/DM | France | TT↑DM | (Otabe S,1999) |
| | Juvenile Obesity | Denmark | No | (Urhammer SA,1998) |
| | Fat/Lean mass | USA women | TT↑ | (Damcott CM,2004) |
| | | | (↓intake) | |
| | BMI/fat mass | Canada-mixed | ↑ T | (Lanouette CM,2002) |
| | BMI | USA | No | (Liu YJ,2005) |
| | Weight loss | Canada [F] | No | (Harper ME,2002) |
| | Resting EE | USA- mixed | ↑ T | (Kimm SY,2002) |
| | | | [Black] | |
| | BMI/Weight loss | Korea | No | (Cha MH,2006) |
| | HDL Cholesterol | Korea | Y | (Cha MH, 2007) |
| | BMI/Weight loss | Korea | No | (Yoon Y, 2007) |
| **Exon 6** | | | | |
| Splice site Mutation | Fat oxidation | Black USA | Yes | (Argyropoulos G,1998) |
| | Growth/ O2 use | YEAST | No | (Brown AM,1999) |
| | BMI/Fat mass | USA Black | No | (Chung WK,1999) |
| | UCP3mRNA | USA Black | UCP3S only | (Chung WK,1999) |
| **Intron 6** | | | | |
| GAIVS6 (microsatellite) | BMI/skin fold | Canada [Cauc] | ↑ wildtype | (Lanouette CM,2002) |
| **Exon 7** | | | | |
| Arg308Trp | Obesity DM | France | No | (Otabe S,1999) |

EE = Energy Expenditure, RQ = Respiratory Quotient, DM = Diabetes Mellitus, Met R =Metabolic rate, [ ] = in this subgroup only, F= females.

**Table 1.6** The association of the -55C>T variant in *UCP3* with Obesity and Diabetes phenotypes.

| Phenotype | Population | Association | Risk | Reference |
|---|---|---|---|---|
| **mRNA** | | | | |
| Decreased | PIMA | Y | C | (Schrauwen P,1999) |
| **Obesity** | | | | |
| WHR | Germany | Y | TT↑ | (Herrmann SM,2003) |
| Obesity | Spain | Y | T | (Ochoa MC,2007) |
| WHR | South Indian[F] | Y | T↑ | (Cassell PG,2000) |
| WHR | UK White[F] | Y | T↑ | " |
| Obesity | France | Y | TT ↑BMI | (Otabe S,2000) |
| BMI | USA Caucasians | Y | C ↑ | (Liu YJ,2005) |
| Juvenile Obesity | UK | Y | C ↑ BMI | (Halsall DJ,2001) |
| % Fat/VO max | UK | N | | " |
| Obesity/ weight gain | Denmark | N | | (Dalgaard LT,2001b) |
| Weight loss | Canada [F] | N | | (Harper ME,2002) |
| Resting EE | USA mixed | N | | (Kimm SY,2002) |
| BMI | France DM | N | | (Meirhaeghe A,2000) |
| Weight Gain | Denmark | N | | (Berentzen T,2005) |
| Obesity | Korea | N | | (Cha MH,2006) |
| Obesity | Taiwan | N | | (Wang TN, 2007) |
| Obesity | PIMA | N | | (Schrauwen P,1999) |
| **T2DM** | | | | |
| Family study | South Indian | N | | (Cassell PG,2000) |
| Case-control | South Indian | N | | " |
| Family study | UK | N | | " |
| Case-control | France | Y | C↑DM | (Meirhaeghe A,2000) |
| Insulin resistance | Spain | Y | T | (Ochoa MC,2007) |
| **Misc** | | | | |
| Cholesterol | France DM | Y | ↑TT | (Meirhaeghe A,2000) |
| Neuropathy | Germany DM | Y | ↑ CC | (Rudofsky G,2006) |

EE= Energy Expenditure, DM = Subjects with diabetes mellitus, [ ] = in this subgroup only, F= females

## 1.6    Study Aims

The aim of this study was to use a genetic approach to determine the role of the Uncoupling Proteins 2 and 3 in the development of type 2 diabetes mellitus.

Hypothesis:

Uncoupling Proteins 2 and 3 influence the risk of development of type 2 diabetes through the modulation of oxidative stress.

Specific Aims

1) Confirmation that identified genetic variants in the *UCP2-UCP3* gene cluster are associated with type 2 diabetes using a prospective study design.

2) Examining the association of common variants in the *UCP2-UCP3* gene cluster traits with markers of oxidative stress.

**CHAPTER TWO**

**MATERIALS AND METHODS**

**CONTENTS OF CHAPTER TWO**

**2.1 Study Samples**

2.1a Japanese American Family Study (JAM)

The JAM Study was organised by the Department of Epidemiology, University of Washington. The study was designed to investigate risk factors for coronary heart disease, diabetes, and the insulin resistance syndrome in Japanese American families (Austin MA,2004a). The study had full Institutional Review Board approval from The University of Washington.

Probands were Nisei (2nd generation) participants in the Japanese American Community Diabetes Study (JACDS) who had a spouse of Japanese descent and children, and who were non-diabetic at the time of the first community-wide survey conducted in 1983. Members of the Nisei generation now range in age from approximately 65 to 75 years. Because most Nisei married other Japanese Americans, the Sansei (3rd generation) is predominately of Japanese descent, with ages now ranging from about 30 to 55 years. Probands were contacted by letter and those interested were asked to give permission to contact family members.

Eligible family members included parents, spouses and offspring of the proband, siblings, spouses of siblings, and nieces and nephews of the proband, age 18 years and over, residing anywhere in the United States, who were not pregnant and not too ill to participate. Thus, the study sample consists primarily of two-generation extended Nisei and Sansei kindreds. Each proband and relative was contacted individually by letter and phone, and asked to participate by providing a fasting blood sample and medical history questionnaire. For relatives living outside of the Seattle metropolitan area, blood samples were shipped by overnight mail to the University of Washington. Type 2 diabetes was defined as fasting glucose >125 mg/dl, taking known medication for diabetes, self-reported diabetes, and self-report of taking insulin or oral antidiabetic pills by questionnaire (Austin MA,2004a) The study participants all provided written, informed consent. I would like to thank Mellissa Austin, principal Investigator, for the use of JAM samples.

2.1b The Second Northwick Park Heart Study Cohort (NPHSII)

The Second Northwick Park Heart Study (NPHSII) was recruited by the Medical Research Council Cardiovascular group at The Wolfson centre for Preventative Medicine. In brief, 3012 unrelated healthy Caucasian middle-aged male subjects (mean age $56.1\pm3.5$ years) recruited from nine UK general practices, detailed elsewhere were prospectively followed for up to 15 years (Gable DR,2006b). The study was approved by the institutional ethics committees and performed in accordance with the declaration of Helsinki. All subjects gave written informed consent.

Baseline characteristics were ascertained by means of a questionnaire at entry into the study. Exclusion criteria at baseline were a history of myocardial infarction, 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. Cases of Type 2 Diabetes at baseline were identified by self report. Exclusion criteria precluded subjects requiring insulin or oral hypoglycaemics from entry into NPHS II. New cases were identified by practice note search for physician diagnosed and treated T2DM according to current national guidelines. To date, 288 coronary heart disease events have occurred in 2775 of the subjects with DNA available for analysis. There were 76 cases of Type 2 Diabetes at baseline and by 15 years a further 169 cases had been identified.

## 2.1c Hypercoagulability and Impaired Fibrinolytic Function Mechanisms Study (HIFMECH)

The HIFMECH study was designed to study genetic and environmental mechanisms contributing to the higher cardiovascular risk in Northern compared to southern Europe (Juhan-Vague I,2002). The study samples of Caucasian male first myocardial infarction survivors below 60 years of age were recruited from four European centres (Northern European-*Stockholm, London*; Southern European-*Marseille, San Giovani Rotondo*) (n=598). Subjects with familial hypercholesterolemia and insulin-dependent diabetes mellitus were excluded. A selection of randomly-selected age-matched healthy controls were also recruited from each catchment area (n=653). The study was performed in accordance with the guidelines in The Declaration of Helsinki and approved by local ethics committees. Written informed consent was obtained from all subjects. The patients and control subjects were examined in parallel in the early morning after an overnight fast, and a blood sample was also obtained. Post-infarction patients were investigated 3 to 6 months after the acute event.

## 2.1d UCL Diabetes and Cardiovascular Disease Study (UDACS)

The 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. The subjects were recruited by Dr Jeffrey Stephens (Cardiovascular Genetics, UCL and Diabetes and Endocrinology, University College London Hospitals NHS Foundation Trust) 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 and clinic measurements of blood pressure (supine and lying), weight and height were measured on all subjects and routine clinic

biochemistry was also recorded.  All patients had diabetes according to WHO criteria current at the time of diagnosis.  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'.   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 free from acute illnesses at the time of recruitment.

## 2.2 Genotype Determination

Leukocyte DNA for genotyping had previously been extracted for all studies from whole blood using the "salting out" method (Miller SA,1988). Briefly, the cell lysis  is achieved with a sugar lysis buffer followed by nuclear lysis using the defined nuclear lysis buffer.  De-proteinisation is performed with sodium perchlorate.  The DNA was extracted using chloroform and then precipitated from the aqueous phase using ethanol.  The extracted DNA was dissolved and stored in TE Buffer. All samples were carefully logged and entered into a database with a unique identifier in order to preserve the anonymity of individuals in the study. This stock DNA is used to prepare working 96 well arrays for genotyping.

Working DNA was standardized to a concentration of 15ng/$\mu$l.  This was achieved by calculating the volume of DNA required to be added to 750$\mu$l of dH$_2$O to achieve this concentration on the basis of the absorbance of a 10$\mu$l sample of stock DNA. Stock arrays were created in labelled 96-well Beckman's array.  These were stored at -20$^o$C. To create working arrays, 100$\mu$l of each sample was removed from the stock array, and transferred to another labelled 96-well array.

2.2a Polymerase chain reaction & Restriction Digest (RFLP)

The technique of Polymerase chain reaction (PCR) relies on double stranded DNA being denatured into single strands by heat, consequently annealing with oligonucleotides, and with the addition of DNA polymerase and nucleotide bases, the synthesis of a double strand on cooling. This process leads to binary replication, generating large quantities of DNA in a short period of time. The first step is therefore a short period of high temperature to denature or 'melt' the DNA. This is followed by cooling in the presence of oligonucleotides that are complementary to the DNA either side of the sequence to be studied. These oligonucleotides anneal, and a DNA polymerase adds nucleotides base by base, thus replicating the DNA (Fig. 2.2). 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.

**Fig. 2.1**: Schematic representation of the polymerase chain reaction (PCR). Denaturation of double stranded DNA occurs at 95°c (melting). Annealing of oligonucleotides varies according to the relative amounts of the four bases present. Polymerisation with *Taq* usually occurs at 72°c (polymerisation).
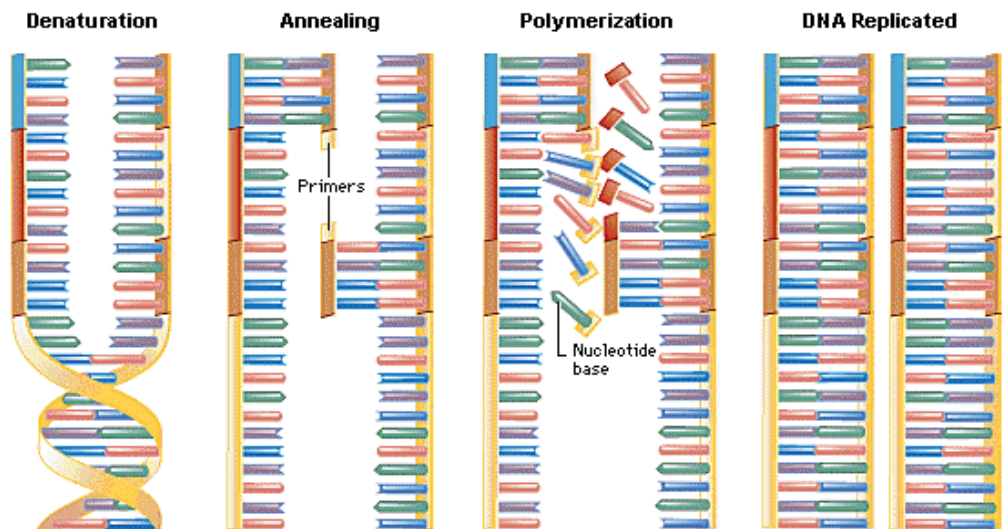
Sample Preparation for Polymerase Chain Reaction

The DNA samples were prepared for PCR by centrifuging the DNA working-array at 200g for one minute to ensure that all the DNA dilutions were at the bottom of their respective wells, reducing the possibility of cross-well contamination when the array lid was removed. The appropriate amount of DNA (see 2.2c) was then removed from each array and transferred into a standard 96-well PCR plate from Corning Inc. (Hemel Hempstead, UK) using a multichannel dispenser.  Life 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.

A bulk mix of reagents was made up for each PCR, allowing adequate volume for the planned number of reactions, with an additional 10% added to ensure that the mix would not run short. PCR oligonucleotides and *Taq* polymerase were kept on ice and added just before the commencement of the reaction. PCRs were performed in a total volume of $20\mu l$ made up with distilled water. Each reaction contained 1x concentration of appropriate polmix, $MgCl_2$, 8pmol of each oligonucleotide and 0.4U of *Taq* polymerase.

The PCR mix was added to each well of the PCR plate using an automatic repeating dispenser. Each sample was overlaid with $20\mu l$ of mineral oil to prevent evaporation. The microtitre plate was then sealed with a clear sticky plastic lid and carefully labelled. 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.   A description of the individual primers and conditions for each variant can be found in section 2.2c.

## Detection of DNA (Agarose gels)

In order to check the successful amplification and size of PCR products, agarose gels were utilised.  For a 2% gel, 2 grams of agarose  was mixed with 100ml of 1×TBE solution containing 10µL of Ethidium bromide (10µg/µL). A microwave oven was used to heat the mixture and dissolve the agarose. The melted agarose was poured into a plastic gel tray (10 × 14cm) and a comb inserted. Solid gels were placed into an electrophoresis tank containing 750ml of 1xTBE buffer solution. $2\mu l$ of MADGE loading dye was added to 5µL PCR product and the entire volume was mixed thoroughly and placed in the separate, submerged wells of the gel. 2µl of a 1Kb ladder (Invitrogen, Paisley UK) was pipetted into the central well in order to size relevant products. All agarose gels were run at 100 volts (v) for a minimum of 30 minutes.

## 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 restriction enzyme digest mix with the recommended buffer system 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.  $5\mu l$ of digestion mix was then added to $8\mu l$ 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 were mixed well. The PCR/digestion mix was then incubated overnight at the recommended temperature. The specific conditions and possible product sizes for each genotyping assay are detailed in section 2.2c.

Microtitre Array Diagonal Gel Electrophoresis (MADGE)

The DNA fragments produced by restriction enzyme digest were separated by electrophoresis on a non-denaturing polyacrylamide gel, using Microtitre Array Diagonal Gel Electrophoresis (MADGE)(Day IN,1995). This technique makes it possible to electrophorese all the 96 wells of a standard PCR plate on a single gel, by allowing the samples to run diagonally. Use of MADGE allowed the 96 well DNA array format to be retained throughout the screening process.

MADGE consists of an open arrangement of 8x12 wells each 2mm deep. The wells are arranged at an angle of $71.2^{o}$ to the short axis of the array, but perpendicular to the long-axis of the Perspex formers used (Fig. 2.2A).   Before making the mix, glass plates of appropriate size (160 x 100 x 2mm) were rigorously cleaned and hand dried. 5 drops of $\gamma$-methacryloxypropyltrimethoxysilane ('sticky' silane) were spread across the plates and left to air-dry. Polymerisation of the MADGE mix was initiated by the addition of ammonium persulphate and the solution was mixed and quickly poured into the three-dimensional former. A glass plate was then gently placed over the mould (silane 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 prised away from the plastic former.

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 10$\mu$l EtBr for 20 minutes.   MADGE loading dye (2$\mu$l) was added to each well of a new, round-bottomed, loading tray, followed by 5$\mu$l of each digested sample, using a multi-channel pipette to pick up the samples from under the oil in the plates. 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 750ml of 1xTBE buffer solution, a multi-channel pipette was used to transfer 5$\mu$l 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-labelled. The gel was electrophoresed at 150v for a minimum of 30 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. Figure 2.2B illustrates a typical pattern obtained for the polymorphisms genotyped.

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 observers.

**Fig. 2.2:** Perspex former (A) and visualised MADGE gel (B) for the UCP2-866G>A and UCP3 -55C>T gene variants.

**A**



Perspex former is used to create angled Indentations in the gel.

B



MADGE gel of digest in 92 samples with 4 blank controls

**For UCP2 -866**

GG   AA   GA

**For UCP3 -55**

CC   TT   CT

Both wild type genotypes were cut by their respective enzymes. Arrow at the bottom of the gel represents the direction of electrophoresis.
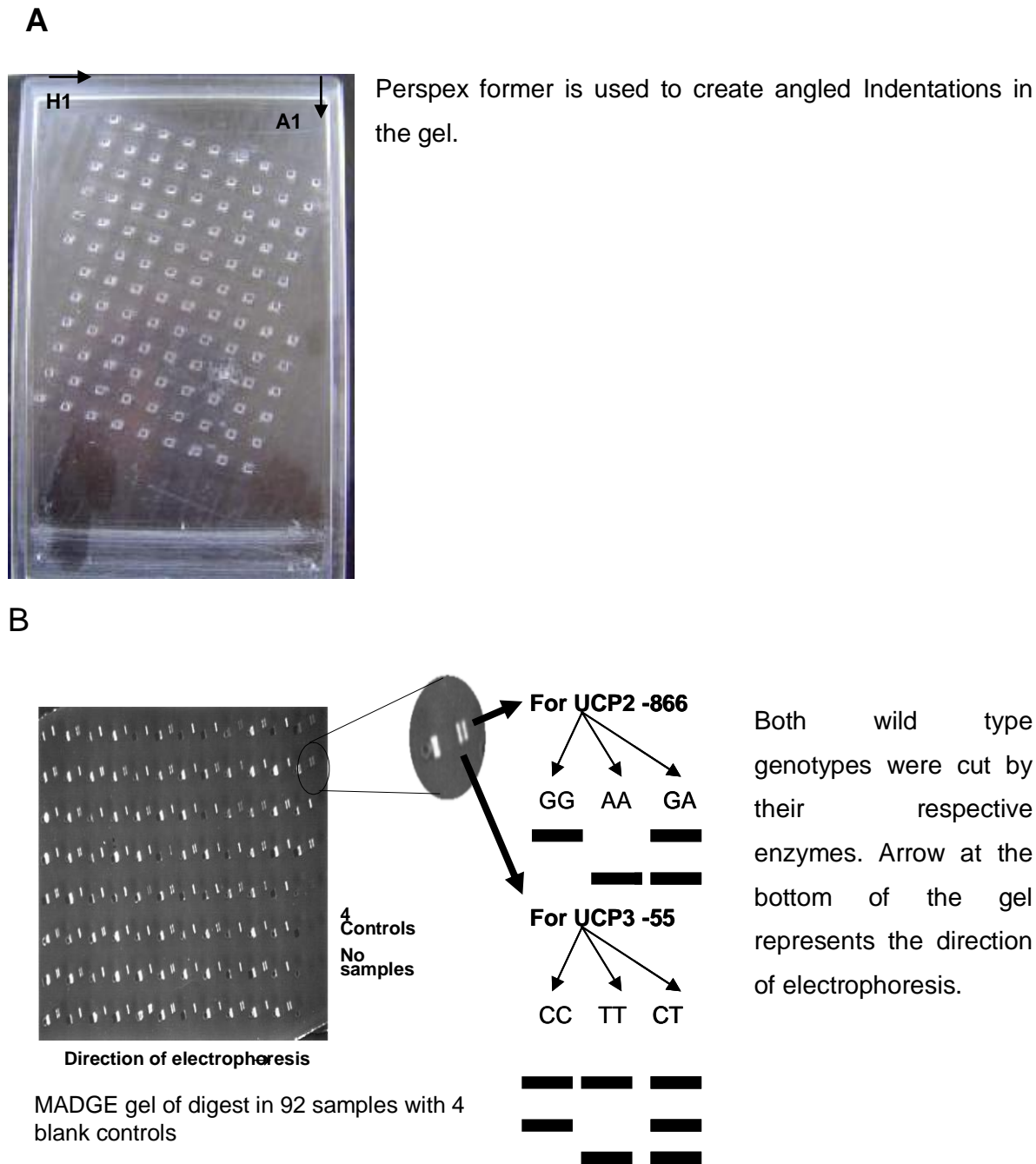
2.2b Taqman® genotyping

The principle behind the Taqman® reaction is described in Fig. 2.4. The method involves the inclusion of two fluorescent, dye-labelled probes for each allele of a specific variant.  The allele specific probes each contain a short sequence of DNA, a reporter dye (Labelled VIC™ or FAM™ depending upon the allele) at the 5' end, and a non-fluorescent quencher (NFQ) dye at the 3' end. During the PCR process, forward/reverse oligonucleotides as well as the labelled probes, anneal to the DNA of interest (Fig. 2.4.A). Amplitaq Gold® DNA polymerase is able to replicate the single strand of DNA until it reaches the labelled probe. Any non-specific binding results in a weakened interaction of the labelled probe with the DNA and displacement of the intact probe (no cleavage of the dye from the quencher) (Fig.2.4.B). If the probe is entirely complementary (hence allele specific probes) to the annealed DNA sequence, the 5' to 3' exonuclease activity of the enzyme results in the cleavage of the 5' dye from the rest of the probe (Fig. 2.4.C). The close proximity of the dye to the quencher usually prevents any significant fluorescent emission. However, once the dye is cleaved, fluorescence increases with each round of DNA replication (Fig.2.4.D). A 7900HT Sequence Detection machine (Applied Biosystems, California USA) is then able to determine the relative levels of either the VIC or FAM dyes, thereby determining the specific genotype.

Preparing the DNA

In contrast to traditional PCR and MADGE based technology, the Taqman® system enables high throughput genotyping in a 384 well format. In order to use this system, DNA was first standardised to an optimal concentration of 1.25ng/µL using the same methodology described in section 2.2. A Biomek 2000 robot (Beckman-Coulter, High Wycombe UK) was used to aliquot 4µL of standardised DNA from a 96 well stock array into a 384 well plate (5ng total) with 16 wells left blank to act as negative controls. A data sheet was also compiled in order to

identify each well to patient ID number. The plates were dried out overnight at room temperature in sterile paper bags and stored until use.

**Fig. 2.3:** Schematic of the Taqman® assay system. A. represents the annealing of the fluorescent probes and oligonucleotides. B. If the fluorescent probe is not identical to the DNA sequence, the probe is displaced by the Taq. C. Successful annealing leads to 5'-3' exonuclease of the probe. D. The VIC™ and FAM™ labels fluoresce and are picked up by the Taqman® machine.
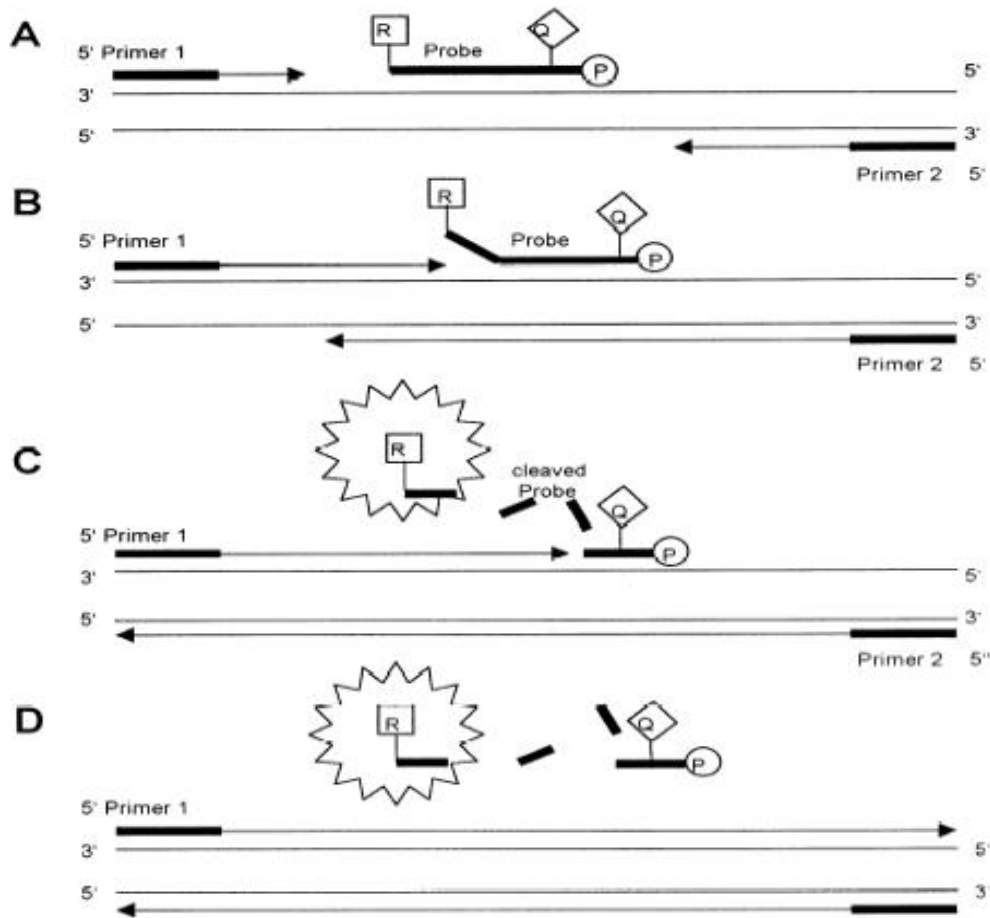
Taqman master mix

Forward/reverse oligonucleotides and labelled probes were ordered using the 'Assay by demand' service available on the Applied Biosystems website (www.appliedbiosystems.com). Section 2.2c lists all the assays successfully designed by Applied Biosystems for this thesis. For each 384 well plate, a master mix was made of the ABgene QPCR Rox mix, the individual assay mix, and distilled sigma water. 4µL of mix was then applied to each well of the 384 plate using a manual Eppendorf 300, 8 channel multi-dispensing pipette and centrifuged (Sigma 4-15) at 200g for thirty seconds. A clear plastic lid (ABgene, Surrey UK) was applied to seal the plate.

**Fig. 2.4:** Screen shot of a typical allelic discrimination plot. The three different coloured dots represent each individual genotype: Blue and red represent homozygotes with the green dots representing heterozygotes. Light blue crosses show the negative controls. Those dots which were not tightly clustered (circled in the figure) to a particular group were re-genotyped.
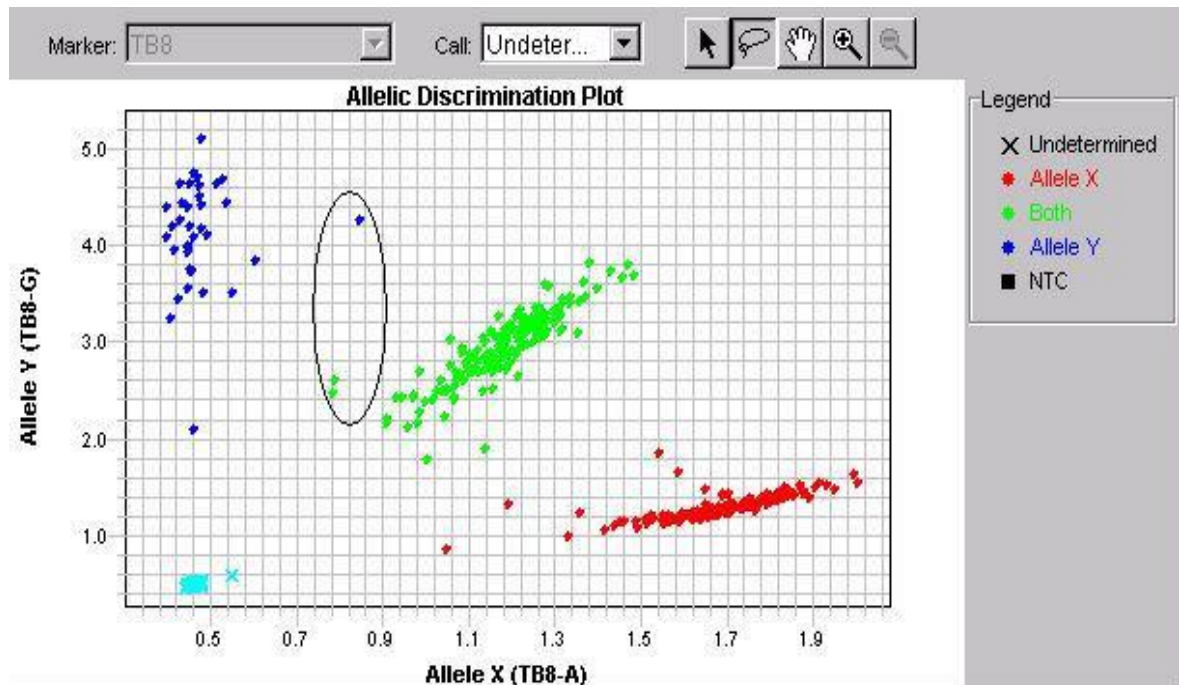
Reading and entering of genotypes

A standard two step heat cycle program on a Thermohybaid (Basingstoke, UK) 384 well, heated block was used to initiate the PCR reaction (95°C for ten minutes, followed by 40 cycles of 95°C for 15s and 60°C for 1min), and all plates were then read on a 7900HT Sequence Detection machine (Applied Biosystems). The 7900HT Taqman® machine uses SDS software 2.1 (Applied Biosystems) in order to differentiate the different genotypes (Fig. 2.6). SDS 2.1 produces an allelic discrimination plot as well as assigning genotypes automatically to an excel file containing patient ID numbers according to array position. To ensure no incorrect inputting of data, a second researcher validated all the genotypes before the data was finally entered into the analysis database.

2.2c Specific Gene variants

UCP2 -866G>A; rs659366 and UCP3 -55C>T; rs1800849

These variants were genotyped in NPHSII using the RFLP method with the help of Dr Jeff Stephens and by using Taqman® genotyping in HIFMECH and JAM with the help of Kah Wah Li.  Dr Jeff Stephens kindly provided the RFLP conditions. The details of the Oligonucleotides and PCR and digest conditions can be found in table 2.1a and 2.1b repectively.  The Taqman oligonucleotides and probes can be found in table 2.1c.

**Table 2.1a** PCR conditions for genotyping by RFLP

| Variant | Oligonulceotides | DNA (μl) POLMIX BSA MG(mmol) | Conditions (°C) | Fragment Length (bp) |
|---|---|---|---|---|
| UCP2 -866 | CACGCTGCTTCTGCCAGGAC AGGCGTCAGGAGATGGACCG | 1.5 NH4 YES 1.5 | 4 MINS @ 95 45s @ 90 30s @ 60 60s @ 72 (30 CYCLES) 5 MINS @ 72 | 360 |
| UCP3 -55 | GGATAAGGTTTCAGGTCAGGC AAGGGATGAGGGAGGAGAAA | 1.5 NH4 YES 1.5 | 4 MINS ' 95 40s @ 95 55s @ 30 60s @ 72 (30 CYCLES) 5 MINS @ 72 | 194 |

**Table 2.1b** The digest conditions for genotyping by RFLP

| Variant | Allele | Enzyme | Buffer BSA Incubation (°C) | Fragment Length (bp) |
|---|---|---|---|---|
| UCP2 | G A | Mlu1 | NEB Buffer 2 NO 37 | 290 + 70 360 |
| UCP3 | C T | HaeII | NEB Buffer 2 NO 37 | 110 + 64 + 20 110 + 84 |

All enzymes and buffers were supplied by New England Biolabs.

**Table 2.2** Taqman assays design for all variants

| Variant | Primers | Probes |
|---------|---------|--------|
| UCP 2 | GCCAGAGGGCCCAATTGTT | VIC-CACGC**G**TCAGTTAC-NFQ |
| | GGGCCTGGTTCGCTTTAATT | FAM-TTCACGC**A**TCAGTTAC-NFQ |
| UCP3 | GCTGTCAACCAACTTCTCTAGGATA | VIC-TCTTATACACAC**G**GGCTGA-NFQ |
| | ACTGTTGTCTCTGCTGCTTCTG | FAM-TCTTATACACAC**A**GGCTGA-NFQ |

## 2.3 Biochemical assays

For all biochemical assays blood was collected in EDTA-containing tubes and centrifuged immediately (unless specified). The plasma was aliquoted into microtubes and placed into dry ice. The samples were stored at -80$^0$C until use. All assays were performed on plasma not previously thawed. All the assays were performed by myself with the help of Jasmin Matin in the JAM study. The Plasma F$_2$-isoprostanes were measured by myself in the laboratories of Professor Kevin Moore in the Centre for Hepatology at the Royal Free campus of UCL.

2.3a Total Antioxidant Status (in House) (TAOS)

Plasma TAOS was measured by a photometric microassay previously described by Sampson *et al* (Sampson MJ,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. There are two arms to the assay (Figure 2.5),

the test arm and a control arm with phosphate buffered saline (PBS) instead of plasma. Plasma TAOS is therefore inversely proportional to the degree of oxidative stress in the sample.

**Fig 2.5** Measurement of plasma TAOS
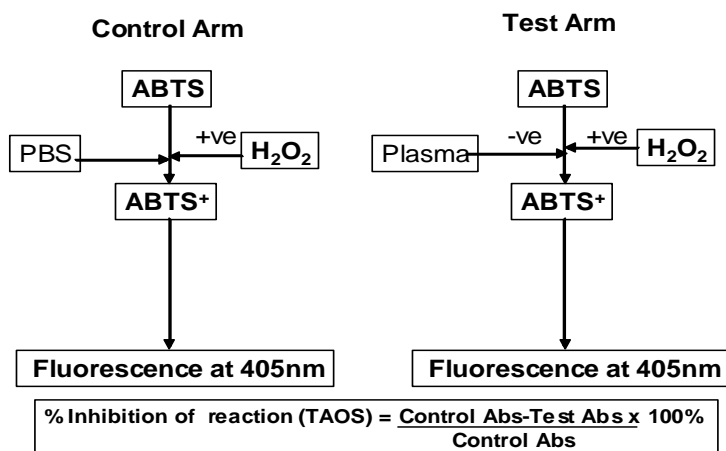


% Inhibition of reaction (TAOS) = $\frac{\text{Control Abs-Test Abs}}{\text{Control Abs}} \times 100\%$

Method

Plasma (2.5$\mu$l) was placed in duplicate into 90 wells of a 96 well Nunc Immuno Maxisorp 96 well-plate. 2.5$\mu$l 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:-

-20$\mu$l of ABTS (20mmol/l).

-20$\mu$l of HRP (30mU/ml).

-40$\mu$l PBS (pH 7.4).

The reaction was then initiated by the addition of 20$\mu$l of $H_2O_2$ (final concentration 0.1 mmol/l). At the end of ten minutes the absorbance due to the accumulation of $ABTS^+$ in the test sample was read, along with the control (containing 2.5$\mu$l of PBS instead of plasma). This was performed using a Tecan GENios plate reader (TGPR) utilising the Magellan 3 software package. 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.

## 2.2b Total Antioxidant Status (Randox, Ireland) (TAS)

Plasma TAS is based on a similar principle to TOAS, in that the sample anti-oxidant capacity is derived from the ability of the sample to inhibit the peroxidase-mediated formation of the $ABTS^+$ radical. The TAS kit uses the peroxidase metmyoglobin rather than horseradish peroxidase which gives a radical cation that is measured by the measurement of absorbance at 600nm (Fig 2.6). The assay is calibrated using a 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid standard (Randox Ireland) and a PBS blank. Quality control is possible by the inclusion of a control human serum sample of known TAS.

**Fig 2.6** The creation of the ABTS radical in the Plasma TAS kit

$$HX\text{-}Fe^{+++} + H_2O_2 \longrightarrow X\text{-}[Fe{+}{+}{+}{+} =0] + H_2O$$

$$ABTS^{®} + X\text{-}[Fe^{++++} =0] \longrightarrow ABTS^{®+} + HX\text{-}Fe^{+++}$$
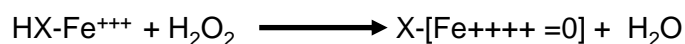
$HX + Fe^{+++} =$ Metmyoglobin

$X\text{-}[Fe^{++++} =0] =$ Ferrylmyoglobin

$ABTS^{®} = 2,2'$-Azino-di-[3-ethylbenzthiazoline sulphonate]

$ABTS^{®}$ is a registered trademark of Boehringer Mannheim

Method.

1. Cuvette method

20$\mu$l of sample, standard or control is added to the cuvette followed by 1ml of "chromagen". The absorbance is read at 600nm (A1) and 200$\mu$l of "substrate" was added and incubated at $37^0$C for 3 minutes. Absorbance was read a second time (A2). All experiments were completed in duplicate.

2. Microplate Method

2.0$\mu$l of sample standard or control was placed in duplicate into 90 wells of a 96 well Nunc Immuno Maxisorp 96 well-plate. Using an automatic Biohit repeating dispenser, 100$\mu$l of "chromagen was added to each well. The TGPR was used to read absorbance (A1) and then 20$\mu$l of "substrate" was added and the plate maintained at $37^0$C for 3 minutes within the TGPR. After 3 minutes the absorbance was read again (A2).

The results were exported to an Excel spreadsheet. Sample Absorbance ($\Delta$A) was calculated A2-A1. For each set of solutions a factor was derived from which the TAS could be calculated (Fig 2.7)


**Fig 2.7** The calculation of TAS

Total Antioxidant Status:

$$\text{Factor} = \frac{\text{conc of standard}}{[\Delta A \text{ blank} - \Delta A \text{ Standard}]}$$

mmol/l = Factor x [$\Delta$A blank – $\Delta$A Sample]

2.3c Bioxytech® AOP-490$^{TM}$ (Oxis International Inc, USA)

The AOP-490$^{TM}$ assay measures the combined antioxidant action of the sample by assaying its ability to reduce the $Cu^{++}$ to $Cu^{+}$. The chromogenic reagent (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) forms a 2:1 complex with $Cu^{+}$ which has a maximum absorbance at 490nm. The assay is calibrated using a standard curve derived from 5 uric acid standards and a deionized water control.

Method

The standard was prepared by adding 1.5ml of water to the "standard vial" and then performing four further dilutions of 500$\mu$l of standard to 500$\mu$l of water. The samples or standard were diluted by adding 15$\mu$l to 585$\mu$l "R1" which contained the chromagen. 200$\mu$l of this solution was then added, in duplicate, to a well in the provided microplate. The absorbance was measured at 490nm in the TGPR (A1) and 50$\mu$l of "R2" (containing the copper ions) was added to each well using an automatic Biohit repeating dispenser. The plate was incubated at room temperature for 3 minutes and the 50$\mu$l of stop solution was added to each well and repeat absorbance measured (A2). The absorbance of the sample was calculated as A2-A1. The absorbance of a sample was derived from a standard curve and the calculation of a linear regression equation (SPSS 12.0) and the anti-oxidant action reported as mM uric acid equivalents (Fig 2.8).

**Fig 2.8** A typical standard curve for the AOP-490 $^{TM}$ assay

Standard Curve



$y = 0.301x + 0.084 \quad r^2 = 0.999$

Absorbance @ 490 nm
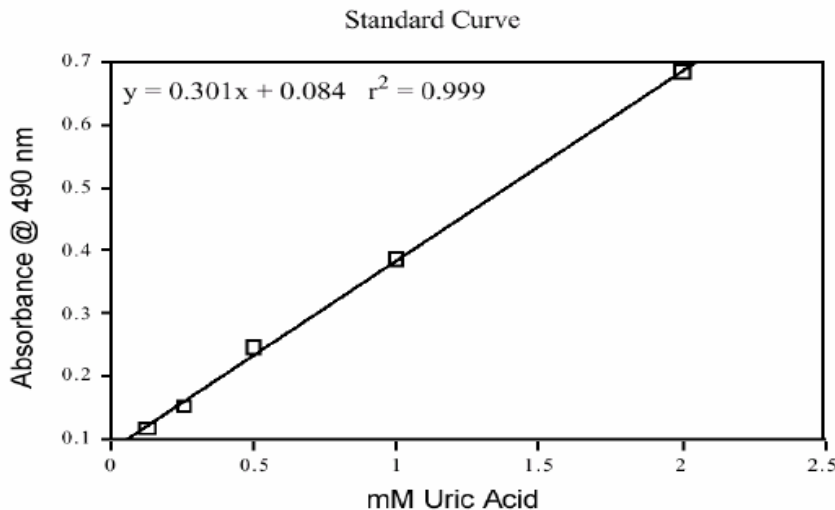
mM Uric Acid

Determine the Uric acid equivalents for each sample from the curve and sample net absorbance by solving for "x".

[Example provide in technical specifications AOP-490 $^{TM}$]

2.3d Glutathione/ Reduced Glutathione Ratio (Calbiochem,UK)

Reduced glutathione (GSH) is a major antioxidant in human tissues, becoming oxidized (GSSG) during the conversion of hydrogen peroxide or lipid hydroperoxides to water and the respective alcohol. When exposed to oxidative stress the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation, which makes this a useful indicator of oxidative stress. Accurate measurement of GSSG requires the prevention of further oxidation of GSH. This is achieved by the addition to the sample, before storage, of 1-methyl-2-vinylpyridium trifluoromethane-sulfonate (M2VP), a thiol scavenger reagent that leaves only GSSG. A second sample is stored without M2VP to assay GSH + GSSG. The detectable product is derived from the reaction of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid [DTNB]) with GSH (Fig 2.9). The GSSG in the sample is converted to GSH by the action of Nicotinamide adenine dinucleotide phosphate (NADPH) catalyzed by Glutathione reductase (GR).

**Fig 2.9** The measurement of GSH/GSSG (see text for explanation of abbreviations)

A) GSSG            B) Total GSH
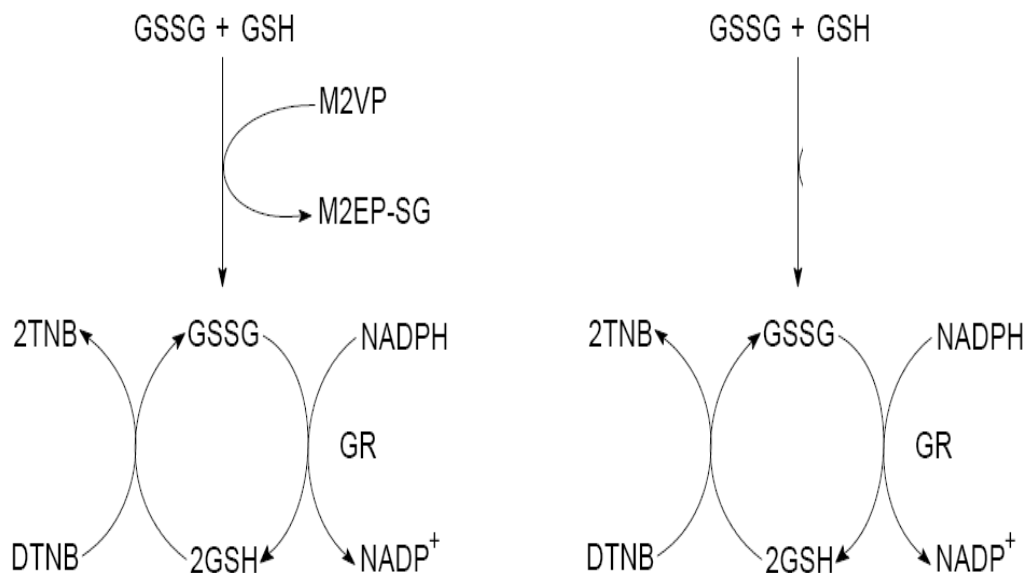


Each GSSG molecule is equivalent to 2 GSH molecules.

Method

The samples and reagents were prepared as follows

1. Blood for GSH - 50$\mu$l of whole blood collected in an EDTA containing tube was placed in a microtube in dry ice and then stored at -80$^0$C until use.

2. Blood for GSSG - 100$\mu$l of whole blood collected in an EDTA containing tube was placed in a microtube containing 10$\mu$l of provided M2VP and frozen in dry ice and then stored at -80$^0$C until use.

3. GSH buffer – The provided vial was reconstituted in 650mls of deionized water.

4. NADPH – The provided vial was reconstituted in 7.5mls of assay buffer on the day of use and discarded after 24 hours

5. 5% Metaphosphoric acid (MPA) was prepared daily.

## 1. GSSG assay

The sample was prepared by mixing the thawed sample with 290μl of ice cold MPA followed by centrifuge at 1000x *g* for 10 minutes. The extract was diluted in the GSSG assay buffer provided (50μl extract + 700μl buffer) and stored on ice until use. The low concentration standards were used for GSSG measurement (0,0.1,0.25,0.50 μM GSH).

## 2. GSH assay

The sample was prepared by mixing the thawed sample with 350μl of ice cold MPA followed by centrifuge at 1000x *g* for 10 minutes. The extract was diluted in the GSH assay buffer as above (50μl extract + 3ml buffer) and stored on ice until use. The high concentration standards were used for GSH measurement (0,0, 1.5,3.0 μM GSH).

The reagents were then added, in duplicate, to the Nunc Immuno Maxisorp 96 well-plate in an identical method for both the GSH and the GSSG assay.

1. 50μl of sample, standard or blank
2. 50μl of chromagen (Ellaman's reagent)
3. 50μl of Enzyme (GR)
4. Mix and incubate at room temperature for 5 minutes
5. 50μl of NADPH
6. Record the change of absorbance at 412nm for 3 mins

Calculation

The TGPR automatically calculated the gradient of the accumulation of chromagen based on 5 absorbance readings over the 3 minutes. Standard curves were

generated for the standards based on these reaction rates in a similar way to the AOP-490$^{TM}$ kit (Fig 2.8). The actual concentrations were calculated allowing for dilution by multiplying the total GSH by a dilution factor of 488 and the GSSG by a factor of 30. Once the calculations of the concentration of total GSH and GSSG are made the ratio can be calculated. GSH is calculated by total GSH – GSSG(x2) and the ratio GSH/GSSG can then be calculated.

2.3e  Plasma $F_2$-isoprostanes

Measurement of isoprostane was performed by Gas Chromatography Mass Spectroscopy (GC-MS) after extraction, purification and derivitisation. The ability of the sample to withstand oxidative stress was also assessed by the measurement of isoprostanes in a 200$\mu$l sample of plasma incubated with 3-Morpolinosyndomimime chloride (SIN-1) at 37$^0$C for six hours (Ferraro B,2003). SIN-1 is a peroxynitrate releasing compound generating superoxide and nitric oxide.    The final concentration of SIN-1 was 11mmol (100$\mu$l of 2.2mg/ml added).

Extraction

Prior to extraction the phospholipids underwent alkaline hydrolysis. To either 800$\mu$l of sample or the 200$\mu$l SIN-1 sample 10$\mu$l of 10mg BHT in 1ml methanol and 40$\mu$l of 8.8mg Trolox in 1 ml methanol was added. An equal volume of 25% Potassium hydroxide was then added with $D_4$-PGF2$_\alpha$ standard. This was then incubated at 37$^0$C for 45 minutes. The sample was adjusted to pH 3 using 1M HCL.

Initial extraction of the prostaglandin-like compound was performed using tC18 columns. 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. 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.

Derivatization- Step 1. Preparation of pentaflourbenzyl ester

The pentafourobenzyl ester was prepared by the addition of 20$\mu$l 10% DIPEA (N,N-di-isopropylethylamine in acetonitile (AcN) and 40$\mu$l 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.

Purification

The samples then underwent an additional purification with thin layer chromatography (TLC). TLC plates (Whatman 60A, Linear K6D, 5x20cm, 250$\mu$m 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 40$\mu$l 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. 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.

Derivatization-Step 2. trimethylsilyl derivative

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

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% (Personal Communication, Dr Ali Reza, Department of Hepatology, UCL).

2.3f  Plasma Non-Esterified Fatty Acids

Plasma Non-esterified fatty acids (NEFA) were measured by an *in vitro* enzymatic colormetric assay (Wako Chemicals GmBH, Neuss, Germany).   The test utilizes three steps.  Firstly, the generation of Acyl-Coa from NEFA using the enzymre Acyl-CoA Synthase.   Secondly the generation of Hydrogen peroxide by the addition of Acyl-CoA oxidase.  Thirdly, the generation of colour from the reaction

between 4-aminophanazone and the hydrogen peroxide.  Oleic acid (282mol/L) was used as the standard and distilled water as negative control.

Method

The assay was performed, in duplicate, in a Nunc Immuno Maxisorp 96 well-plate as follows.

1. 5μl sample, standard or control
2. 100μl Reagent A (dissolved in solvent A)
3. Mix well and leave for 10 minutes @ 37$^{o}$C
4. 200μl Reagent B (dissolved in solvent B)
5. Mix well and leave for 10 minutes @ 37$^{o}$C
6. Read in the Tecan Plate reader @ 550nm.

The result is calculated as follows

$$\text{Free fatty acids (mg/dL*)} = \text{Absorbance of sample} \times \frac{\text{Concentration of standard (mg/dL)*}}{\text{Absorbance of Standard}}$$

*concersion factor: mg/dL x0.035=mmol/L

## 2.4 Clinical Protocols

The clinical protocols were all recruited and performed by myself.   All the studies have been approved by committee alpha, Combined UCL/UCLH local ethics committee and for the fatty meal a site-specific assessment was also approved for the Institute of Child Health, UCL.   Healthy subjects were recruited from laboratory

and clinic staff while subjects with diabetes were recruited from the UDACS study. Excluded were those with clinically manifest CVD, smokers, those with connective tissue or other inflammatory disease and those with malignancy or other terminal illness. Those on anti-inflammatory medications such as steroids or NSAID's were also excluded. Medication was omitted on the day of the study but patients were encouraged to take any medication after the study was completed. Written consent was obtained and all subjects were given an information leaflet regarding the study. A letter was sent to the General Practitioner of each consenting patient to inform them of the patient's participation in the study. A small snack was provided for all patients before they left.

2.4a Oral Glucose tolerance test

The subjects attended after an overnight fast. Blood was drawn from a cannula placed in the left ante-cubital vein and collected at ten minute intervals in relation to the ingestion of a standard 75g oral glucose load (394ml Original Lucozade, Glaxosmithkline (GlaxoSmithKline,2005)). Glucose was collected in oxalate tubes (grey top) and assayed on a YSI 2000 glucose analyzer (CV 0.9%). Blood was also collected into EDTA-containing tubes and prepared for the measurement of TAOS.

2.5b Meal rich in used cooking oil

The subjects attended after an overnight fast. Blood was drawn from a cannula placed in the left ante-cubital vein for assessment of lipid profile, renal function and glycated haemoglobin, and analysed using routine methods by Chemical Pathology Department, Middlesex Hospital. Blood was also taken for glucose and oxidative stress markers as above. The subject was then given a prepared meal rich in used cooking oil. Blood was taken for glucose and oxidative stress markers every hour for four hours.

Used cooking oil meal

The meal consisted of 100g of vanilla ice cream (Tesco value soft serve), 200ml of skimmed milk, 50ml evaporated milk, 10g Yoghurt (Onken Natural), 50g of tinned apricots (no syrup), one egg and 46g of used cooking oil obtained from a local commercial restaurant (Potion Bar and Restaurant). The cooking oil was always obtained at the same time every week, on a Friday afternoon. The oil was changed by the restaurant on Saturday morning so had been used for one week. The fat was stored at $-20^0$C and protected from light until use. The meal was blended and presented as a milkshake with chocolate syrup added as flavour if required. This provided 64.4g of fat of which 30g was saturated. The meal also contained 62.5g of carbohydrate and 20.5 g of protein (Williams MJA,1999). The subjects were requested to drink the meal over a period of 15 minutes and the meal was well tolerated.

## 2.5 Statistical Analysis

Statistical analysis was performed using SPSS (version 12.1, SPSS Inc., Chicago) or 'Intercooled STATA' package (version 7.0, STATA Corporation, Texas). The analysis was performed by Jackie Cooper, Fotios Drenios and myself. For gene association analysis, data are reported for those individuals amongst whom high-throughput genotyping was successful.

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.

Specific details are include in the relevant section but in general, allele frequencies are shown with the 95% confidence interval. Analysis of variance (ANOVA) was

used to assess the association between genotypes and baseline characteristics on normally distributed data, or on other data after appropriate transformation. Chi-squared tests were used to compare differences in categorical variables by genotype.

No adjustment was made for multiplicity of testing. However, analysis always followed an *a-priori* hypothesis or design. In all cases a P value of less than 0.05 was considered statistically significant.

In NPHSII, survival analysis with respect to the prospective risk of cardiovascular disease or type 2 diabetes was carried out using Cox proportional hazards model, 'failure' being the first CHD event or diagnosis of type 2 diabetes. Results are presented as hazard ratios (HR) with their corresponding 95% confidence interval (CI). 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.

## 2.6 Reagents and commonly used stock solutions and equipment

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: $D_4$-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, 250$\mu$m thick). All other reagents were supplied by Sigma-Aldrich (Poole, UK).

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

Polymerase chain reaction (PCR): PCR oligonucleotides and *Taq* polymerase were supplied by Invitrogen Ltd (Paisley, UK). 50mM $MgCl_2$ supplied by Bioline (London, UK). All necessary restriction enzymes were supplied by New England Biolabs Inc (Hertfordshire, UK) or Roche Diagnostics (Lewes, UK). dNTPs were supplied by Pharmacia Biosystems Ltd (Milton Keynes, UK). 96 well PCR plates were obtained from Corning Inc. (Hemel Hempstead, UK). All other reagents supplied by Sigma-Aldrich Ltd. (Poole, UK).

Gels: 19:1 30% 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). Agarose was supplied by Helena Biosciences (Sunderland, UK) and 10× Tris Borate EDTA (TBE) was supplied by Severn Biotech Ltd (Worcestershire, UK). 1kb ladder was supplied by Invitrogen Ltd (Paisley, UK).

Taqman assays: All Taqman assays were obtained from Applied Biosystems (California, USA). Taqman Absolute QPCR Rox mix and 384 well plates were supplied by ABgene (Surrey, UK).

Commonly used stock solutions

Ammonium persulphate solution (APS): 0.25g APS dissolved in 1ml distilled water.
MADGE Loading Dye: 0.0015% bromophenol blue; 0.015% xylene cyanol; 10% glycerol; 10mM EDTA

$NH_4$ polmix buffer: 16mM $[NH_4]_2$ $SO_4$; 67mM Tris-HCL pH8.4; 0.01% Tween 20; 2mM dATP; 2mM dTTP; 2mM dGTP; 2mM dCTP

Sticky Silane: 0.5% v/v glacial acetic acid; 0.5% v/v γ methacryloxy-propyl-trimethoxy-silane

TBE buffer: 0.04M Tris-borate; 1mM EDTA pH7.4

Reagent A (sucrose lysis buffer): 0.32M sucrose; 5mM $MgCl_2$; 10mM Tris-HCl ph7.5; 1% Triton-X-100

Reagent B (nuclei lysis buffer): 10mM Tris-HCl pH8.2; 0.4M NaCl; 2mM Na2EDTA pH 8.0
TE Buffer: 10mM Tris-HCl; 1mM EDTA pH 7.6

Equipment

Automatic pipettes

Manual Eppendorf 300, 8 channel pipette (Eppendorf, Hamburg, Germany).
Eppendorf multipette Pro repeating dispenser (Eppendorf, Hamburg, Germany).
Finnipipette multichannel dispenser (Life Sciences, Basingstoke, Hants, UK).
Automatic Biohit repeating dispenser (Alpha Laboratories, UK)

Plate Reader
Tecan GENios (Tecan Group Ltd, Switzerland)

## 2.7 Declaration of activity

Chapter 3 NPHSII

Standardised DNA was available to me.  Genotyping was performed by a mixture of RFLP and Taqman.  I performed this with the help of Dr J. Stephens, Miss J. Matin and Ka-Wah Li.  Statistical analysis was directed by me with the help of Ms J. Cooper.

Chapter 4 HIFMECH

Standardised DNA was available to me.   I performed genotyping by Taqman with the help of Miss Ka-Wah Li.  Statistical analysis was directed by me with the help of Ms J Cooper and Ms E. Dawe.

Chapter 5

Markers of Oxidative stress in JAM

I performed the selection and assessment of different assays to measure plasma markers of oxidative stress.  I performed the assays on the samples from the JAM study with the help of Miss J. Matin.  I directed the statistical analysis with the help of Dr Brendan Pearce (University of Washington).

JAM

Standardised DNA was available to me.   I performed genotyping using Taqman with the help of Miss Ka-Wah Li.  Statistical analysis was directed by me with the

help of Dr Brendan Pearce (University of Washington) who performed the hereditability calculations.

Chapter 7 Clinical Studies

The patients and normal volunteers were recruited by me. I prepared and administered the glucose drink and test meal. Venous sampling, plasma glucose assay, oxidative stress marker and NEFA assays were performed by me. I measured plasma F2-isoprostanes in the Department of Hepatology, UCL [Professor K. Moore] with the help of Dr Ali Reza. Statistical analysis was performed by me with the help of Dr F. Drenos.

# CHAPTER THREE

## PROSPECTIVE RISK OF TYPE 2 DIABETES IN THE NORTHWICK PARK
## HEART STUDY II.

**Association of risk with the *UCP2-866G>A* and *UCP3-55C>T* Variants**

**CONTENTS OF CHAPTER THREE**

## 3.1 Prospective Risk of Type 2 Diabetes in NPHSII

3.1.1 Introduction

As described in chapter 1, the rate of mitochondrial oxidative metabolism appears to be important in the development of T2DM. UCP2 and UCP3, as part of the mitochondrial carrier protein superfamily are, therefore, good candidates for having a role in the pathogenesis of T2DM. The *UCP2* and *UCP3* genes are located in a cluster within 8kb of each other on chromosome *11q13*. A variant associated with altered mRNA levels has been identified in both these genes (*UCP2-866G>A; UCP3-55C>T*).

As seen in chapter 1, these variants have been associated with both obesity and T2DM traits which suggest that they are important in their pathogenesis (Table 1.4c and 1.6). The *UCP2-866A* is associated with higher mRNA levels and was associated with reduced insulin secretion or T2DM in Austrian (Krempler F,2002), Italian (D'Adamom M,2004) and Japanese samples (Sasahara M,2004), however, the -866A allele was also associated with lower subcutaneous adipose tissue mRNA, but not with T2DM in a mixed race sample from the USA (Wang MY,1999). Although 50% lower UCP3 protein has been described in subjects with T2DM (Schrauwen P,2001a), the *UCP3-55C>T* variant, which is associated with altered UCP3 mRNA levels (Schrauwen P,1999), has not consistently been associated with T2DM. The *UCP3-55T* allele was associated with reduced risk of T2DM (Meirhaeghe A,2000), but this was not replicated in other studies (Cassell PG,2000; Halsall DJ,2001) .

3.1.2 Aims

The purpose of this research was to clarify the inconsistencies identified in cross-sectional gene association studies, and to further elucidate the role of identified genetic variation in the UCP2-UCP3 gene cluster in the development of T2DM.

3.1.3 Methods

The Second Northwick Park Heart Study is a prospective study of over three thousand men who have now been followed for 15 years. The men were healthy at recruitment, based on a definition that excluded men with cardiovascular disease, malignancy or taking regular medication. Therefore, men with diet controlled T2DM, were not excluded from the study. Analysis excludes these men unless stated otherwise. New cases were identified by practice note search for physician diagnosed and treated T2DM according to current national guidelines. Obesity was defined as BMI over 30kg/m$^2$. Population attributable fraction (PAF) represents the impact of the variant on the sample in question, and is a composite of the exposure to the risk and the magnitude of the risk and is estimated as pd * (Hazard ratio -1/ Hazard ratio), where pd is the proportion of cases exposed to the risk factor (Greenland S,1993).

## 3.2. Baseline Characteristics Predicting the Development of Type 2 Diabetes

The NPHSII contains a total of 3012 eligible men. There were a total of 76 (2.5%) of men with T2DM at baseline. At 10 years 86 (2.9%) new cases had been recorded and at 15 years 169 (5.6%) new cases were recorded. The total incidence at ten years was 5.4% and 8.1% at 15 years.

The baseline characteristics associated with the development of T2DM are shown in table 3.1a including baseline subjects with diabetes, and in table 3.1b excluding baseline subjects with diabetes. The factors associated with diabetes are similar in both analyses [blood pressure, lipid parameters and CRP], with age being only significantly associated with diabetes if baseline subjects with T2DM are included.

**Table 3.1a** Baseline characteristics and their association with the development of T2DM over 15 years-including baseline subjects with T2DM

| | No diabetes N=2767 | With diabetes N=245 | HR* (95% CI) | P value |
|---|---|---|---|---|
| Age (years) | 56.0 (3.5) | 56.7 (3.4) | 1.31 (1.08-1.58) | .005 |
| SBP[†](mmHG) | 136.3 (18.7) | 142.3 (18.8) | 1.32 (1.16-1.51) | <0.0001 |
| DBP(mmHG) | 84.4 (11.4) | 85.6 (11.1) | 1.08 (0.94-1.23) | 0.26 |
| BMI[†]($kg/m^2$) | 26.0 (3.3) | 28.5 (4.0) | 1.95 (1.71 – 2.22) | <0.0001 |
| Smoking | 791 (28.6%) | 68 (27.8%) | 0.96 (0.72-1.29) | 0.80 |
| Cholesterol(mmol/L) | 5.72 (1.01) | 5.85 (1.09) | 1.15 (1.01-1.31) | 0.04 |
| Triglyceride[†](mmol/L) | 1.75 (0.92) | 2.31 (1.2) | 1.69 (1.48-1.93) | <0.0001 |
| Fibrinogen[†](g/l) | 2.71 (0.52) | 2.76 (0.51) | 1.06 (0.93-1.21) | 0.37 |
| CRP[†](mg/l) | 2.92 (3.44) | 3.96 (4.32) | 1.27 (1.10-1.46) | 0.001 |

See Table 3.1b for key

**Table 3.1b** Baseline characteristics and their association with the development of T2DM-excluding baseline subjects with T2DM

| | No diabetes N=2767 | With diabetes N=169 | HR* (95% CI) | P value |
|---|---|---|---|---|
| Age (years) | 56.0 (3.5) | 56.3 (3.0) | 1.16 (0.93-1.46) | 0.19 |
| SBP[†](mmHG) | 136.3 (18.7) | 141.3 (19.3) | 1.30 (1.11-1.52) | 0.001 |
| DBP(mmHG) | 84.4 (11.4) | 86.2 (11.3) | 1.14 (0.98-1.34) | 0.09 |
| BMI[†]($kg/m^2$) | 26.0 (3.3) | 28.6 (3.7) | 1.86 (1.65 – 2.10) | <0.0001 |
| Smoking | 791 (28.6%) | 54 (32.0%) | 1.29 (0.93-1.79) | 0.13 |
| Cholesterol(mmol/L) | 5.72 (1.01) | 5.90 (0.98) | 1.20 (1.03-1.39) | 0.03 |
| Triglyceride[†](mmol/L) | 1.75 (0.92) | 2.27 (1.7) | 1.55 (1.34-1.80) | <0.0001 |
| Fibrinogen[†](g/l) | 2.71 (0.52) | 2.78 (0.53) | 1.17 (1.00-1.36) | 0.11 |
| CRP[†](mg/l) | 2.92 (3.44) | 4.05 (4.36) | 1.37 (1.16-1.61) | <0.001 |

†geometric mean (approx sd)
CRP measurements made after diabetes was recorded are excluded from the analysis (n=2)
 +age and practice adjusted hazard ratio for 1 sd increase in all variables except smoking (current:non), obesity (>30:<30)  and age (5 year increase).

Excluding the subjects with T2DM at baseline, BMI, C-reactive protein (CRP), triglyceride, cholesterol and blood pressure were all associated with increased risk of development of T2DM, with BMI conferring the highest risk (HR 1.86 [1.65-2.10:p=<0.0001 per increase of 1 SD]. A stepwise model was used to determine which of these variables were independently associated with T2DM (table 3.2) Cholesterol and blood pressure were no longer associated with T2DM, and BMI remained the most significant predictor.

**Table 3.2** Baseline Characteristics with an independent association with the risk of development of type 2 diabetes (stepwise model)

|  | HR+ (95% CI) | P value |
| --- | --- | --- |
| BMI (kg/m$^2$) | 1.72 (1.49-1.99) | <0.0001 |
| Triglycerides(mmol/L) | 1.27 (1.08-1.49) | 0.004 |
| CRP (mg/l) | 1.21 (1.01-1.46) | 0.04 |

+age and practice adjusted hazard ratio for 1 sd increase.

When the men were divided between those whose BMI at baseline was high enough to warrant a diagnosis of obesity (>30kg/m$^2$), the rates of T2DM were much higher than in the non-obese men (Fig. 3.1).  Obese men were 3.96 [2.87-5.47] times more likely to go on to develop T2DM (p<0.0001) than non-obese men.

**Fig.3.1:** Kaplan-Meier plot for the development of type 2 diabetes by the presence or absence of obesity.
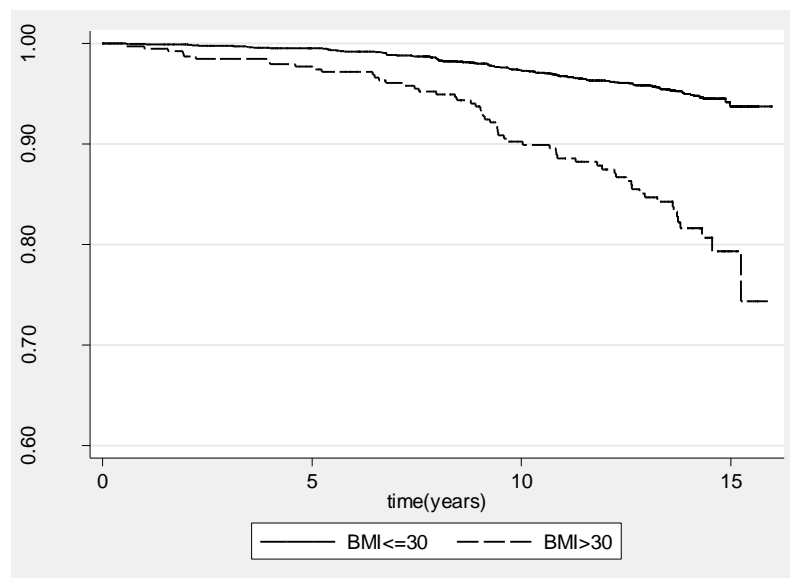
### 3.3 *UCP2-866G>A* and prospective risk of Type 2 Diabetes

3.3.1 Genotype Characteristics

DNA was available for 2775 [92%] of men, and genotype was obtained in 2695 [97%] of men and the frequencies were in Hardy Weinberg Equilibrium (p=0.85). The allele frequency for the -866A allele were 0.365 (0.35-0.38). There was no difference in BMI, Systolic blood pressure, triglycerides or CRP between genotypes (Table 3.3).

**Table 3.3.** Baseline characteristics by *UCP2 -866* genotype

| | *UCP2* Genotype | | | |
|---|---|---|---|---|
| | GG | GA | AA | P value |
| | N=1088 | N=1245 | N=362 | |
| BMI (kg/m$^2$) | 26.3 (3.4) | 26.2 (3.4) | 26.5 (3.5) | 0.26 |
| Blood Pressure (mmHg) | 137.2 (18.9) | 136.8 (18.5) | 138.2 (19.8) | 0.45 |
| Cholesterol (mmol/L) | 5.71 (1.02) | 5.73 (1.00) | 5.78 (1.01) | 0.59 |
| Triglycerides (mmol/L) | 1.78 (0.94) | 1.78 (0.93) | 1.88 (1.03) | 0.20 |
| CRP (mg/L) | 3.04 (3.56) | 2.91 (3.42) | 3.31 (3.82) | 0.20 |

All Geometric means (approximate sds) except Cholesterol.

3.3.2 Risk of Type 2 Diabetes

The genotype frequency did not differ significantly between those who developed diabetes and those who did not (no diabetes v diabetes [GG/GA/AA%]: 40/47/13 v 42/40/18:p=0.16) but a trend to a higher frequency of the AA genotype was seen when the baseline subjects with T2DM were included (40/47/13 v 42/40/18: p=0.06). The Kaplan-Meier plot (Fig. 3.2) indicates that the *UCP-2* AA genotype

only appears to be associated with the development of diabetes about 5-10 years earlier but there is a "catch up" in incidence in the GG+GA men by 15 years.

**Fig 3.2:** Kaplan-Meier plot for the development of T2DM by *UCP2-866G>A* genotype.
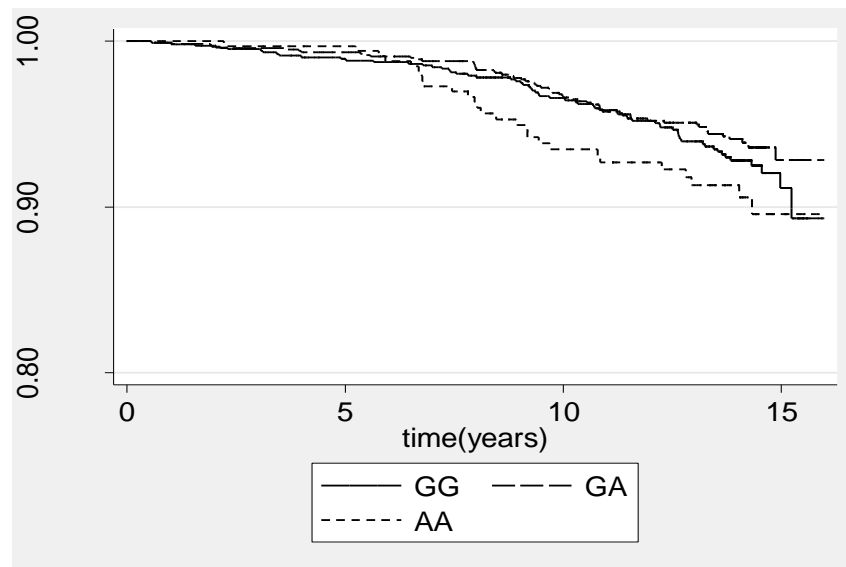


When 15 year risk of T2DM was examined using this recessive model the AA genotype was associated with an increased risk of 1.47 (0.97-2.23:p=0.07) excluding baseline cases, and 1.49 (1.03-2.14:p=0.03) including baseline cases, compared to the GG+GA men adjusted for age and recruitment site.  This risk effect was maintained after adjustment for BMI, blood pressure, cholesterol, triglycerides and CRP, with an increased 15 year risk of T2DM of 1.59 times (1.03-2.45:p=0.04) in AA homozygotes.  The acceleration of onset of T2DM associated was also seen in this recessive model (Fig 3.3).  This was confirmed by examining the risk of diabetes at 10 years. The genotype difference for risk of diabetes at 10 years was highly significant (HR [AA v GG/GA] 1.94[1.18-3.19]: p=0.009).

**Fig.3.3:** Kaplan-Meier plot for the development T2DM by *UCP2AA* genotype based on a recessive model
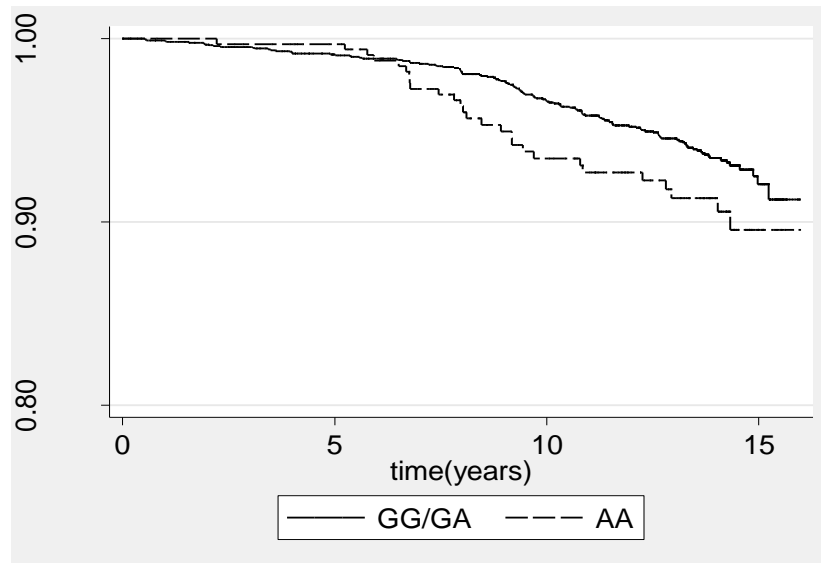


### 3.3.3 Interaction with Obesity

As obesity was such a significant risk factor for T2DM the influence of obesity on the *UCP2-866G>A* variant was examined. The risk of diabetes at 15 years associated with the AA genotype increased from 1.35 [0.79-2.32; p=0.34] to 5.55 [2.95-10.45; p=<0.0001] in obese AA men (Fig. 4.4). There was no evidence of interaction, with the effect being purely additive (p=0.85). This remained the case if BMI tertiles were used instead of obesity (p=0.80) or as a continuous variable (p=0.26). There was no difference in absolute (GG+GA v AA Median [IQR]: 0.20[-0.34 to 0.72] v 0.20 [-0.3 to 0.88]: p=0.60) or percentage change in weight by genotype (0.25 [-0.42 to 0.91] v 0.23 [-0.38 to 1.09]:p=0.63) [Fig 3.7].

**Fig 3.4:** The Hazard ratio for the development of type 2 diabetes by *UCP2-866* genotype stratified by the presence or absence of obesity.
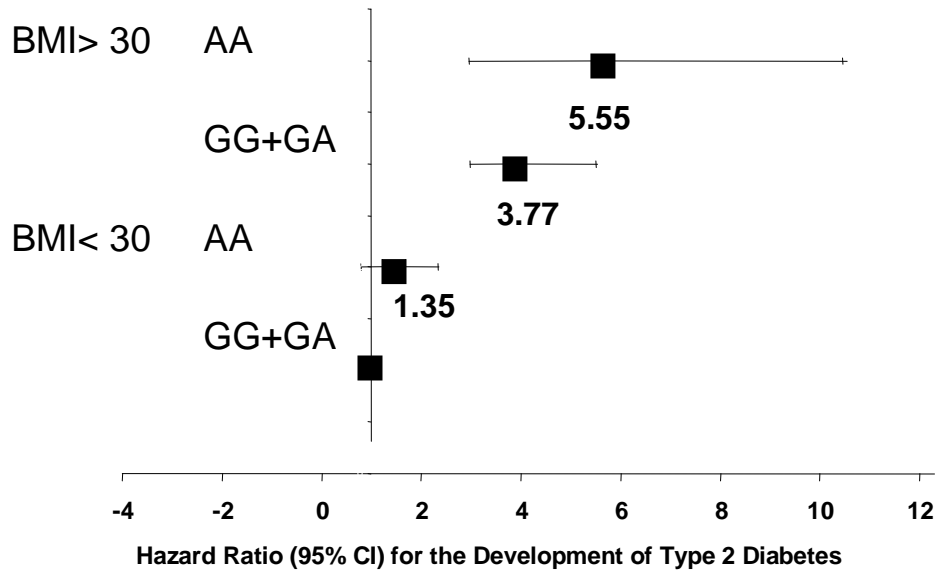


## 3.4 *UCP3-55C>T* and prospective risk of Type 2 Diabetes

3.4.1 Genotype Characteristics

Genotype was available as for *UCP*2. The genotype frequencies were also in Hardy Weinberg equilibrium (p=0.16) with the frequency of the rare *UCP3-55T* allele being 0.23 [0.21-0.24]. The baseline characteristics by genotype are shown in table 3.3 with no significant difference for any variable.

**Table 3.4:** Baseline characteristics by *UCP3 -55* genotype

| | *UCP3* Genotype | | | |
|---|---|---|---|---|
| | **CC** **N=1634** | **CT** **N=905** | **TT** **N=155** | **P value** |
| BMI (kg/m$^2$) | 26.3 (3.4) | 26.2 (3.4) | 25.9 (3.3) | 0.38 |
| Blood Pressure (sys) (mmHg) | 137.1 (18.8) | 136.9 (18.8) | 138.0 (19.0) | 0.78 |
| Cholesterol (mmol/L) | 5.70 (1.0) | 5.77 (1.0) | 5.79 (1.0) | 0.22 |
| Triglycerides (mmol/L) | 1.79 (0.95) | 1.81 (0.94) | 1.77 (0.96) | 0.90 |
| CRP (mg/L) | 3.02 (3.56) | 3.09 (3.60) | 2.67 (2.80) | 0.37 |

All Geometric means (approximate sds) except Cholesterol

### 3.4.2. Risk of Type 2 Diabetes

There was no difference in genotype frequencies between those with and those without type 2 diabetes at 15 years (% with genotype CC/CT/TT without diabetes v diabetes; 60.5/33.9/3.6 v 63.0/28.6/8.4:p=0.16), with no difference if the subjects with diabetes at baseline were included (60.6/33.9/5.5 v 61.8/27.0/11.2:p=0.19). However, the Kaplan-Meier plots for development of diabetes by genotype (Fig 4.5) and  using a recessive model (Fig 3.6) indicate that *UCP3* genotype also appears to accelerate date of onset of diabetes.   As seen for *UCP2*, the TT genotype was associated with a significant hazard ratio [95%CI] for the development of type 2 diabetes at 10 years (HR[95%CI]; 2.06 [1.06-3.99]: p=0.03) but not at 15 years (1.50 [0.85-2.66], p=0.16).

**Fig 3.5:** Kaplan-Meier plot for the development of T2DM by *UCP3-55C>T* genotype.
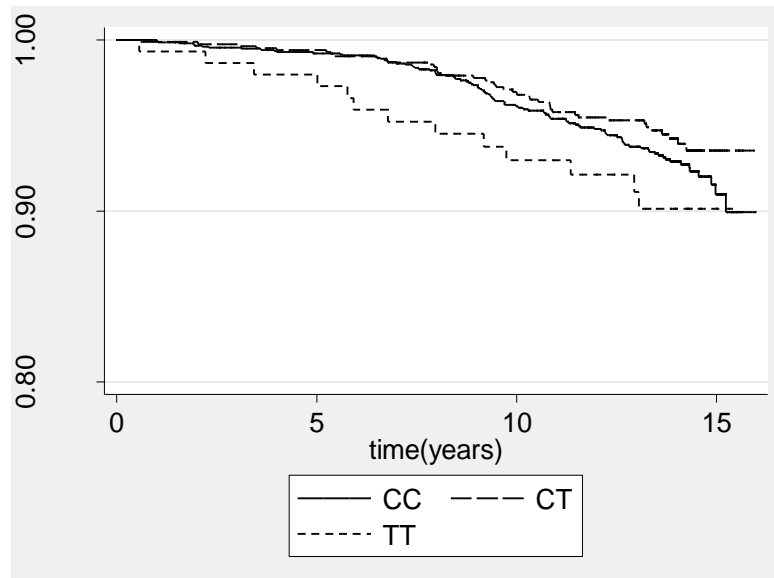


**Fig.3.6:** Kaplan-Meier plot for the development T2DM by *UCP3TT* genotype based on a recessive model
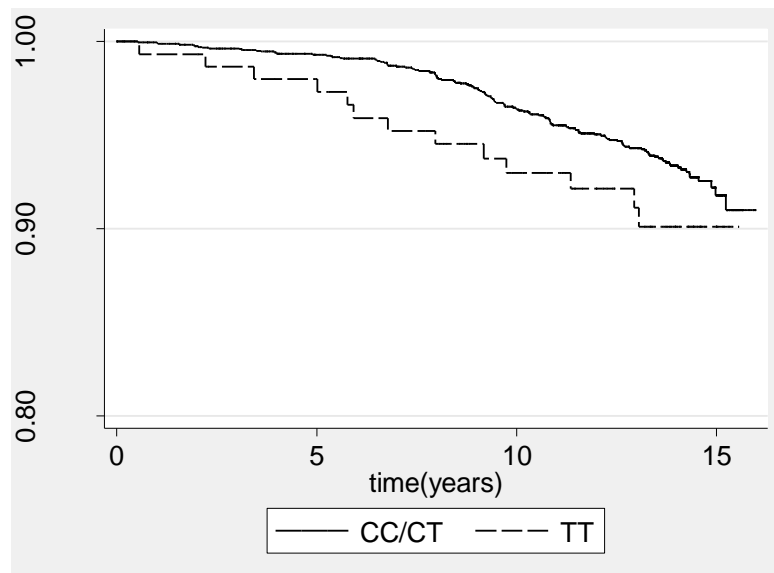
3.4.3.Interaction with Obesity

Risk of T2DM was again exacerbated by obesity, with the risk associated with *UCP3*TT genotype increasing to 5.65 ([2.07-15.46]: p<0.001) at 15 years in the obese men (Fig.3.8). The differences were again purely additive, with no evidence of interaction if this was considered as BMI tertile, obesity present or not, or BMI as a continuous variable (p=0.91, 0.38 and 0.76 respectively). The increase in risk of diabetes could again not be explained by weight gain with no difference between genotypes in absolute (CC+CT v TT Median [IQR]: 0.20[-0.34 to 0.74] v 0.13 [-0.32 to 0.80]: p=0.38) or percentage weight change (0.25[-0.42 to 0.91] v 0.16[-0.42 to 0.80]: p=0.45)[Fig. 3.7].

**Fig.3.7** The absolute and percentage change in weight by *UCP2* and *UCP3* genotype in the NPHSII study at five years.
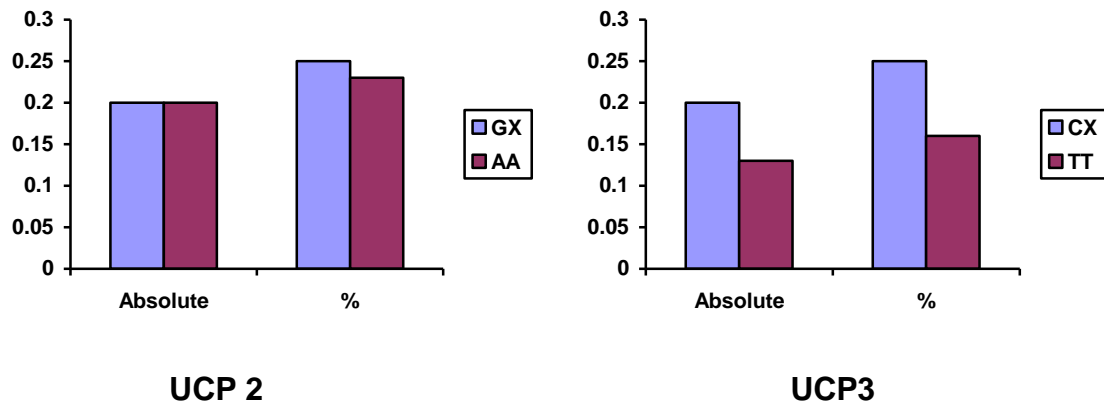


**UCP 2**          **UCP3**

**Fig 3.8:** The Hazard ratio for the development of type 2 diabetes by *UCP3-55C>T* *g*enotype stratified by the presence or absence of obesity.



Hazard Ratio (95% CI) for Development of Type 2 Diabetes
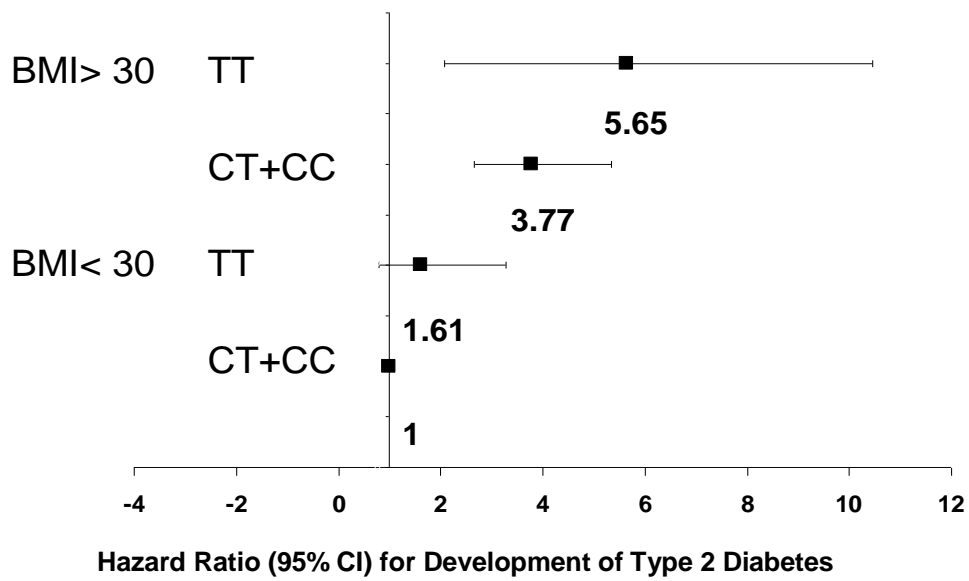
## 3.5    Combined Genotypes and Risk of type 2 Diabetes

### 3.5.1. *UCP2-UCP3* Haplotypes

The two variants showed weak but significant positive linkage disequilibrium (D'=0.28, p<0.001). As shown in Table 3.5 all four haplotypes were present in this sample. As would be predicted by the single SNP analysis, only the -866A/-55T combination was associated with increased risk of diabetes at 10 years (AT v GC 1.63[1.12-2.36]: p=0.01), and this effect was no longer statistically significant at 15 years (1.27[0.93-1.73]: p=0.13).    The relatively low frequency of this haplotype (9%), and the recessive nature of their effect precluded a robust estimate of the additive effect with obesity.

**Table 3.5:** *UCP2/UCP3* haplotypes and risk of type 2 diabetes at 10 and 15 years

| Haplotype | Frequency in those without diabetes | Frequency in those with diabetes | Hazard Ratio [95%CI] | P value |
|---|---|---|---|---|
| **10 YEARS** | | | | |
| GC | 0.50 | 0.47 | 1.00 | |
| GT | 0.14 | 0.10 | 0.74 [0.47-1.17] | 0.19 |
| AC | 0.28 | 0.28 | 1.09 [0.76-1.55] | 0.64 |
| AT | 0.09 | 0.15 | 1.63 [1.12-2.36] | 0.01 |
| **15 YEARS** | | | | |
| GC | 0.51 | 0.53 | 1.00 | |
| GT | 0.13 | 0.10 | 0.73 [0.50-1.05] | 0.09 |
| AC | 0.27 | 0.25 | 0.94 [0.72-1.22] | 0.64 |
| AT | 0.10 | 0.13 | 1.27 [0.93-1.73] | 0.13 |

3.5.2 *UCP2-UCP3* combined genotypes and Type 2 Diabetes Risk

The percentage of men developing T2DM by combined *UCP2-UCP3* genotype is shown in Table 3.6*.* After adjustment for age and place of recruitment, men homozygous for both rare alleles (the  AA-TT genotype) were the only group showing a significant increased risk of T2DM at ten years (HR 4.20[1.70-10.37]:p=0.002) or at 15 years (2.37[1.07-5.2]:p=0.03

**Table 3.6:** Combined *UCP2-UCP3* genotypes and risk of type 2 diabetes at 10 and 15 years

| *UCP2* | *UCP3* | 10 YEARS | | 15 YEARS | |
|---|---|---|---|---|---|
| | | % with diabetes (number) | HR(95% CI)* | % with diabetes (number) | HR(95% CI)* |
| GG | CC | 3.2 (24/741) | 1.00 | 6.8% (50/741) | 1.00 |
| | CT | 2.3 (6/265) | 0.59 (0.24-1.44) | 4.5% (12/265) | 0.54 (0.28-1.03) |
| | TT | 9.4 (3/32) | 3.00 (0.89-10.04) | 9.4% (3/32) | 1.36 (0.42-4.37) |
| GA | CC | 3.8 (25/663) | 1.09 (0.62-1.91) | 5.4% (36/663) | 0.77 (0.50-1.18) |
| | CT | 2.2 (10/461) | 0.63 (0.30-1.31) | 5.0% (23/461) | 0.69 (0.42-1.14) |
| | TT | 1.4 (1/71) | 0.38 (0.05-2.81) | 4.2% (3/71) | 0.56 (0.17-1.81) |
| AA | CC | 3.7 (6/161) | 1.14 (0.46-2.79) | 6.8% (11/161) | 1.05 (0.54-2.02) |
| | CT | 5.7 (8/141) | 1.64 (0.73-3.67) | 6.4% (9/141) | 0.93 (0.45-1.89) |
| | TT | 13.6 (6/44) | 4.20 (1.70-10.37) | 15.9% (7/44) | 2.37 (1.07-5.25) |

*age and practice adjusted

In order to obtain a more robust estimate of the effect of the combined genotypes, the subjects were combined into four groups on the basis of homozygosity for the variant alleles (*UCP2-UCP3*:1= GG+CC, 2= GG+GA-TT, 3= AA-CC+CT, 4= AA-TT).  Risk of T2DM increased across the groups with increasing numbers of rare

alleles, progressing from no variant homozygotes to 2 variant homozygotes (1.5% of men) at 10 years (p=0.002) and 15 years (p=0.04)(Fig.3.9).

**Fig 3.9:** Hazard ratios for the development of type 2 diabetes by combined UCP2-UCP3 group genotype at A) 10 years and B) 15 years of follow up.

A) 10 years

B) 15 years
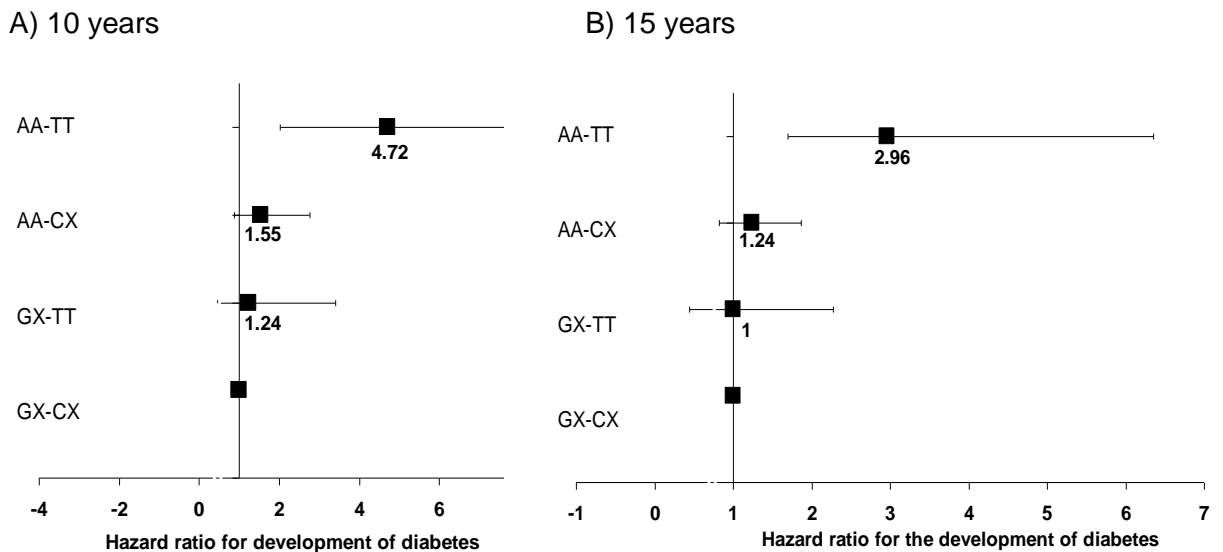


### 3.5.3. Interaction with Obesity

When the effect of the combined *UCP2-UCP3* genotype was combined with obesity, non-obese AA-TT men showed an increased risk of T2DM of 2.50(0.91-6.86: p=0.08) (Figure 3.10) while obese AA-TT men had an increased risk of 19.23 (5.83-63.39: p<0.001). The effects of the individual genotypes were additive with no evidence of an interaction between the effect of *UCP2* and *UCP3* genotype (p= 0.15). There was also no significant evidence of interaction between the effect of combined genotype group and obesity (p=0.83) or with BMI as a continuous variable (p=0.54). A Cox regression model with the two genotypes and BMI (as tertiles) showed hazard ratios [95%CI] of approximately similar magnitude for the two genotypes although the UCP3 risk estimate just crossed one (*UCP2* 1.74[1.05-2.88]:p=0.03; *UCP3* 1.92[0.98-3.76]: p=0.06). These are slightly lower than the single genotype unadjusted hazard ratios and are lower than the risk of developing

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type 2 diabetes associated with the middle or upper tertile of BMI (4.13[1.70-10.05] and 9.61[4.14-22.32] respectively: p<0.0001 for both).

**Fig.3.10:** The hazard ratio for the development of type 2 diabetes at 15 years of follow up by combined *UCP2-UCP3* genotype in those above with and without obesity.
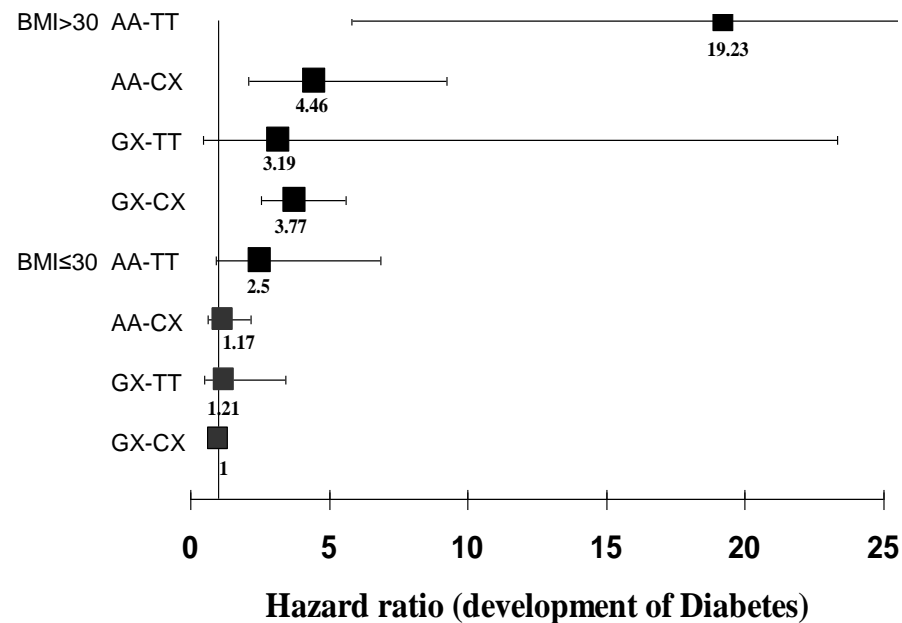


### 3.5.4 Population attributable Fraction

To determine the impact of genotype on the population using the Population attributable fraction (PAF) was calculated. The PAF [95% CI] after 15 years of follow up for variation in the *UCP2-UCP3* gene cluster was 5.3% [-1.1 to 11.4]. When obesity was added to this model the PAF was similar at 4.7% [-1.6 to 10.7]. Obesity had a much larger influence, with a PAF of 21.2% [14 to 57]. The influence of variation in the gene cluster as a whole was examined using the individual genes (Fig. 3.11A). Variation in *UCP2* genotype explained 6 (PAF 3.8% [-2 to 9]) cases, variation in *UCP3* 3 and 8 (1.9% [-1 to 5]) if adjusted for BMI which

is much lower than the 36 (21 [14 to 28]) cases of type 2 diabetes that would be explained by obesity.

If PAF is examined at 10 years, then as expected, the influence of genotype is higher (11.6% [0 to 21.7]). The influence of obesity is similar (25.6% [13 to 36]). However, as there are fewer cases of new onset Type 2 Diabetes at this time the number of cases due to obesity is lower [22]. The extra cases present due to genotype was 8 and 4 for *UCP2* variation and *UCP3* variation respectively (PAF 8.5% [-2 to 18] and 5.5%[-1 to 12] respectively [Fig: 3.11]).

**Fig.3.11:** Population attributable risk for *UCP2-866G>A, UCP3-55C>T* and obesity at A) 15 years B) 10 years.
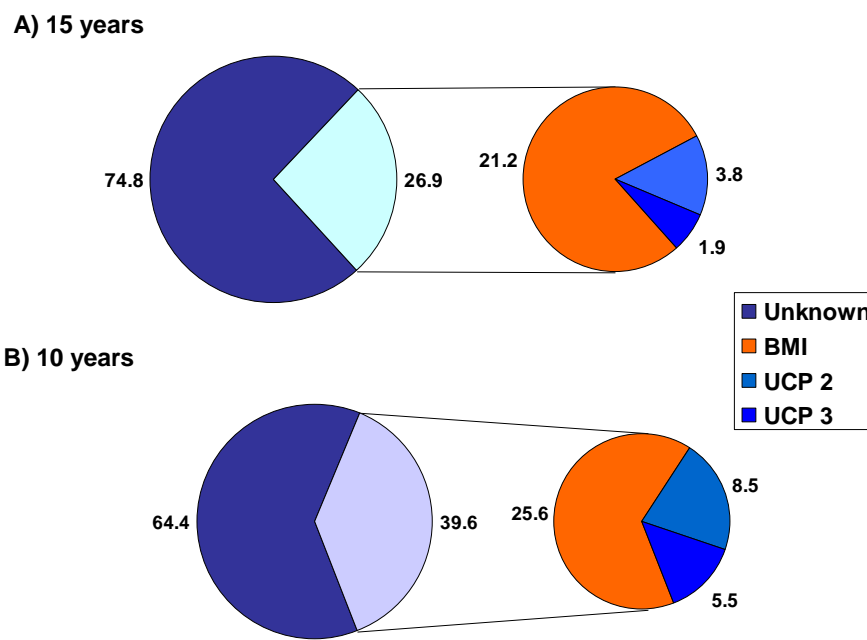


(PAF %)

## 3.6 Discussion

This 15 year study of the prospective risk of T2DM in healthy middle aged Caucasian men confirmed the significance of obesity as a major risk factor for diabetes, and also found a significant impact on risk of developing T2DM associated with variants in the genes for *UCP2* (-866A) and *UCP3* (-55T). Subjects who developed T2DM also had higher blood pressure, triglycerides, cholesterol and C-reactive protein (CRP) at baseline, although only BMI, triglycerides and CRP were associated independently with increased risk.  The association with CRP and triglycerides may have arisen because subjects going on to develop T2DM were already more insulin resistant at baseline (Duncan BB,2003) but this could not be confirmed as other measures of insulin resistance such as HOMA were not collected at recruitment.  The risk associated with BMI was over a third higher than any other metabolic parameter and the size of the risk associated with obesity, defined here as BMI>30kg/m$^2$, was approximately four times that associated with the genetic variants.  In effect, after 10 years of follow up,1 out of 4 cases of T2DM would have been prevented if there were no obesity, BMI being the best marker of adiposity in the group as waist circumference was not collected at baseline, present in the sample, while 1 out of 10 cases would be prevented if there were no genetic variation at *UCP2*-866 or *UCP3*-55.  The additive effect of the combination of obesity and the presence of the gene variants increased the risk to extremely high levels, although such subjects were rare in the sample.  In obese subjects homozygous for either variant the risk of T2DM increased to over 5 times that of non-obese subjects, non-homozygous for either variant.  Homozygotes for both variant alleles had a risk of diabetes from 2.5 times higher in the non-obese, to nearly 20 times higher in obese subjects.

The *UCP2*-866A allele has consistently been associated with risk of T2DM in cross-sectional studies in European subjects, and the mechanism of this appears likely to be due to increased UCP2 expression in the pancreas.  The location of the

-866G>A variant, within a multifunctional *cis* regulatory site involving putative binding sites for pancreatic and hypoxia-induced transcription factors, suggests that it is likely to be functional (Esterbauer H,2003). Consistent with this, a promoter construct of the -866A allele was associated with 1.2 fold higher expression vs. -866G in INSE-1 cells, derived from rat β-cells (Krempler F,2002). Putative mechanisms are examined in later chapters but it is likely that UCP2 expression uncouples ATP production from glucose metabolism, reducing ATP production, and since insulin secretion depends on the ATP/ADP ratio as a marker of glucose metabolism in the pancreatic β-cell (Erecinska M,1992), secretion is reduced as a result. The higher risk of developing T2DM associated with variation in the *UCP2* gene is thus likely to be due to a pancreatic effect on insulin secretion. Risk is exacerbated by obesity, as obese subjects are already likely to be insulin resistant and will require higher insulin secretion to maintain normal glucose homeostasis.

A different mechanism appears likely for the UCP3 risk effect. As discussed in chapter 1 although *UCP3* expression has been associated with higher uncoupling *in vitro* (Jaburek M,1999), *UCP3* expression is increased both by fasting and by an iso-caloric high fat diet, neither of which increases metabolic rate nor uncoupling. Also, an increase in expression does not change mitochondrial membrane potential. These findings suggest that UCP3 does not have a conventional uncoupling function in man (Schrauwen P,2004). Overexpression of *UCP3* in mice which protects them from diabetes, with a 68% increase in mitochondrial palmitate oxidation. This effect is specific to UCP3, as there is no increase in glucose oxidation, change in oxygen consumption or mitochondrial membrane potential, which is seen with increased uncoupling *per se* (MacLellan JD,2005). *UCP3* expression will thus protect the mitochondria, by exporting the fatty acid anions and peroxides from the mitochondrial matrix, protecting mitochondrial function and preventing the development of T2DM (Schrauwen P,2003).

Since the *UCP3*-55T variant is associated with risk of diabetes in the men studied here, a decrease in function would be predicted for the T allele. The variant was originally mapped near a TATA box (6bp) (Schrauwen P,1999) where it may affect transcription, but a recent report (Acin A,1999) places the variant in the '5UTR near a PPAR responsive element (4bp) which also suggests it could modify regulation of *UCP3* expression. A reduction in *UCP3* function in -55T carriers has been seen in some but not all previous cross sectional studies. *UCP3* mRNA expression was higher in CT/TT male non-diabetic Pima Indians, but this study included only 24 subjects and was based on only seven copies of the T variant from 48 alleles (Schrauwen P,1999). The T allele was associated with higher BMI or WHI ratio, consistent with decreased function, in French and German Caucasians, South Asian Indian parent-offspring trios, South Asian Indians and the British Diabetic Association Warren 2 trios collection (Cassell PG,2000; Halsall DJ,2001; Herrmann SM,2003; Otabe S,2000). However, one study showed a lower BMI in TT subjects in a United Kingdom sample (Halsall DJ,2001) and increased function is also suggested with the association of protection from diabetic neuropathy (Rudofsky G,2006). In these studies, association with T2DM was examined only once and a relationship not found. The variant allele was found to be protective against T2DM in two French cohorts although also associated with an atherogenic lipid profile (Meirhaeghe A,2000). The reason for this difference is not clear; the French sample does not appear to be very different to the NPHSII sample although the NPHSII sample is nearly three times larger, which may be important when the impact of an individual variant is small.

The overall prevalence of T2DM in this sample is 2.5% at baseline and 8.1% after 15 years of follow up. This is lower than that reported by the Joint Health Surveys Unit (Health Survey for England 2003,2004), however, the increase in prevalence of both diagnosed and undiagnosed diabetes between the age range 55-64 years and 65-74 years is similar, from 10.4%, to 18.8%. The lower prevalence is in part due to the exclusion of those with diabetes on treatment at the beginning of the study. Although a small number of subjects with diabetes managed by dietary

modification alone were included in the study the findings were consistent if they were included in the analysis. The method of identification of the men with T2DM, by the medical record search, is unlikely to include any false positive diagnosis, but in the absence of a full recall for fasting glucose testing some affected subjects may be missed. This would then result in a small underestimate of the 15 year incidence of T2DM, but would not confound the genetic association observed. This is the first prospective study to look at risk of T2DM and, overall prospective gene-association studies are more powerful than the case-control design (Humphries SE,2003) but further replication is required to resolve these apparent discrepancies.

# CHAPTER FOUR

## THE *UCP2-866G>A* AND *UCP3-55C>T* VARIANTS IN THE HYPERCOAGULABILITY AND IMPAIRED FIBRINOLYTIC FUNCTION MECHANISMS STUDY (HIFMECH)

# CONTENTS OF CHAPTER FOUR

## 4.1 Hypercoagulability and Impaired Fibrinolytic Function Mechanisms Study (HIFMECH)

### 4.1.1 Introduction

The previous chapter demonstrated the association of variants in the uncoupling proteins 2 and 3 with prospective risk of type 2 diabetes. The study, however, did not address the potential mechanisms that underlie this association. The HIFMECH study is described in detail in chapter 2. The measurement of a number of metabolic markers gives the opportunity to investigate the mechanism by which these gene variants influence risk of type 2 diabetes. Chapter 1 describes how there may be an overlap in the underlying pathophysiology driving cardiovascular disease and type 2 diabetes. The case-control nature of this study allows for the association of these variants with myocardial infarction to be studied, determining if the pathway of increased risk of type 2 diabetes is likely to be shared with cardiovascular disease, or is independent of it.

### 4.1.2 Aims

To examine the *UCP2-866G>A* and *UCP3-55C>T* variants in the HIFMECH study to determine if the increase in prospective risk of type 2 diabetes is due to an association with adverse metabolic markers.

To estimate the effect of the *UCP2-866G>A* and *UCP3-55C>T* variants on risk of myocardial infarction.

### 4.1.3 Methods

The HIFMECH study was designed to study genetic and environmental mechanisms contributing to the higher cardiovascular risk in Northern compared to Southern Europe (Juhan-Vague I,2002). The study sample of Caucasian male first

myocardial infarction survivors below 60 years of age were recruited from four European centres (Northern European-*Stockholm, London*; Southern European-*Marseille, San Giovani Rotondo*) (n=598). A selection of randomly-selected age matched healthy controls was also recruited from each catchment area (n=653). To reduce the risk of error due to multiple testing statistical analysis followed the *a-priori* design, to study the association of risk factors associated with cardiovascular risk in relation to the North and South of Europe, combining data from the two North and two Southern centres for a more statistically robust comparison. There was no difference between common allele homozygotes and heterozygotes for prospective risk of type 2 diabetes so a recessive model was used for both genotypes. The metabolic markers were studied in controls, as they are most similar to the participants of NPHSII. In keeping with the original design, risk of myocardial infarction associated with genotype was also estimated. Associations were estimated in cases only for the purpose of assessing confounding of any differences detected in cardiovascular risk associated with genotype.

## 4.2.    Baseline Characteristics

The baseline characteristics of the HIFMECH study are shown in table 1. There are significant differences between both cases and controls and between subjects in the North and South of Europe. Those in the South had a better lipid profile but were more likely to smoke. There was no difference between BMI between the North and South but waist:Hip ratio (WHR) was higher in the North than in the South (Controls 1 v 0.95,p<0.001; Cases 0.98 v 0.93, p<0.001). As expected, cases had higher rates of smoking and type 2 diabetes, whilst although triglycerides were higher in cases there was no clear difference in blood pressure and lipids. This is likely to be due to post-infarct treatments. The frequencies of the *UCP2-866* and *UCP3-55* genotype were as expected from Hardy-Weinburg proportions in both the North and South sample (*UCP2-866*: North p=0.72, South

p=0.96. *UCP3-55*: North p=0.49, South p=0.45). The frequency of the rare *UCP2-866A* was 0.34[0.32-0.37], with no difference between the North and South (p=0.95). By contrast, there was a difference between the frequency of the rare *UCP3-55T* between the North (0.28[0.25-0.31]) and the South (0.18[0.15-0.20];p=0.001).

**Table 4.1.** The baseline characteristics of the subjects determined by case/control and North/South status.

| | NORTH | | | | SOUTH | | | | North V South | Cases V Controls |
| | Cases | | Controls | | Cases | | Controls | | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age (yrs) | 53.0 | 5.1 | 52.7 | 5.1 | 51.0 | 5.6 | 50.5 | 5.6 | <0.01 | 0.17 |
| BMI (Kg/m$^{2)}$) | 27.2 | 3.4 | 25.8 | 3.1 | 27.0 | 3.3 | 26.4 | 3.3 | 0.20 | <0.01 |
| Blood Pressure (mmHg) - Systolic | 129.3 | 17.7 | 130.4 | 15.8 | 126.9 | 16.2 | 126.4 | 13.3 | <0.01 | 0.87 |
| - Diastolic | 82.3 | 9.8 | 84.0 | 8.5 | 81.5 | 10.6 | 84.4 | 8.7 | 0.82 | <0.01 |
| Smoking-Current/Ex (%) | 76.8 | 2.0 | 61.9 | 2.3 | 86.9 | 1.4 | 61.2 | 2.0 | <0.01 | <0.01 |
| Diabetes-Type 2 (%) | 11.6 | 1.5 | 0 | - | 11.5 | 1.4 | 0 | - | 0.67 | <0.01 |
| Cholesterol (mmol/L) | 5.65 | 1.22 | 5.68 | 0.96 | 5.18 | 1.11 | 5.39 | 0.93 | <0.01 | 0.06 |
| Triglyceride (mmol/L) | 1.99 | 0.83 | 1.52 | 0.58 | 1.79 | 0.72 | 1.39 | 0.60 | <0.01 | <0.01 |

The blood pressure and lipid parameters include those on anti-hypertensive and lipid lowering agents respectively

There was no difference in the characteristics of those with and without genotype in any of the four groups (data not shown).

## 4.3    *UCP2-866G>A*

4.3.1 Metabolic Markers by Genotype in controls

There was no difference between genotype (Analysis followed recessive effect identified in NPHS; AA v GX) for any of the lipid parameters (cholesterol, triglycerides, or apoB) (Table 4.2) in either the North, the South or with both samples combined.

**Table 4.2**: The differences between lipid profiles in healthy subjects for the *UCP2-866G>A* variant in the HIFMECH study.

*adjusted for centre [1]log transformed [2]square root transformed

|  | UCP2-866 | North | South | Combined* |
|---|---|---|---|---|
| **Cholesterol (mmol/l)** | **GG/GA** | 5.67 (0.98) 182 | 5.40 (0.92) 265 | 5.70 (0.95) 447 |
|  | **AA** | 5.69 (0.85) 31 | 5.34 (1.00) 36 | 5.67 (0.93) 67 |
|  | **P value** | 0.93 | 0.70 | 0.81 |
| **Triglycerides (mmol/l)** | **GG/GA** | 1.53 (0.58) 182 | 1.38 (0.59) 265 | 1.52 (0.62) 447 |
|  | **AA** | 1.49 (0.63) 31 | 1.41 (0.67) 36 | 1.52 (0.68) 67 |
|  | **P value** | 0.74 | 0.76 | 0.97 |
| **apoB (mmol/l)** | **GG/GA** | 97.8 (23.2) 182 | 94.1 (20.2) 265 | 98.1 (21.7) 447 |
|  | **AA** | 92.8 (20.0) 31 | 93.1 (20.3) 36 | 95.2 (20.4) 67 |
|  | **P value** | 0.25 | 0.79 | 0.30 |

Although BMI ($Kg/m^2$) was lower in AA subjects in the North (GX v AA 26.0 [3.1] v 24.9 [3.2]; p=0.06) there was no difference in the South (26.4[3.2] v 27.0[3.0]; p=0.25).  However, as shown in Fig 4.1a, WHR was lower in AA homozygotes compared to G carriers in both the North, South and all controls combined (North: 1.00[0.06] v 0.98[0.07]; p=0.03: South 0.95 [0.05] v 0.93 [0.07]; p=0.04: combined 1.00[0.06] v 0.98[0.07]; p=0.003).  There were also significant differences in fasting insulin between genotypes with the previously identified genotype for prospective risk of type 2 diabetes (UCP2-866AA) associated with significantly lower insulin levels (pmol/l) in the North and in all controls combined but not significant in the South although the same trend was seen (North: 40.4[26.5] v 30.2[13.8]; p=0.02: South 37.4[23.5] v 32.9[23.0]; p=0.37: combined 40.3[24.6] v 32.3[18.6]; p=0.02)(Fig 4.1b).  This difference was no longer significant in the North or South

once adjusted for BMI, but remained so in all the controls combined (North: 42.6[25.6] v 36.1[13.8]; p=0.15: South 39.1[21.7] v 32.2[22.1]; p=0.14: combined 42.6[24.6] v 35.6[18.6]; p=0.03)(Fig 4.1b).

**Fig 4.1**: The association of the *UCP-886AA* genotype with a) lower waist hip ratio b) lower insulin secretion (unadjusted) and lower insulin secretion  adjusted for BMI
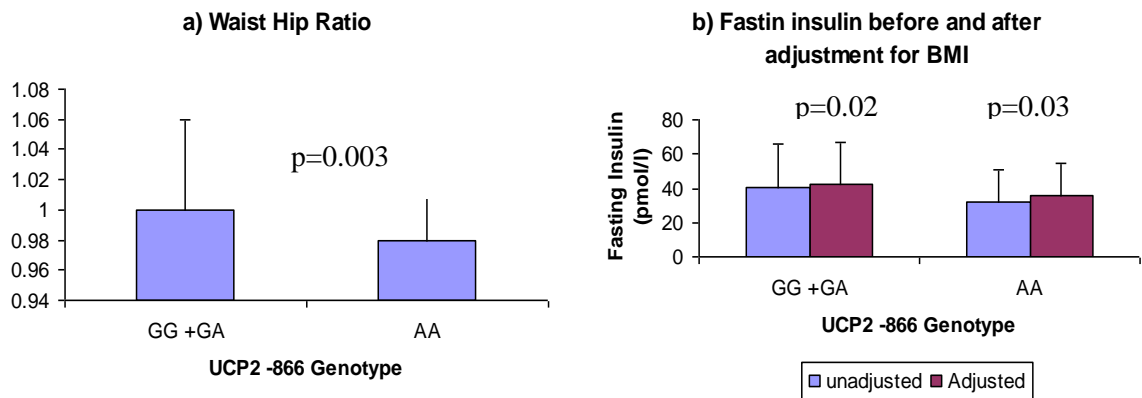


4.3.2. Risk of cardiovascular disease and genotype association in cases

The odds of being a case by *UCP2-8AA*, compared to GG+GA, using a conditional logistic regression model were a modestly higher odds ratio for Myocardial Infarction in the North of 1.30 [95%CI 0.76-2.23] but not in the South 1.00 [0.60-1.68].    The risk for North and South combined was 1.19 [0.81-1.75]; p=0.38(Fig 4.2).    In cases, the AA genotype was associated with significantly lower than cholesterol in the South [p=0.04] and higher in the North [p=0.05] but in the combined group no significant difference was seen.  Triglycerides were lower in AA subjects in both North and South and in the combined group the difference was statistically significant [p=0.05]. These differences may have occurred by chance but would be likely to weaken any association between the AA genotype and cardiovascular disease.  There were no other statistically significant associations in cases between metabolic markers and genotype [p=0.17-0.78]. (Table 4.3)

**Fig 4.2:** The Odds ratio for Myocardial Infarction associated with the *UCP2-886G>A* genotype in HIFMECH
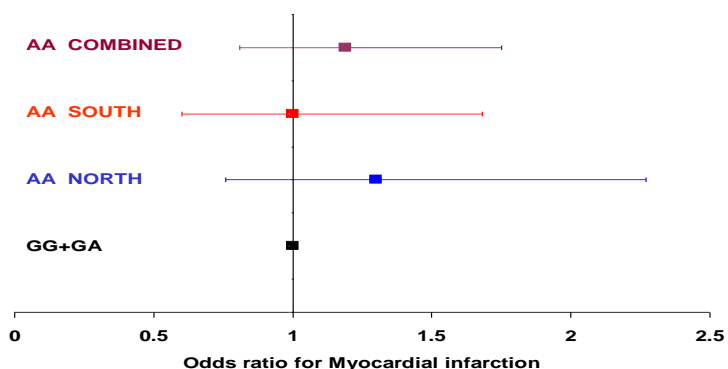


**Table 4.3**: The association of the *UCP2-866G>A* genotype and metabolic markers in cases in the HIFMECH study.

| | UCP2 | North | South | Combined* |
|---|---|---|---|---|
| **BMI[1] (Kg/m^{2})** | **GG/GA** **AA** **P value** | 27.3 (3.4) 182 26.8 (3.0) 39 0.36 | 27.1 (3.4) 249 26.5 (3.0) 33 0.31 | 27.3 (3.4) 431 26.8 (3.0) 72 0.17 |
| **WHR** | **GG/GA** **AA** **P value** | 0.98 (0.06) 182 0.99 (0.05) 39 0.19 | 0.95 (0.06) 232 0.95 (0.07) 31 0.87 | 0.98 (0.06) 414 0.98 (0.06) 70 0.40 |
| **Cholesterol (mmol/L)** | **GG/GA** **AA** **P value** | 5.58 (1.21) 170 6.00 (1.24) 37 0.05 | 5.23 (1.12) 237 4.81 (0.91) 32 0.04 | 5.65 (1.16) 407 5.67 (1.15) 69 0.90 (N/S Interaction p=0.005) |
| **Triglycerides[1] (mmol/L)** | **GG/GA** **AA** **P value** | 2.02 (0.83) 170 1.87 (0.86) 37 0.31 | 1.82 (0.72) 237 1.59 (0.70) 32 0.08 | 2.02 (0.81) 407 1.82 (0.81) 69 0.05 |
| **apoB[2] (mmol/L)** | **GG/GA** **AA** **P value** | 106.3 (28.9) 170 111.8 (25.0) 37 0.29 | 97.3 (22.3) 237 90.1 (20.9) 32 0.08 | 107.6 (25.9) 407 106.7 (24.1) 69 0.78 |
| **Insulin[1] (pmol/L)** | **GG/GA** **AA** **P value** | 45.8 (30.7) 169 38.1 (25.2) 36 0.14 | 57.8 (40.6) 145 56.3 (45.9) 17 0.88 | 45.9 (31.4) 314 40.1 (28.5) 53 0.19 |
| **Insulin[1] (BMI adjusted)** | **GG/GA** **AA** **P value** | 42.0 (23.8) 166 37.2 (21.0) 36 0.24 | 56.6 (37.7) 145 57.7 (45.7) 17 0.91 | 42.0 (25.8) 311 38.9 (25.0) 53 0.41 |

*adjusted for centre,[1]log transformed,[2]square root transformed

## 4.4    *UCP3-55C>T*

### 4.4.1  Metabolic Markers by Genotype in controls

*UCP3-55C>T* genotype was not associated with differences in waist hip ratio, BMI or insulin.   The lipid parameters were unremarkable except the TT genotype was associated with higher apoB in the South (Table 4.4).

**Table 4.4:** The association of the *UCP3-55C>T* genotype and metabolic markers in controls in the HIFMECH study.

| | UCP3 | North | South | Combined* |
|---|---|---|---|---|
| BMI[1] (Kg/m$^2$) | CC/CT | 25.8 (3.1) 221 | 26.4 (3.2) 309 | 25.8 (3.1) 530 |
| | TT | 25.4 (3.5) 22 | 26.2 (2.3) 11 | 25.4 (3.1) 33 |
| | P value | P=0.51 | P=0.81 | P=0.49 |
| WHR | CC/CT | 1.00 (0.06) 220 | 0.95 (0.06) 200 | 1.00 (0.06) 420 |
| | TT | 1.01 (0.06) 21 | 0.94 (0.09) 6 | 1.00 (0.06) 27 |
| | P value | P=0.54 | P=0.65 | P=0.71 |
| Cholesterol (mmol/L) | CC/CT | 5.74 (0.99) 204 | 5.37 (0.93) 308 | 5.71 (0.95) 512 |
| | TT | 5.44 (0.96) 20 | 5.84 (1.01) 11 | 5.70 (1.03) 31 |
| | P value | P=0.19 | P=0.10 | P=0.92 |
| Triglycerides[1] (mmol/L) | CC/CT | 1.51 (0.58) 204 | 1.39 (0.62) 308 | 1.52 (0.64) 512 |
| | TT | 1.63 (0.78) 20 | 1.42 (0.51) 11 | 1.61 (0.70) 31 |
| | P value | P=0.40 | P=0.85 | P=0.46 |
| apoB[2] (mmol/L) | CC/CT | 98.3 (23.1) 204 | 93.9 (20.1) 308 | 98.1 (21.6) 512 |
| | TT | 95.8 (21.5) 20 | 102.8 (23.6) 11 | 99.8 (22.7) 31 |
| | P value | P=0.51 | P=0.03 | P=0.68 |
| Insulin[1] (pmol/L) | CC/CT | 39.9 (25.5) 205 | 36.1 (22.6) 202 | 39.3 (24.9) 407 |
| | TT | 37.3 (26.5) 20 | 42.4 (27.3) 9 | 39.7 (27.2) 29 |
| | P value | P=0.65 | P=0.45 | P=0.94 |
| Insulin[1] (BMI adjusted) | CC/CT | 43.0 (24.6) 205 | 37.6 (21.2) 202 | 42.0 (23.8) 407 |
| | TT | 41.2 (21.8) 20 | 45.3 (27.1) 9 | 43.4 (24.0) 29 |
| | P value | P=0.75 | P=0.33 | P=0.76 |

*adjusted for centre,[1]log transformed,[2]square root transformed

5.4.2  Risk of Cardiovascular disease and genotype association in cases

There was a lower odds ratio for myocardial infarction associated with TT homozygotes of borderline significance in the North (OR[95%CI] 0.45[0.2-1.05]: p=0.06) but not so in the South (0.51[0.18-1.48]: p=0.22).  The odds ratio for myocardial infarction in the study combined was lower in those with the TT genotype (0.47[0.25-0.92]: p=0.03

**Fig 4.3:**  The Odds ratio for cardiovascular disease associated with the *UCP3-55C>T* genotype in the HIFMECH study.
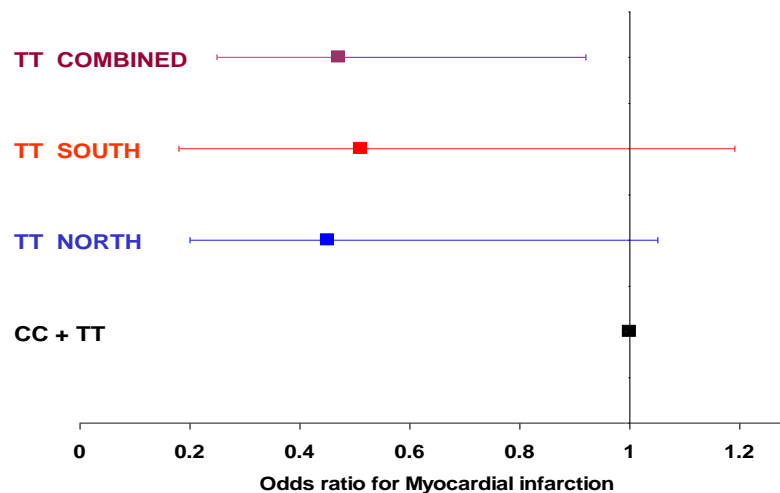


The UCP3-55TT homozygotes had lower mean cholesterol in the North and South, although the result was of borderline significance in the North (Cholesterol (mmol/L) CC +CT v TT: North 5.7[1.2] v 5.1[0.9], p=0.09; South 5.2[1.1] v 4.2[1.1], p=0.04; combined 5.7[1.2] v 4.9[1.0], p=0.01).  A similar association was seen with apoB but in the North the differences were not significant (p=0.22) (Table 4.5).

**Table 4.5** The association of the *UCP3-55C>T* genotype with metabolic markers in cases in the HIFMECH study.

| | UCP3 | North | South | Combined* |
|---|---|---|---|---|
| BMI[1] (Kg/m$^2$) | CC/CT<br>TT<br>P value | 27.2 (3.2) 209<br>27.7 (5.0) 13<br>P=0.57 | 27.0 (3.3) 292<br>25.3 (3.4) 5<br>P=0.24 | 27.2 (3.2) 501<br>27.1 (4.6) 18<br>P=0.87 |
| WHR | CC/CT<br>TT<br>P value | 0.98 (0.06) 210<br>0.98 (0.07) 12<br>P=0.82 | 0.95 (0.06) 272<br>0.95 (0.08) 5<br>P=0.95 | 0.98 (0.06) 482<br>0.98 (0.07) 17<br>P=0.82 |
| Cholesterol (mmol/L) | CC/CT<br>TT<br>P value | 5.70 (1.23) 198<br>5.08 (0.90) 12<br>P=0.09 | 5.20 (1.11) 279<br>4.15 (1.08) 5<br>P=0.04 | 5.68 (1.16) 477<br>4.94 (0.95) 17<br>P=0.01 |
| Triglycerides[1] (mmol/L) | CC/CT<br>TT<br>P value | 1.99 (0.85) 198<br>2.17 (0.64) 12<br>P=0.49 | 1.79 (0.72) 279<br>1.61 (0.45) 5<br>P=0.56 | 1.99 (0.82) 477<br>2.05 (0.60) 17<br>P=0.76 |
| apoB[2] (mmol/L) | CC/CT<br>TT<br>P value | 108.1 (28.5) 198<br>98.0 (24.4) 12<br>P=0.22 | 96.8 (22.2) 279<br>76.3 (20.3) 5<br>P=0.03 | 108.0 (25.6) 477<br>94.4 (23.5) 17<br>P=0.03 |
| Insulin[1] (pmol/L) | CC/CT<br>TT<br>P value | 44.1 (29.6) 198<br>50.3 (33.7) 11<br>P=0.52 | 56.0 (38.8) 170<br>73.8 (66.1) 3<br>P=0.50 | 44.3 (30.1) 368<br>52.0 (35.7) 14<br>P=0.39 |
| Insulin[1] (BMI adjusted) | CC/CT<br>TT<br>P value | 41.5 (23.7) 194<br>42.1 (22.2) 11<br>P=0.93 | 55.3 (36.8) 170<br>76.9 (36.4) 3<br>P=0.40 | 41.3 (25.5) 364<br>44.8 (23.1) 14<br>P=0.63 |

*adjusted for centre,[1]log transformed,[2]square root transformed

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4.5 Discussion

Analysis of the *UCP2-866* variant in the HIFMECH study provides two important observations. Firstly, these data confirm the previous studies in Europe [see table 1.4c] showing that AA subjects are leaner [2% lower WHR] in both the North and the South, and secondly that AA subjects have lower fasting plasma insulin levels. In the healthy subjects the difference in fasting insulin levels persisted when adjusted for body mass index. There was no other relationship between genotype and any of the adverse metabolic markers, suggesting that the effect of genotype variation is not through conventional risk factors for type 2 diabetes. This association with β-cell dysfunction could have been replicated if the variant was

also associated with pro-insulin or insulin split products which are associated with early β-cell dysfunction but these were not available for this study sample.

This study was underpowered to detect an association between genotype and cardiovascular risk. The *UCP2-866AA* was associated with a modestly higher odds ratio for myocardial infarction in the North but not in the South. This is in keeping with other data suggesting that the A allele is associated with risk of cardiovascular disease in healthy men (Dhamrait SS,2004) and carotid atherosclerosis in women (Oberkofler H,2005). The A allele is also associated with higher markers of oxidative stress in men with type 2 diabetes (Dhamrait SS,2004). Why this association is seen more strongly in the North is not clear. The mean WHR [%] was higher in the North [Controls 100 v 95 p<0.001; Cases 98 v 93, p,0.001] and this and other environmental factors such as diet could alter oxidative stress load. The *UCP2* gene promoter is induced by oxidative stress through both hypoxic and toxic pathways, and differences in oxidative stress load could alter the influence of *UCP-2* promoter variants on transcription function (Krempler F,2002; Oberkofler H,2005).

The association of the A allele with higher oxidative stress suggests that this allele is associated with lower "uncoupling". This is in contrast to the obesity and insulin data where the A allele is "protective" and associated with higher uncoupling. This could be explained if the different alleles behave in different ways at baseline and under oxidative stress loads, such as that from cardiovascular risk factors. This is supported by the findings that THP-1 (macrophages) and HUVEC (endothelium) cells (Oberkofler H,2005), compared to INSE-1 (Krempler F,2002) and Paz-6 cells (Esterbauer H,2001) show differences in transcription factor binding. Different effects in different disease processes could also be explained by differential regulation of *UCP2* in different tissues. Indeed, in pancreatic cells [INSE-1] the effect of the UCP2A promoter construct is enhanced transcription under PAX6 stimulation [which would be associated with diabetes], but lower transcription in

COS-7 cells, which would be associated with higher oxidative stress [see Fig 7.2, page 210].

The association of the *UCP3TT* genotype with type 2 diabetes does not seem to be explained by differences in conventional risk factors for type 2 diabetes or by markers of insulin resistance. In healthy controls the only association of an adverse metabolic marker with *UCP3* genotype was an association with higher ApoB in the South. The significance of this finding is uncertain given the number of comparisons performed. The association of the *UCP3 TT* genotype with type 2 diabetes must be explained through a mechanism not measured in the HIFMECH study.

The association of the TT genotype with protection from myocardial infarction is a novel finding. How the genotype associated with type 2 diabetes is also associated with cardiovascular disease is unclear and the association could be a chance finding. The putative role of UCP3 in mitochondrial metabolism, protection from oxidative stress from over-nutrition, would also be expected to protect from cardiovascular disease. This genotype was associated with increased prospective risk of diabetes in NPHSII (see chapter 4) The HIFMECH study is cross sectional in nature, this design is more susceptible to confounding than prospective studies. The association may have occurred as a result of a confounding factor, such as the association of lower cholesterol of 0.75mmol/l with the TT genotype in cases. This association was seen both in the North and the South although was based on only 17 TT homozygotes and was not seen in the NPHSII study. The reduction of cholesterol from 5.68 to 4.94mmol/l (assuming a HDL of 1.0mmol/l) in a 65 year old normotensive, non-smoker reduces the 10 year risk of cardiovascular disease by 13% (http://cvrisk.mvm.ed.ac.uk/calculator/framingham.htm) which would explain, at least in part, some of the lower risk observed. The expression of *UCP3* may also be stimuli or organ specific, as is the case with *UCP2*. There is currently little information on the effect of *UCP3* variants on *UCP3* transcription and is based on small numbers (Schrauwen P,1999) or very rare variants (Chung WK,1999) and do

not provide much evidence of control of *UCP3* expression.  If the association of the *-55UCP3TT* variant with risk of type 2 diabetes and protection from cardiovascular disease is replicated, then further work will be required to understand the mechanisms underlying these data.

# CHAPTER FIVE

# PLASMA MARKERS OF OXIDATIVE STRESS IN THE JAPANESE AMERICAN STUDY

# CONTENTS OF CHAPTER FIVE

## 5.1 Assays of Plasma Markers of Oxidative Stress in The Japanese American Family Study

5.1 Introduction

The association of risk of type 2 Diabetes associated with variation in *UCP2* and *UCP3* has been examined in chapters 3 and 4. The *UCP2* variant is associated with lower insulin secretion but the mechanism for this, and the mechanism for the risk associated with *UCP3*-55TT is as yet unclear. It is possible that these genetic variants are associated with differences in non-classical risk factors for type 2 Diabetes, more specifically the modification of oxidative stress.

As described in chapter 1, living organisms have developed a wide range of complex anti-oxidant systems to counteract the damaging effects of reactive oxygen species. The oxidative stress balance within an organism can be determined by several different methods. A component of one of the anti-oxidant systems can be measured directly or a component of the cell damaged by reactive oxygen species can be assayed. Other methods rely on the concept of anti-oxidant capacity, which is estimated as the capacity of the sample to scavenge free radicals in a test solution, and represents the sum of the anti-oxidant systems. There are two main methods to determine anti-oxidant capacity, based on the ability of the sample to inhibit an oxidizing reaction or to drive a reducing reaction.

The Japanese American Family (JAM) study is a family study to investigate risk factors for coronary heart disease and type 2 diabetes. The Principal investigator of the study is Prof. Melissa Austin and the subjects were recruited in the United States of America, from in or around Washington, and were of Japanese descent (Austin MA,2004a). The subjects are well characterised, and stored unthawed frozen plasma samples were available. The plasma markers of oxidative stress give an indication of the amount of free radical mediated damage present, and enable the examination of the factors that determine oxidative stress burden in this population. The genetic component of type 2

Diabetes may in part be through modification of the body's ability to generate or protect itself from oxidative stress.

5.2 Aims

1. To compare different methods of measuring anti-oxidant capacity with the aim of developing a method of measuring oxidative stress markers that was accurate and capable of handling a large number of samples.

2. To measure markers of oxidative stress in plasma samples of subjects from the Japanese American Family Study.

    i) Examine the factors that predict levels of plasma markers of oxidative stress.

    ii) Determine the genetic contribution to plasma markers of oxidative stress by calculating heritability.

    iii) Examine if genetic variation in the *UCP2-UCP3* gene cluster is associated with variation in levels of oxidative stress markers.

## 5.3    The assessment of different methods to measure plasma markers of oxidative stress burden

5.3.1 Methods

Once the different types of method had been identified, a kit was selected for each method on the basis of practicality, cost and technical specifications. Products were compared from the major suppliers to this laboratory. 1) A glutathione/ reduced glutathione (Calbiochem;354103) assay was chosen as an assay of a single component of the anti-oxidant systems; 2) Total anti-oxidant Status (TAS- Randox; NX2332) as an oxidation reaction based on ABTS, Metmyoglobin and Hydrogen peroxide and 3) AOP-490® (Oxis; 21052) as a reducing reaction based on copper ions.  Detailed methods for each kit are illustrated in chapter 2.  These methods were also compared to the Total Anti-oxidant Status (TOAS) method, based on ABTS, Horseradish peroxidase and

hydrogen peroxide, which was developed within this group from the method of *Sampson et al.*

The plasma used for these studies was provided by members of the group. Blood was collected into EDTA-containing tubes and centrifuged at 3500g for 8 minutes. The plasma was separated and stored at $-80^0$C in microtubes until use. The samples were stored by sample number only, no record was kept that would enable later identification of the sample. The inter-assay coefficient of variation (CV) was calculated by dividing the standard deviation by the mean and is expressed as a percentage. Inter-assay variability was calculated on the basis of one of these samples repeated in the plate up to 40 times. The correlation between the assays was calculated by assaying the same ten samples with each kit. A different aliquot was used for each kit and the assays were performed on the same day.

5.3.2 Results

5.3.2a Calculation of inter-assay variation.

The methods were all performed in accordance with the manufacturers instructions. However, for the TAS kit this required samples to be run individually in 1ml cuvettes. The method was modified to enable the use of the kit in a 96 well plate format by reducing the volume of reagents by 100. This method uses similar volumes to the TOAS assay. The dilution was tested by comparing the values obtained for the TAS control serum by both methods. There was no difference between the two samples (Fig 5.1). The expected value was 1.55 [1.16-1.94] mmol/L .The cuvette method assayed this sample as 1.44 [1.23-1.97], whilst the 96 well plate dilution method assayed this sample as 1.31 [1.25-1.37]. There was no significant difference between the two methods (p=0.86).

**Fig 5.1:** Comparison of the 96 well plate modified method (n=20) of using the TAS reagents with the manufacturers recommended cuvette method (n=8).
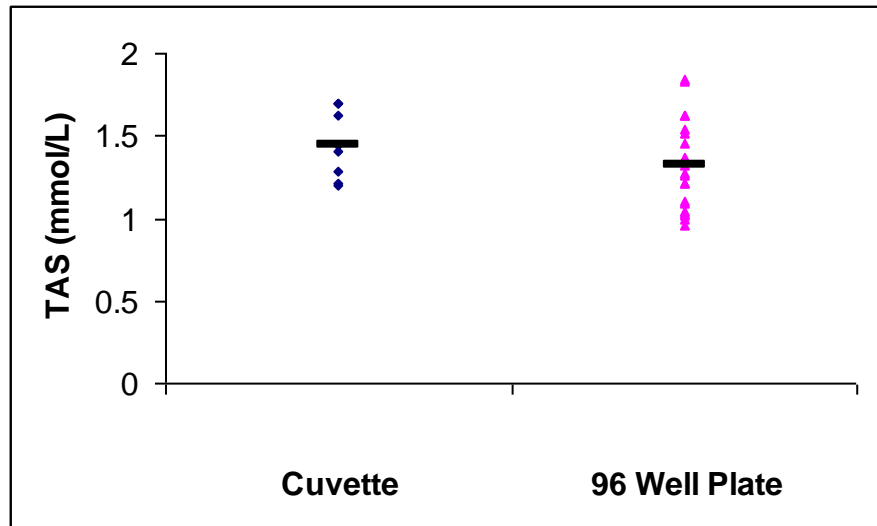


The inter-assay variation for the different methods is shown in Table 5.1. The TAS CV was measured in plasma and for the control serum provided. The Randox kit is supplied with more than one set of reagents. Measurement of TAS for the control serum supplied was repeated alongside the measurement of TAS in the plasma sample. The CV of the assay was lower when the second set of reagents was used (21% v 9%). The time taken to assay 40 samples was also estimated (ie samples in duplicate in a 96 well plate, including controls, blanks, etc). The CV for the TOAS was lower (4.1%) but the glutathione assay had the lowest (1.0%). However, this method required the longest time and the samples needed pipetting and preparation at collection.

**Table 5.1:** The interassay CV for the oxidative stress assays.

| Assay | Sample | N | Mean | Std Deviation | CV(%) | Time for 40 samples |
|-------|--------|---|------|---------------|-------|---------------------|
| TAS (mmol/l) | Control 1 | 20 | 1.31 | 0.28 | 21.2 | 15 mins |
| | Control 2 | 20 | 1.77 | 0.16 | 9.0 | |
| | Plasma | 14 | 1.04 | 0.09 | 9.5 | |
| TOAS(%) | Plasma | 14 | 53.3 | 2.20 | 4.1 | 25 mins |
| AOP-490 (mmol/L) | Plasma | 8 | 0.16 | 0.03 | 21.1 | 35 mins |
| Glutathione Ratio | EDTA Blood | 20 | 21.25 | 0.22 | 1.0 | 60 mins |

5.3.2b Relationship between different methods

A total of 10 volunteers had plasma taken on the same day and stored in separate aliquots for assay with each method. The samples were defrosted at the same time and run on the same plate. Linear regression was used to determine the relationship of the methods. There was no significant correlation between any of the methods (Table 5.2) with the highest value seen being between TAOS and the GSH/GSSG method (r=0.32, p=0.37).

**Table 5.2:** The relationship between different methods of measuring plasma markers of oxidative stress in 10 volunteers. (Pearson correlation)

| | AOP-490 | [p] | TAOS | [p] | TAS | [p] | GSH:GSSG |
|---|---------|-----|------|-----|-----|-----|----------|
| GSH:GSSG | 0.12 | [0.7] | -0.32 | [0.4] | 0.11 | [0.8] | 1 |
| TAS | 0.25 | [0.5] | -0.79 | [0.8] | 1 | | |
| TAOS | 0.16 | [0.7] | 1 | | | | |
| AOP-490 | 1 | | | | | | |

### 5.3.3 Discussion

The assays available for the determination of oxidative stress vary in their cost and ease of use. The difference in oxidative stress levels between subjects with different genotypes is likely to be small, and therefore the chosen method needs to be simple enough to perform on a large number of samples. The 'in-house' TOAS assay showed the least variability, but is the most familiar, and the effect of familiarity is likely to explain the significant improvement in TAS variability between the initial assay and the repeat assay of the provided control serum performed with the assay of the plasma sample. The glutathione assay requires pre-preparation of samples at the sampling site and this makes it unsuitable for large scale studies. The lack of variability may also reflect the fact that oxidized GSH in healthy volunteers is found at the lower level of detection for the kit, and this may reflect lack of ability to discriminate between samples rather than laboratory precision. The AOP-490® test would also be suitable, as the CV is likely to improve with increased familiarity. However, the much higher cost of nearly £1.65 per sample, compared with 22p for TAS and 5p for TOAS, made this impractical to perform in large scale genetic association studies.

The use of any combination of these methods would be desirable as they are detecting different spectrums of anti-oxidant activity, as indicated by the low correlation between them. Therefore, the combination of TOAS and TAS was chosen, even though the only difference in the two tests is the catalytic enzyme. The correlation of value in 10 healthy subjects was essentially negligeable (-0.08) and the spectrum of the anti-oxidant system that each is measuring appears likely to be sufficiently different for them to independently validate findings.

## 5.4 Plasma Oxidative stress markers in the Japanese American Family Study

### 5.4.1 Methods

A previously unthawed plasma sample was used for the plasma markers of oxidative stress. The assays were selected on the basis of the previous work in this chapter. Variables were transformed to normal where appropriate. Heritability was calculated using The Statistical Analysis for Genetic Epidemiology (SAGE) program and was carried out by Brendan Pierce in Washington, using an analytical strategy suggested by myself. Genotype was determined using Taqman© Technology as previously described in chapter 2.

### 5.4.2 Results

Serum was available for 490 subjects. TOAS was successfully measured in 476 subjects and TAS in 490, the discrepancy arises mainly due to the rejection of samples with significant haemolysis which TAS can correct for but TOAS cannot. The analysis was limited to the Nissei and Sansei generations due to the very small numbers in other generations; in total this excluded only four subjects. The characteristics of the subjects are shown in table 5.3. The Nissei generation was nearly thirty years older and as a result had higher blood pressure, lipids and rates of type 2 diabetes. The heritability calculation was based on these subjects recruited from 68 separate Japanese-American families as defined on page 100.

**Table 5.3:** Baseline characteristics of the Japanese American Family Study

|  | Total | | Nissei | | Sansei | |
|---|---|---|---|---|---|---|
| N= | 490 | | 215 | | 275 | |
| Gender [M/F] | 215/275 [43.8%] | | 95/130 [42.2%] | | 120/141 [46%] | |
| Age [years] | 54.5 | [16.5] | 69.7 | [8.8] | 41.6 | [7.9] |
| LDL [mg/l] | 116.9 | [31.2] | 120.6 | [32.8] | 113.75 | [29.6] |
| HDL [mg/l] | 54.0 | [14.9] | 54.5 | [15.5] | 53.6 | [14.6] |
| Triglycerides [mg/l] | 136.4 | [90.2] | 151.0 | [82.9] | 123.6 | [94.8] |
| Systolic BP [mmHg] | 122.0 | [19.1] | 131.1 | [20.7] | 113.9 | [12.9] |
| Diastolic BP [mmHg] | 76.1 | [10.2] | 77.5 | [9.9] | 74.9 | [10.3] |
| BMI [kg/m$^2$] | 24.5 | [3.7] | 24.7 | [3.6] | 24.3 | [3.9] |
| Smokers – Never/Ex | 276/153 | | 103/97 | | 170/56 | |
| Current | 40 [8.5%] | | 14 [6.5%] | | 25 [9.9%] | |
| Diabetes Y/N | 49/441[11%] | | 42/183 [18%] | | 7/254 [2.68%] | |

Mean [SD]

5.4.2a  Relationship between TAS and TOAS

Mean TOAS [SD] was 53.34% [8.5] and mean TAS was 1.36mmol/L [0.22].
TOAS was transformed to the square to normalize the distribution. There was a
weak but significant positive correlation between the two measures (r=0.076,
p<0.001). Therefore, only 7.6% of the variability in either marker can be
explained by a relationship with the other (Fig 5.2).

**Fig 5.2:** The relationship between plasma TAS and TOAS in the Japanese American Family Study
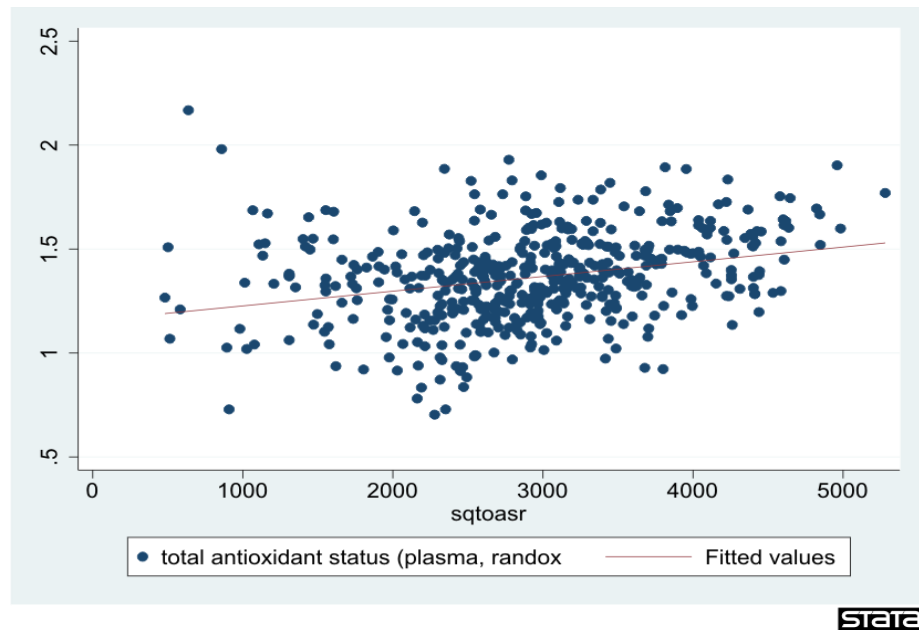


sqtoasr= √ Plasma TOAS

plasma randox = plasma TAS

5.4.2b Oxidative stress markers and biochemical characteristics

The mean TOAS (mean[SD] 54.6[30.8] v 53.3[28.2]: p=0.009) and TAS (1.41 [0.2] v 1.31[0.2]:p= 0.0001) were both lower in women than in men  (Fig 5.3) and TAS but not TAOS was lower in the Sansei generation (6%).  There were no differences for mean TAS or TOAS by Diabetes or smoking status [Table 5.4].

**Fig 5.3:** The mean of two markers of oxidative stress by gender in the Japanese American Family Study. (Mean [SE])

TOAS

TAS



P=0.009

P=0.0001
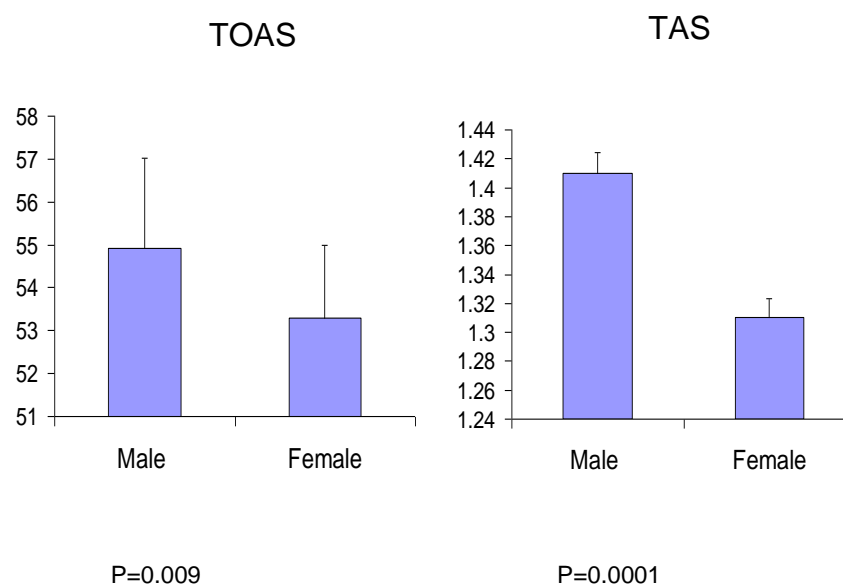
**Table 5.4:** The comparison of mean TOAS and TAS by baseline characteristics in the Japanese American Family Study.

|  | TOAS [%] |  | TAS [mmol/L] |  |
|---|---|---|---|---|
| Diabetes Y (n=49) | 52.81[29.96] | 0.35 | 1.37[.223] | 0.70 |
| N (n=441) | 54.15[32.61] |  | 1.36[.217] |  |
| Generation: Nissei (n=215) | 54.22[30.17] | 0.50 | 1.40[.211] | 0.0013 |
| Sansei (n=275 ) | 53.82[18.43] |  | 1.32[.218] |  |
| Smokers Current (n=40 ) | 53.79[27.55] | 0.92 | 1.33[.235] | 0.29 |
| Ex (n=153 ) | 54.21[30.28] |  | 1.38[.222] |  |
| Never (n= 276) | 53.95[29.10] |  | 1.34[.214] |  |

Mean [SD]

Simple linear regression showed a statistically significant correlation between TAS and triglycerides [ln], HDL cholesterol [Square], LDL cholesterol [Square root], age, Systolic Blood pressure (SBP[reciprocal]) and BMI. A significant

correlation with TOAS was only seen with triglycerides and systolic blood pressure. The relationship in all cases was weak, with the largest $R^2$ of 4% [Table 5.5]. Where a correlation was described with both TAS and TOAS, i.e. for triglycerides and SBP, this was in opposite directions, however, the correlation was weak in both cases and the line of best fit was close to horizontal.

**Table 5.5:** Correlation between oxidative stress markers and metabolic markers in the Japanese American Family Study.

| | TOAS | | | TAS | | | TAS V TOAS |
|---|---|---|---|---|---|---|---|
| | B | $R^2$ | p | B | $R^2$ | p | |
| Triglycerides | -0.11 | 0.03 | 0.0001 | 0.502 | 0.03 | <0.00001 | <0.00001 |
| HDL | 0.007 | 0.001 | 0.16 | -0.857 | 0.03 | <0.00001 | <0.00001 |
| LDL | 0.13 | 0.004 | 0.08 | 0.45 | 0.002 | 0.005 | 0.11 |
| AGE | 0.078 | 0.0004 | 0.37 | 14.81 | 0.04 | <0.00001 | 0.0001 |
| SYS BP | -0.010 | 0.007 | 0.04 | 0.274 | 0.003 | 0.005 | 0.01 |
| BMI | 0.13 | 0.001 | 0.53 | 3.32 | 0.04 | <0.00001 | 0.001 |

The ability of the two assays to measure the same signal was examined by comparing the distribution of the residuals. In all cases these were significantly different. In total 10 variables were examined and the two markers of plasma oxidative stress gave the same answer in 3 only. However, using best subsets regression [Minitab V14] to identify the best multiple regression model the same independent predictors were identified for both TAS and TAOS. The amount of variability in oxidative markers due to the five identified predictors [age, sex, Triglycerides, BMI and systolic blood pressure] was $R^2$ =7.0% for TOAS and 11.9% for TAS [Table 5.6].

**Table 5.6:** The best multiple regression model for both TOAS and TAS as identified by best subsets regression [Minitab v14] in the Japanese American Family Study

|  | TOAS | | TAS | |
|---|---|---|---|---|
|  | **B** | **P** | **B** | **p** |
| Trigs | -332.8 | <0.0001 | 0.05 | 0.009 |
| AGE | 9.14 | 0.001 | 0.003 | <0.0001 |
| SYS BP | -149.4 | 0.008 | -0.038 | 0.007 |
| BMI | 21.25 | 0.061 | 0.007 | 0.008 |
| Gender | -219.8 | 0.007 | -0.091 | <0.0001 |
| $R^2$ = | 0.0699 | | 11.93 | |
| P= | <0.0001 | | <0.0001 | |

5.4.2c Heritability

The familial correlation coefficients for both measures of total anti-oxidant status are shown in table 5.7. There was a significant correlation between parent-offspring and sibling pairs, indicating that there is likely to be some genetic determinant of plasma markers of oxidative stress. However, the highest correlations were between spouses [TAS 0.60 (0.39-0.81); TOAS 0.50 (0.25-0.50)].

**Table 5.7:** Familial correlations for two plasma markers of oxidative stress in the Japanese American Family Study.

|  | TAS (RANDOX) | | | TOAS (In-house) | | |
|---|---|---|---|---|---|---|
|  | Pairs | Correlation Coefficient (95% CI) | | Pairs | Correlation Coefficient (95% CI) | |
|  |  | Unadjusted | Adjusted |  | Unadjusted | Adjusted |
| **Spouse pairs** | 41 | 0.60 (0.39, 81) | 0.58 (0.37,0.79) | 38 | 0.50 (0.25,0.75) | 0.50 (0.25,0.75) |
| **Parent : offspring** | 287 | 0.28 (0.11,0.45) | 0.29 (0.13,0.45) | 266 | 0.26 (0.09,0.43) | 0.27 (0.10,0.44) |
| **Sibling** | 374 | 0.36 (0.20, 52) | 0.32 (0.16,0.48) | 337 | 0.30 (0.14,0.46) | 0.29 (0.13,0.45) |

The genetic variance component [SEM] for TAS was 0.03 [0.0045] and for TAOS was 31.41 [6.98]. After adjustment for age and gender the genetic variance component for TAS was 0.02 [0.004] and 33.35 [7.50] for TOAS. The

heritability estimates for the TAS kit and in-house TOAS were 0.56 [p<$10^{-7}$] and 0.48 [p<$10^{-7}$] respectively.  When adjusted for age and gender these results were essentially unchanged [TAS= 0.54, [p<$10^{-7}$] and TOAS = 0.49 [p<$10^{-7}$]; Fig 5.3].    There was also a significant variance component due to the marital relationship, although less than the genetic component [TAS 0.02 [0.003]; TOAS 27.23 [7.39] and TAS 0.02 [0.003]: TOAS 33.90 [7.76] adjusted for age and gender.   The lower heritability for TOAS may be in part due to estimation of a random variance component  for TOAS 7.25 [9.01] not seen for TAS although this was not significant [p>0.05] when adjusted for age and gender (0.18[9.44]).

**Fig 5.4:**    The heritability of two plasma markers of oxidative stress in the Japanese American Family Study.
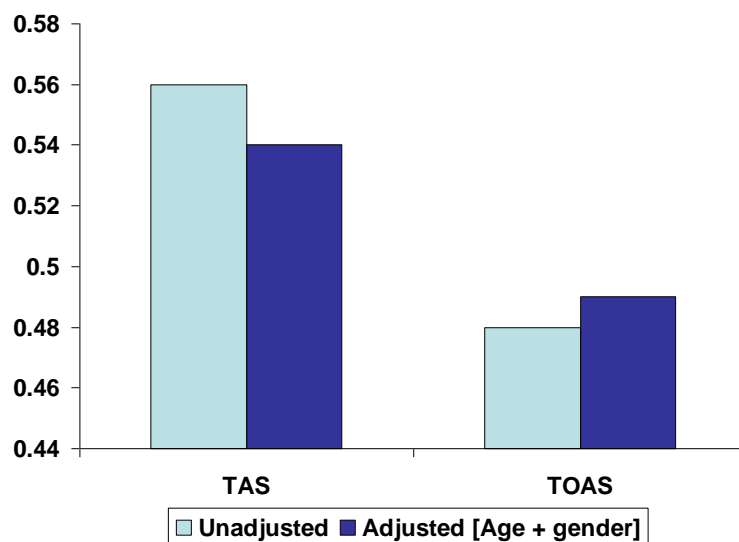


All estimates p<$10^{-6}$

5.4.2d *UCP2-UCP3* genotype and markers of oxidative stress.

*UCP2-866G>A* and *UCP3-55C>T* genotyping was restricted to these subjects in whom plasma was available.   Genotype was obtained in 94% and 95% of subjects for *UCP2-866* and *UCP3-55* respectively.  Genotype frequencies were as predicted from Hardy Weinberg proportions [Table 5.8].   There was no difference in genotype frequencies by generation [p>0.05].

**Table 5.8:** *UCP2 -866G>A* and *UCP3-55C>T* genotype frequencies in the Japanese American Family study.

| UCP2 | GG | GA | AA | Undetermined |
|---|---|---|---|---|
| Total | 142 | 224 | 102 | 12 |
| Ninsei | 63 | 106 | 49 | 2 |
| Sansei | 79 | 118 | 52 | 9 |
| **UCP3** | **CC** | **CT** | **TT** | **Undetermined** |
| Total | 201 | 208 | 52 | 18 |
| Ninsei | 93 | 93 | 26 | 8 |
| Sansei | 108 | 115 | 26 | 9 |

There were no significant differences in any of the baseline characteristics by either genotype [Table 5.9].

**Table 5.9:** Baseline characteristics by genotype in the Japanese American Family Study.

| UCP2 | GG | GA | AA | |
|---|---|---|---|---|
| Age | 52.3 [16.2] | 55.0 [16.1] | 56.4 [16.7] | 0.11 |
| Triglycerides | 132.0[91.1] | 142.48 [96.8] | 136.0 [78.3] | 0.40 |
| HDL | 53.9 [14.6] | 54.0 [15.0] | 52.85 [15.6] | 0.58 |
| LDL | 113.1 [31.4] | 117.3 [31.2] | 121.1 [31.88] | 0.07 |
| BMI | 24.44 [3.75] | 24.69 [3.87] | 24.51 [3.46] | 0.79 |
| SYS BP | 120.7 [17.45] | 123.6 [20.2] | 122.3 [19.0] | 0.67 |
| Gender M:F | 52/50 | 99/125 | 59/83 | 0.33 |
| Nissei/Sansei | 49/52 | 106/118 | 63/79 | 0.52 |
| Diabetes N/Y | 89/13 | 203/21 | 127/15 | 0.64 |
| SMOKING curr/Ex/Never | 3/39/54 | 25/67/123 | 9/44/84 | 0.07 |
| UCP3 | CC | CT | TT | |
| Age | 53.8 [16.1] | 54.4 [16.8] | 55.6 [14.8] | 0.76 |
| Triglycerides | 131.5[85.7] | 142.54 [95.1] | 147.1 [98.8] | 0.53 |
| HDL | 5.41 [15.2] | 52.9 [15.0] | 51.75 [13.6] | 0.17 |
| LDL | 115.9 [32.6] | 116.8 [30.6] | 121.5 [31.28] | 0.56 |
| BMI | 24.11 [3.54] | 24.92 [4.01] | 24.81 [3.29] | 0.08 |
| SYS BP | 122.2 [20.32] | 121.2[18.1] | 126.9 [17.8] | 0.06 |
| Gender M:F | 84/117 | 90/119 | 28/24 | 0.29 |
| Nissei/Sansei | 93/108 | 93/115 | 26/26 | 0.81 |
| Diabetes N/Y | 179/22 | 189/20 | 46/6 | 0.84 |
| SMOKING curr/Ex/Never | 18/53/121 | 15/73/110 | 4/21/26 | 0.24 |

Mean [SD]: Units as in Table 5.3.

There was no relationship between *UCP2-866* genotype and either TOAS [GGvGAvAA: %[SD] : 54.4[29.0] v 53.8[29.0] v 54.0 [30.6];p=0.40] or TAS [mmol/l [SD]: 1.35[.22] v 1.35[.21] v1.38[.22]; p=0.32]. There was also no relationship between *UCP3-55* genotype [CCvCTvTT] and either marker of oxidative stress [TOAS. 54.0[29.6] v 53.8 [28.7] v 54.6 [30.5];p= 0.47: TAS 1.35[.22] v 1.35 [0.21] v 1.40 [0.22]; p=0.24] [Fig 5.4].

Best subset regression analysis has previously identified the best predictive model for both the markers of oxidative stress contained the same five variables. The addition of the genotype data to this model [Table 5.10] made no significant difference in the predictive value of the model if the genotype data was added separately [Model 2 = addition of *UCP2* genotype; Model 3 = addition of *UCP3* Genotype] or combined [Model 4 = Addition of both *UCP2* and *UCP3* genotype]. The largest effect seen was the inclusion of *UCP2* genotype to the TAOS model increased the $R^2$ from 6.99% to 7.5% (p=0.66) There is no association with variants in the *UCP2-UCP3* gene cluster with plasma markers of oxidative stress in the Japanese American Family study.

**Table 5.10:** Multiple regression models and determinants of plasma markers of oxidative stress in the Japanese American Family Study.

| TOAS | Model 1 B | p | Model 2 B | p | Model 3 B | P | Model 4 B | p |
|---|---|---|---|---|---|---|---|---|
| Trigs | -332.8 | <0.0001 | -345.4 | <0.0001 | -349.5 | <0.0001 | -380.7 | <0.0001 |
| AGE | 9.14 | 0.001 | 9.77 | 0.01 | 9.58 | 0.001 | 9.07 | 0.002 |
| SYS BP | -149.4 | 0.008 | -148.6 | 0.03 | -146.9 | 0.009 | -133.4 | 0.021 |
| BMI | 21.25 | 0.061 | 21.39 | 0.10 | 21.11 | 0.067 | 21.98 | 0.059 |
| Gender | -219.8 | 0.007 | -224.2 | 0.01 | -221.4 | 0.008 | -180.4 | 0.034 |
| *UCP 2* | | | | | | | | |
| GA | | | -61.9 | 0.54 | | | -18.6 | 0.86 |
| AA | | | 14.6 | 0.89 | | | 106.7 | 0.42 |
| *UCP 3* | | | | | | | | |
| CT | | | | . | 0.25 | 0.99 | 49.71 | 0.60 |
| TT | | | | | 119.4 | 0.37 | 178.4 | 0.23 |
| $R^2$ = | 0.0699 | | 0.075 | | 0.0697 | | 0.0705 | |
| P= | <0.0001 | | <0.0001 | | <0.0001 | | <0.0001 | |
| V Model 1 | | | 0.66 | | 0.64 | | 0.67 | |

| TAS | Model 1 B | p | Model 2 B | p | Model 3 B | P | Model 4 B | p |
|---|---|---|---|---|---|---|---|---|
| Trigs | 0.05 | 0.009 | 0.46 | 0.19 | 0.05 | 0.011 | 0.45 | 0.023 |
| AGE | 0.003 | <0.0001 | 0.003 | <0.0001 | 0.003 | <0.0001 | .0031 | <0.0001 |
| SYS BP | -0.038 | 0.007 | -0.332 | 0.19 | -0.040 | 0.006 | -0.368 | 0.012 |
| BMI | 0.007 | 0.008 | 0.007 | 0.008 | 0.007 | 0.009 | 0.007 | 0.009 |
| Gender | -0.091 | <0.0001 | -0.85 | <0.0001 | -0.081 | <0.0001 | -0.828 | 0.000 |
| *UCP 2* | | | | | | | | |
| GA | | | -0.26 | 0.31 | | | -0.02 | 0.45 |
| AA | | | -0.003 | 0.89 | | | 0.009 | 0.78 |
| *UCP 3* | | | | | | | | |
| CT | | | | | -0.0002 | 0.99 | 0.009 | 0.69 |
| TT | | | | | 0.32 | 0.35 | 0.38 | 0.32 |
| $R^2$ = | 11.93 | | 11.12 | | 10.79 | | 10.59 | |
| P= | <0.0001 | | <0.0001 | | <0.0001 | | <0.0001 | |
| V Model 1 | | | 0.49 | | 0.61 | | 0.63 | |

5.5 Discussion

The examination of plasma markers of oxidative stress in the Japanese American Family study confirmed the observation in the kit comparison work that the two methods show a low correlation even though they use similar reactions. This is likely to be due to differences in affinity of the components of the anti-oxidant system and the enzyme involved, in this case horseradish peroxidase and metmyloglobin peroxidase. This difference has led some authors (Block G,2006: Schlesier K, 2002) to suggest that two markers should be reported to confirm the effect of a studied variable on plasma markers of oxidative stress.

The use of this strategy in the Japanese American Family study was, in part, successful. A high heritability was seen for both TAS and TOAS markers and familial correlations suggested that some of this may be in part due to similar diet and environment, as the highest familial correlation was seen in spouse pairs. This high level of heritability is in keeping with a previous study using an ABTS and metmyoglobin based method (Wang XL,2001). This study was also a family study with a Mexican American sample, although they had not yet developed cardiovascular disease. In subjects with a higher environmental oxidative stress burden, a higher percentage of the unexplained variation in TAS was explained by genetic factors. The slightly higher estimate of heritability for the Japanese American Family study may be in part due to the higher oxidative stress burden increasing the genetic influence.

However, the comparison of correlation with metabolic parameters recorded was weak and not consistent between the two methods. The failure to demonstrate likely associations with smoking and diabetes status may be due to study power or the use of therapy for primary or secondary prevention, which would be expected to lower oxidative stress burden. Targets for blood pressure and lipid lowering therapy are more demanding in patients with type 2 diabetes, and a number of these drugs, including statins, aspirin and ace inhibitors have been shown to reduce the oxidative stress burden (Ceriello A, 2006). The lack of association with oxidative stress markers and smoking is based on only forty

[8%] current smokers and no stratification of amount of smoking was included in the study or an assessment of how recently participants had become ex-smokers. These factors would weaken the strength of an association present and may explain why none was found. There was also no stratification of severity of diabetes to indicate how much hyperglycaemia is present, as it is this that is likely to be associated with plasma markers of oxidative stress per se. The plasma marker of oxidative stress has been associated with diabetes medication but not the diagnosis of diabetes, suggesting that in this case that diabetes medication is acting as a surrogate for diabetes severity. This is the fourth study across a variety of samples selected from Finland (Alho H,1999), China (Woo J, 1997) and Mexican Americans (Wang XL,2001) that describes lower plasma markers of oxidative stress in women despite lower risk of cardiovascular disease. Lower levels of anti-oxidant capacity in women despite lower risks of coronary artery disease may be due to the protective effect of oestrogen being through anti-oxidant components of low affinity with the assays used, or through other mechanisms. In men the anti-oxidant levels present may have been stimulated to higher levels given an increased burden of oxidative stress. This paradoxical difference is worthy of further study.

Multiple regression demonstrated the best model for predicting oxidative stress was based on the five variables of triglycerides, age, systolic blood pressure, BMI and female gender. However, the variation in these factors only explained 11.9% of the variation in TAS and 7.0 % of TAOS suggesting that other non-measured factors are important. These are likely to be diet and exercise, alcohol consumption and use of medication.

The addition of genotype data did not improve the model and mean TAS or TOAS did not differ by genotype, including with the use of a recessive model. There are a number of possible reasons for this.

This is a family study and we have made no allowance for the fact that many of the subjects share a relationship with others as this would have also reduced the power of the study. The *UCP2 -866A* variant is known to function differently under different conditions (Oberkofler H, 2005) and the effect on plasma

markers of oxidative stress is greater when there is a higher environmental load of oxidative stress production. Forty three percent of the sample is from a younger generation with lower blood pressure and less adverse lipid profiles as well as low rates of diabetes. Under this low burden the differences in function between the two alleles may be too small to detect. We have used two different plasma markers of oxidative stress and they have correlated poorly with each other and with adverse metabolic markers. These markers in effect measure what is left of the anti-oxidant systems in plasma as a surrogate for the production of ROS. Uncoupling proteins are found in the mitochondrial membrane and changes in mitochondrial oxidative stress may have significant effects on mitochondrial function without detectable changes to other areas of the cell of the extracellular matrix. This will make the differences between genotype undetectable using this method at early stages in the disease process, whilst it is possible to detect them later such as when cardiovascular disease has developed (Dhamrait, 2004). The number of rare homozygotes is low, especially for the *UCP3-55C>T* variant with only 52 (10.6%) rare homozygotes in the study. This current sample size gives only a 65% percent chance of detecting an association at 80% power [p=0.05] based on these means.

There is also the possibility that these variants do not behave in the same way in subjects of Japanese descent. Although, the frequency of the UCP2-866A allele is consistent across a number of European samples [0.30-0.40] as well as the Middle East [Iran (Akrami SM, 2007)], North India (Rai E, 2007) and Taiwan (Wang TN, 2007) the rare allele frequency in other ethnic groups including Korean (Yoon Y, 2007) and Japanese samples (Ji Q,2004) has been consistently higher [0.45-0.50]. In these samples the -886A has not been associated with type 2 diabetes although it has been found in association with hypertension and triglycerides as well as lower weight loss on a low calorie diet. The frequency of the rare allele differs between the Northwick Park Heart Study and the Japanese American Family study [0.37 v 0.46;p<0.01]. The relationship between *UCP2* genotype and plasma markers of oxidative stress was reported in a sample from the Northwick Park Heart study (Dhamrait S, 2004) and such an association may not be present in Japanese Americans.

The *UCP3-55T* has been less widely studied in Japanese samples and there seems to be no difference in rare allele frequency between European and Korean samples. However, the rare allele frequency in the Northwick Park Heart Study is lower than the Japanese American Family Study [0.22 v 0.34; p<0.05] suggesting that the populations are distinct at the *UCP2-UCP3* gene cluster. Further work is required to determine which are the important variants in samples of Japanese ancestry are and to determine if variation at the UCP2-*UCP3* also is associated with type 2 diabetes.

In summary, plasma markers of oxidative stress are highly influenced by genetic variation and this may explain the failure of anti-oxidant interventions in clinical settings. Environmental influences on plasma markers of oxidative stress are poorly understood and expected factors, such as diagnosis of diabetes, are not always associated with changes in plasma markers of oxidative stress. The finding of an association with variation in the *UCP2-UCP3* gene cluster was not replicated in a sample of Japanese descent.

**CHAPTER SIX**

**FUTURE WORK – DEVELOPING A MODEL TO STUDY GENETIC INFLUENCES ON THE MODIFICATION OF OXIDATIVE STRESS**

# CONTENTS OF CHAPTER SIX

## 6.1    The study of genetic influences on the modification of oxidative stress

### 6.1.1 Introduction

Genes that modify oxidative stress, either through the augmentation of anti-oxidant defenses, or through promoting reactive species production, would be potential candidates for being important in the aetiology of type 2 diabetes or cardiovascular disease or both.   Gene association studies that identify such candidates do not give information on the direction of causality or on the potential mechanisms. Further investigation is required to demonstrate that the gene variant is associated with a change in gene function.   Investigation of the pathophysiological effect of this change in function can give insight into the mechanisms of these complex disorders and open up new therapeutic windows.   The evidence from previous chapters suggests the *UCP2-866G>A* and *UCP3-55C>T* variants are good examples of variants with an influence in gene function, and that both are associated with complex diseases and are associated with differences in markers of oxidative stress.   The investigation of how individuals with different variants handle an oxidative stress load would give us further understanding of their role in health and disease.

### 6.1.2  Aims

To develop an experimental model that will enable the study of the effect of genetic variation on oxidative stress burden.    The differences between genotype are expected to be small, therefore, the number of subjects required to demonstrate a difference between two genotypes would require a model that is easy to perform on a number of subjects.

## 6.2 Oral Glucose Tolerance Test

### 6.2.1 Methods

An oral challenge was thought to be the simplest and most practical way of inducing an oxidative stress burden. Oxidative stress markers provide the most efficient way of assessing this. A glucose load has previously been shown to induce an increase in markers of oxidative stress (Sampson MJ,2002). Volunteers were recruited from the group and staff of the department of diabetes and endocrinology (UCLH). A 75g oral glucose tolerance test was performed, with the glucose administered in the form of lucozade. Plasma was sampled at 10 minute intervals for 3 hours to study the glucose and oxidative stress [ROS] response further, and identify an ideal time point for sampling when comparing genotypes.

### 6.2.2 Results

A total of 10 volunteers, age range 25-57 attended for the study. Fasting glucose was 5.2 mmol/l (range 4.6-6.5mmol/l) and peaked at 20 minutes (8.4mmol/l[6.1-12.6]) returning to baseline at 20 minutes (Fiq. 6.1).

**Fig 6.1:** Mean glucose in healthy volunteers over 3 hours following a 75 g oral glucose load
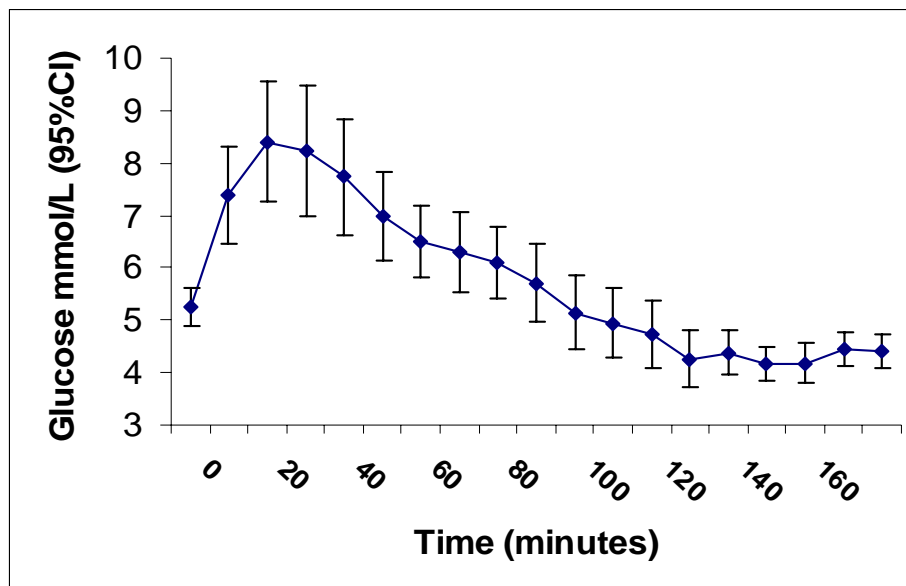
There was no association between time and plasma TAOS (Kendalls Tau test p=0.82) (Figure 6.2)

**Fig. 6.2:** Plasma TAOS over 3 hours after a 75g oral glucose load in healthy volunteers.
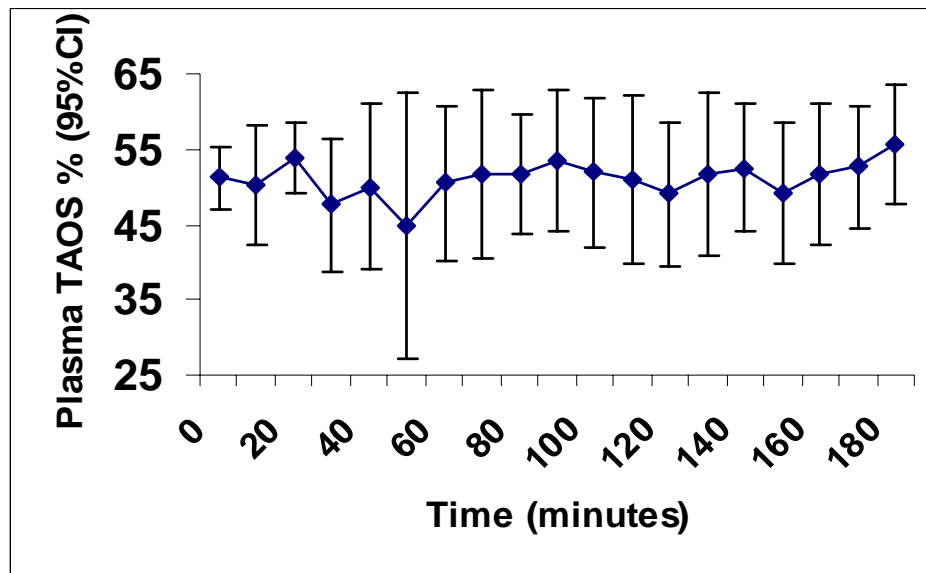


To determine if the failure to demonstrate a change in markers of oxidative stress was due to the marker being used, a subgroup of subjects (n=3) were used to measure $F_2$-isoprostanes by gas chromatography-mass spectrometry before and after *in-vitro* stimulation with SIN-1.  This is a peroxynitrate releasing compound generating superoxide and nitric oxide which stresses the anti-oxidant components of the plasma and will generate further $F_2$-isoprostanes, especially if the anti-oxidant components have already been depleted by a recent challenge (Ferraro B,2003).  There was no change in $F_2$-isoprostanes over time (Fig. 6.3) in the first three samples and no further subjects were assayed.

**Fig. 6.3:** Plasma $F_2$-isoprostanes at baseline and after SIN-1 exposure at 20 minute intervals over 3 hours after 75g oral glucose load in healthy volunteers.
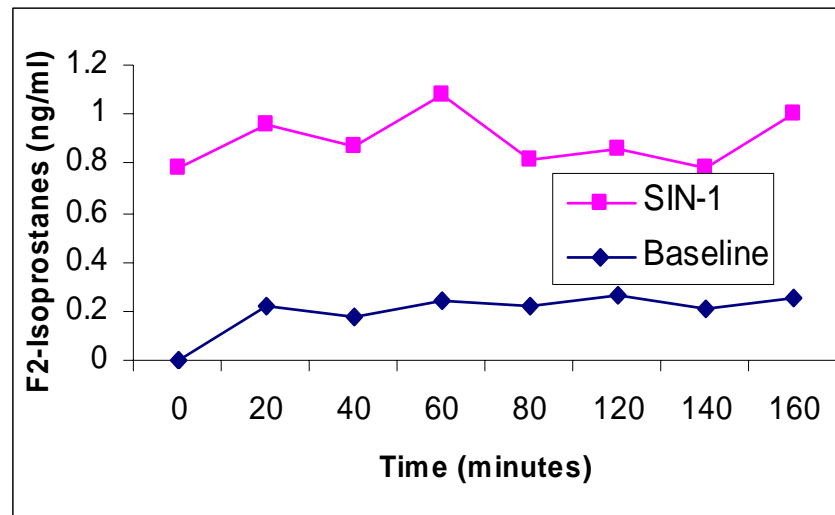


6.2.3 Discussion

The oral glucose tolerance test did not induce a measurable change in oxidative markers in healthy volunteers.  This challenge would, therefore, not be useful to study genetic differences.   There are a number of reasons why the oral glucose tolerance test failed to induce a change.   The age range of the volunteers was wide but the majority were young (mean 34.4) and a number undertake regular exercise (n=5[50%]).  Although, after exercise there are higher levels of oxidative stress, exercise also stimulates the expression of anti-oxidant defenses, and after repeated bouts of exercise the anti-oxidant systems adapt, with higher *de novo* synthesis of anti-oxidant defences, so these individuals may not show increased oxidative stress under this stimulus (Ji LL,2002). This also means that they are likely to be relatively insulin sensitive, and the peak glucose, and the area under the curve for the glucose excursion would be low, so that the impact of the challenge would be less.  The oral glucose tolerance test has induced a change in $F_2$-isoprostanes in subjects with type 2 diabetes but the peak glucose in this study was 21.1mmol/L (Sampson MJ,2002).

The appearance of altered membrane components after oxidation takes about six hours. The shorter time course may mean that the altered cellular anti-oxidant components has not yet had time to equilibrate with plasma components and that this is why no difference was detected. The use of SIN-1 to stress the plasma should overcome this but this still made no difference (Ferraro B,2003).


**6.3    Meal rich in used cooking oil**

6.3.1 Methods

The failure to induce adequate oxidative stress with a glucose load led to the examination of a fat-based challenge. Review of the literature led to the selection of a meal rich in used cooking oil, which when given to healthy volunteers induced endothelial dysfunction. This was not induced when unmodified cooking oil was used, suggesting that used cooking oil provides the strongest challenge (Williams MJA,1999). The failure to show a difference in healthy volunteers may have been in part due to the failure to induce significant metabolic abnormalities. To overcome this, a sample of patients with type 2 diabetes was selected from the UDACS study. Subjects attended fasting and were given the meal as detailed in chapter 2. Blood was sampled every hour for four hours for glucose, NEFA and markers of oxidative stress. To improve the characterization of changes in anti-oxidant capacity it was decided to use more than one oxidative stress marker (Block G,2006).

6.3.2 Results

The baseline characteristics for the subjects that attended the fatty meal challenge are shown in table 6.1. The subjects are older than the previous study and show a wide range of metabolic derangement. They show a wide range of metabolic

control of both glucose and lipid parameters.  Baseline markers of oxidative stress also showed a wide range (TOAS 50%-70% and TAS 1.26-1.93 mmol/L).

**Table 6.1:** Baseline characteristics of the subjects in the fatty meal pilot study (n=9)

|  | Mean ± | Standard Deviation | Range |
|---|---|---|---|
| Age (years) | 62.5 | 6.1 | 54-74 |
| BMI (kg/m$^2$) | 26.6 | 4.5 | 21.7-32.6 |
| Glucose (mmol/l) | 10.3 | 5.3 | 7.2-24.1 |
| Hba1c (%) | 8.1 | 1.5 | 6.3-11.3 |
| HDL cholesterol (mmol/L) | 1.8 | 0.8 | 0.9-3.2 |
| LDL cholesterol (mmol/L) | 2.1 | 1.0 | 0.3-3.8 |
| Triglycerides (mmol/L) | 4.6 | 1.1 | 2.8-5.9 |
| NEFA (mmol/L) | 0.46 | 0.26 | 0.07-0.99 |
| Creatinine (mmol/L) | 100.7 | 60.4 | 61-258 |
| TAS (mmol/l) | 1.5 | 0.2 | 1.3-1.9 |
| TOAS (%) | 60.9 | 9.4 | 50.0-70.4 |

The meal contained 68g of carbohydrate, and the change in plasma glucose over the four hours is shown in fig. 6.4.  Glucose increased from a baseline of 10.3 mmol/l [SD 3.5] and peaked at 16.3mmol/l [5.5] at 3 hours.  The glucose was significantly higher than baseline throughout the study (p=0.05,0.006,0.004,0.02 at 1,2,3 and 4 hours respectively).  The changes in NEFA are shown in fig 6.5. NEFA levels were high at baseline, falling to significantly lower levels at t=60 and 120mins, reaching a nadir at t=120, with no significant change to levels following this time.

**Fig. 6.4:** Mean (± SEM) plasma glucose in subjects with type 2 diabetes after a meal rich in used cooking oil (N=9)



*p<0.05, p<0.001
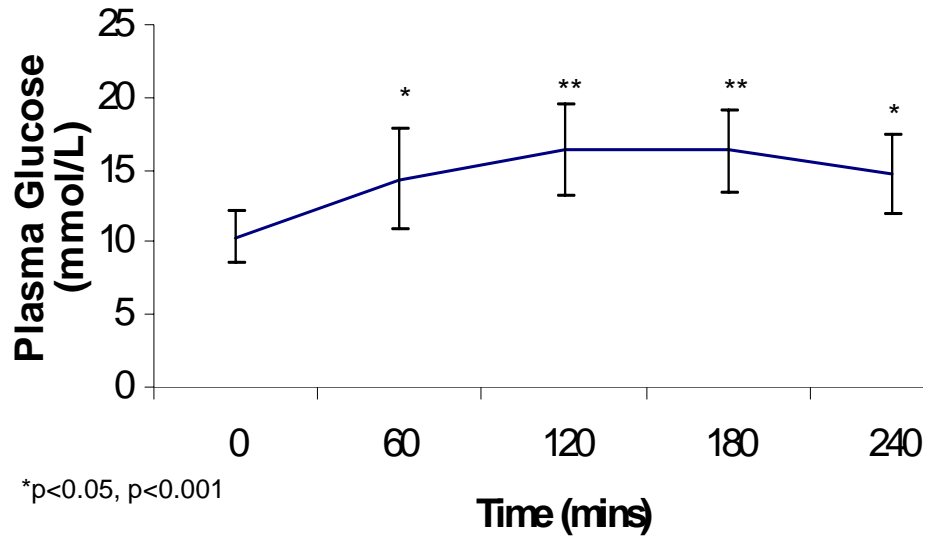
**Fig 6.5:** Mean (± SEM) plasma Non-esterified fatty acids in subjects with type 2 diabetes after a meal rich in used cooking oil
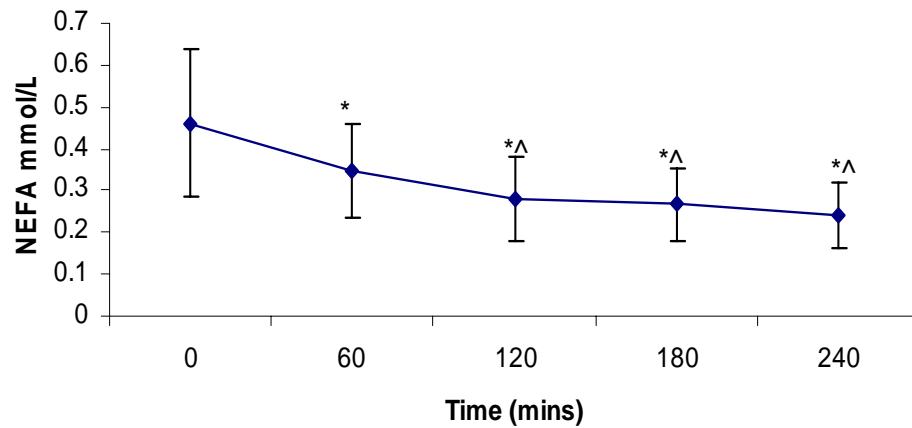


*P<0.05 v T=0,
(n=9) ^P<0.05 v T=60

There was a small decrease [5.4%] in plasma TOAS (Fig. 6.6) across four hours from (mean [SD]) 60.6% [9.4] to 57.4% [8.4]. This was not statistically significant (p=0.31 at 240 mins). TAS was also measured during the study (Fig.6.7). There

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was a significant reduction in plasma TAS of 13% at 2 hours (p=0.005) and 15% at 3 hours (p=0.13) from (Mean[SD]) 1.50mmol/l[0.19] to 1.30[0.14] and 1.27[0.15] respectively. TAS had begun to recover at four hours increasing to 1.37[0.19] although this was just still significantly different to baseline (p= 0.054).

**Fig 6.6:** Mean (± SEM) TOAS in subjects with type 2 diabetes after a meal rich in used cooking oil.
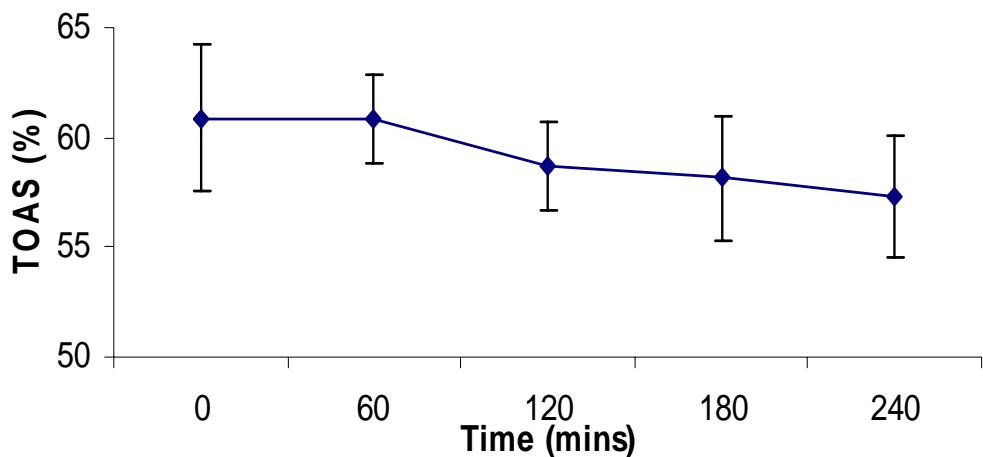


**Fig 6.7:** Mean (± SEM) TAS in subjects with type 2 diabetes after a meal rich in used cooking oil.
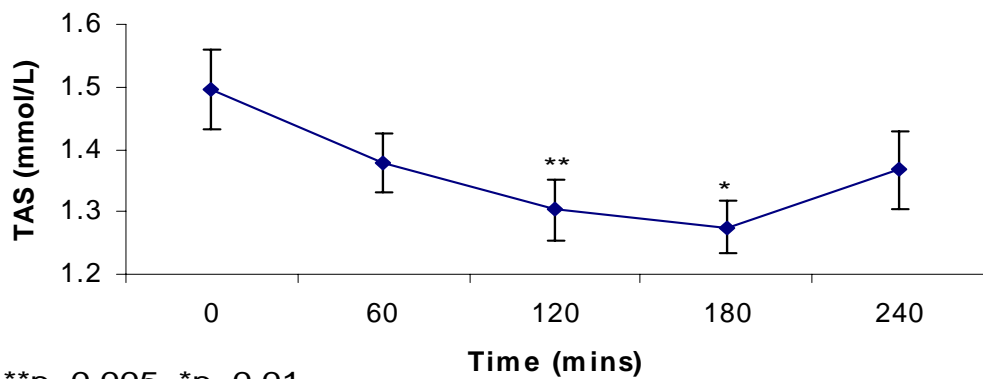


**p=0.005, *p=0.01

6.3.2 Discussion

The meal rich in used cooking oil did have an effect on oxidative stress markers when given to subjects with type 2 diabetes. There was a small reduction in plasma TOAS of 5% [p=0.31] and a statistically significant fall in TAS that was detectable at two hours and persisted for a further hour. This was associated with an increase in plasma glucose and a fall in plasma NEFA.

Plasma NEFA are known to fall immediately after a meal because insulin sensitive lipase activity is inhibited by post –prandial insulin secretion (Frayn KN,1994). The levels return to pre-meal levels after approximately three hours (Austin MA,2004b). The NEFA response to an oral challenge may vary with the fat content of a test meal (Jackson KG,2005), although the results of studies has been variable (Burdge GC,2006). This may have been because the early response does not differ between subjects and any differences are seen in the recovery phase (Burdge GC,2006).

This model is more promising in delivering a method that is capable of being used to study oxidative stress generation. The model is simple and could be limited to three hours for subject convenience. The method requires the availability of a source of used oil that has been exposed to similar cooking and temperature wear. This model could be used to study the influence of *UCP2* and *UCP3* variants on oxidative stress by comparing the magnitude of the change in oxidative stress markers between wildtype and variant homozygotes. However, the study is time consuming and on the basis of a difference in TAS at three hours of 1.49 mmol/L [SD 1.93] with a 25% difference between AA and GG homozygotes (Dhamrait SS,2004) a total of 385 patients per genotype would have to be recruited to give the study 80% power at $p<0.05$ to detect a difference between genotypes [www.stat.ubc.ca/~rollins/stats/sssize/N2.html]. This number of subjects would raise a number of practical difficulties. The number of patients to screen to identify the required number of rare homozygotes would be large. There would also be

difficulties in procuring a large volume of standardized oil. A commercial source would be inconsistent and the oil would have to be prepared in-house to a standard use protocol, and this prepared oil would also require further study to ensure it replicates the results obtained with this commercial oil preparation. The practicality of this study is limited by the ability to accurately measure acute change in ROS production. This type of study is only likely to become more practical once the measurement of oxidative stress improves.

The two methods of oxidative stress used are both based on the inhibition of an oxidative reaction by the plasma sample. The TAS assay is based in a commercial kit whilst the TOAS is based on in house reagents and this could produce differences in precision but the CV for both assays are similar [9.0 v 4.2 %]. The assays differ only in the enzyme used [Horseradish peroxidase in TOAS and peroxidase metmyoglobin in TAS]. This almost certainly explains that while both assays changed in the same direction only TAS reached statistical significance. There is no current data to suggest which components of the anti-oxidant defences show higher affinity with these enzymes but this does suggest that the used cooking oil does affect some components more than others and identification of these specific components could lead to a more specific assay of the change in oxidative stress burden with the used cooking oil and improve the power of the model to detect differences between genotype.

**CHAPTER SEVEN**

**DISCUSSION**

# Chapter 7 Discussion

## 7.1 Summary of Data

The role of modification of oxidative stress by uncoupling proteins 2 and 3 in the aetiology of type 2 diabetes was examined using variation in Uncoupling Protein 2 and 3 Gene cluster. A prospective study of over 3000 men confirmed the association of variants within the gene cluster with type 2 diabetes. A second study demonstrated that uncoupling protein 2 may play a role in insulin secretion. *UCP2* and *UCP3* gene variants were not associated with any conventional risk factors for type 2 diabetes in healthy subjects. Markers of oxidative stress can be used to indicate levels of oxidative stress burden, not all methods are suitable for large scale genetic studies. Two internally assessed methods were used to study heritability of oxidative stress burden in a family study and robust estimates of heritability were obtained. However, in this study *UCP2-UCP3* gene variants were not associated with levels of oxidative stress markers. Finally, a model was developed that suggests that oxidative stress markers could be used to measure genetic influences on ROS production.

### 7.1.1 Chapter 3, The Northwick Park Heart Study II

Subjects who developed T2DM also had higher blood pressure, triglycerides, cholesterol and C-reactive protein (CRP) at baseline, although only BMI, triglycerides and CRP were associated independently with increased risk. This study confirmed the significance of obesity as a major risk factor for diabetes, and also found a significant impact on risk of developing T2DM associated with variants in the genes for *UCP2* (-866A) and *UCP3* (-55T). The population attrtibutable faction at 10 years was 25.6% for obesity and 11.6% for genetic variation in the *UCP2-UCP3* gene cluster.

### 7.1.2 Chapter 4 The Hypercoagulability and Impaired Fibrinolytic Function Mechanisms Study

Data from this study confirmed previous studies in Europe showing that *UCP2-866* AA subjects are leaner. Secondly that AA subjects have lower fasting

plasma insulin levels.   In the healthy subjects the difference in fasting insulin levels persisted when adjusted for body mass index.   There was no other relationship between genotype and any of the adverse metabolic markers for either genotype.

### 7.1.3  Chapter 5 The Japanese American Family Study

The assays available for the determination of oxidative stress vary in their cost and ease of use.   Methods of measuring plasma markers of oxidative stress with very small differences in the assay reaction correlate poorly suggesting they measure different spectrums of the anti-oxidant system.   A high heritability was seen for both TAS and TOAS markers and familial correlations suggested that some of this may be in part due to similar diet and environment as the highest familial correlation was seen in spouse pairs. The comparison of correlation with metabolic parameters recorded was weak and not consistent between the two methods with independent associated factors explaining a low proportion of the variation on plasma markers of oxidative stress in both cases.

### 7.1.4  Chapter 6 The study of genetic influences on the modification of oxidative stress

An oral glucose tolerance test did not induce a measurable change in oxidative markers in healthy volunteers.   A meal rich in used cooking oil did have an effect on oxidative stress markers when given to subjects with type 2 diabetes. This was associated with an increase in plasma glucose and a fall in plasma NEFA.

## 7.2    Conclusions

### 7.2.1   Uncoupling Protein 2

These data suggest the association of the *UCP2-866 A* variant with type 2 diabetes in Caucasian men, in keeping with data from previous European samples.    This variant was also associated with a leaner phenotype in a number of previous studies and also in data from chapter 4.  The mechanism of action cannot, therefore, be through conventional risk factors for type 2 diabetes which are dominated by obesity (chapter 3).    Investigation of the UCP2 knockout mouse suggests that the mechanism of action may be through a direct effect on glucose dependant ATP production in the pancreatic β-cell itself.   A defect in insulin secretion is associated with the *UCP2-866A* (chapter 4), even when corrected for BMI.    This paradox has led to the description UCP2 as a "diabetes devil and adiposity angel" and suggests that *UCP2-886A* is associated with higher transcription, more energy use and less insulin secretion [Fig 7.1].

**Fig 7.1** Mechanism of action the *UCP2-866A* variant associated with higher transcription in the β-cell.



1)GLUCOSE ENTRY VIA GLUT 2

2)GLYCOYSIS

4)INSULIN SECRETION

3)ACTIVATION OF $K_{ATP}$ CHANNEL

HEAT    ATP

$H^+$

UCP2    ADP

$H^+$

**Increased UCP2**

**1 and 2. Reduced production of ATP from glucose**

**3.  Reduced potassium influx**

**4. Reduced insulin secretion**
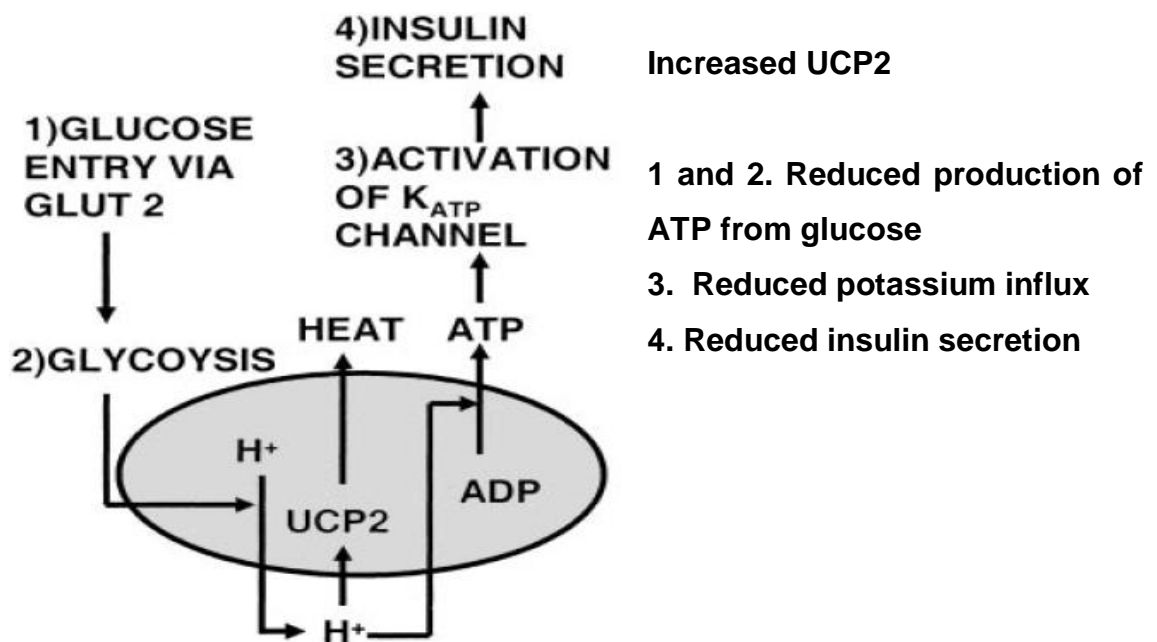
However, *UCP2-886A* has also been associated with higher plasma markers of oxidative stress and cardiovascular disease, an association also suggested in chapter 4. This would be in keeping with reduced transcription or at least reduced "uncoupling". This identifies a further paradox, where in certain conditions or organs the *UCP2-866A* variant behaves differently and supports the differences found in transcription factor binding. The regulation of the *UCP2* promoter is certainly tissue specific, with PAX-6 associated with higher transcription from the variant promoter in INSE-1 cells and lower in COS-7 cells. A model of *UCP2* regulation is shown in Fig 7.2.

The failure to find an association with oxidative stress in a Japanese-American Family study is not consistent with other studies. There are a number of reasons for this (Chapter 5).

**Fig 7.2** A model of tissue specific regulation of *UCP2*
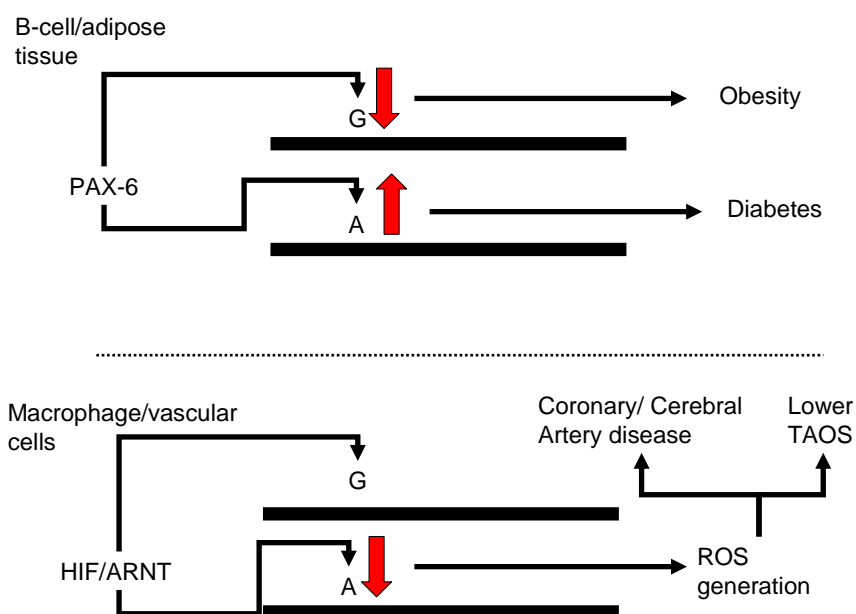
In summary, these data suggest that UCP2-*866AA* gene variant is accelerating the onset of type 2 diabetes by a direct effect on reducing insulin secretion in the β-cell of the pancreas.

## 7.2.2  Uncoupling Protein 3

Overexpression of *UCP3* in mice protects them from diabetes, with a 68% increase in mitochondrial palmitate oxidation.  Since, the *UCP3*-55T variant is associated with risk of diabetes in the men studied here (chapter 3) a decrease in function would be predicted for the T allele.  There is little data examining *UCP3* mRNA expression from different alleles.  The only study in 24 subjects suggested higher levels in CT/TT male non-diabetic Pima Indians, but this was based on only seven copies of the T variant from the total 48 alleles.  Clinical studies have been more consistent, with the association of the -55T variant with higher BMI in most but not all previous studies.   The association with type 2 diabetes of the *UCP3-55TT* has not previously been described although the variant has been described to be protective from type 2 diabetes in two French cohorts.  This could be because of sample size or timing.  The Kaplan-Meier plot suggests that the presence of the variant *accelerated* the onset of type 2 diabetes rather than being associated with new cases of type 2 diabetes.  This observation is important as it would be necessary to include this information in any model that used this variant to predict onset of type 2 diabetes.  It would also explain why genetic prediction is as yet yielding only small improvements above conventional risk factors (Humphries SE. 2007).

The association of the *UCP3 TT* genotype with type 2 diabetes must be explained through a mechanism not measured in the HIFMECH study (Chapter 4).   This includes most conventional risk factors including BMI, lipids and insulin secretion.   The physiological role of UCP3 is poorly understood but these findings are consistent with our current understanding of pathophysiology of type 2 diabetes with lower fat oxidation and reduced UCP3 protein found in subjects in type 2 diabetes.  Variation in *UCP3* was not found to be associated with two different plasma markers of oxidative stress (Chapter 5).  They are also

consistent with the *UCP3-55T* allele as the variant associated with reduced function.

In summary, these data suggest that the *UCP3-55C>T* variant accelerates the onset of type 2 diabetes by an as yet unidentified pathway that does not appear to affect other traditional risk factors for the development of type 2 diabetes. This is consistent with our current understanding of the role of UCP3 [Fig 7.2].

**Fig 7.3** Suggested mechanism of action the *UCP3-55T* variant associated with lower transcription .
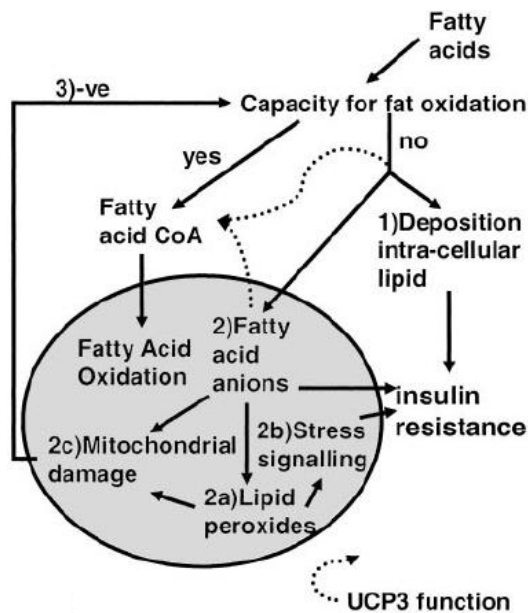


**Lower UCP3**

**1 and 2. Adverse consequences of fatty acid over supply**
**2a-c. Consequences of prolonged exposure to fatty acid anions**
**3. Further impairment if fatty acid oxidation and oversupply**

7.2.3 Oxidative Stress

Measuring plasma markers of oxidative stress offers the promise of helping to understand the pathophysiology of a number of human diseases including type 2 diabetes. However, as demonstrated (Chapter 5) there is a significant difference in methods even when the difference in the assay reaction is small. The heritability of two plasma markers of oxidative stress was high but as correlation between spouses was the highest the conclusion that there is a significant genetic influence on oxidative stress burden cannot be made with

confidence. The variability in oxidative stress burden does not, however, appear to be explained by variation in conventional adverse metabolic indicators, suggesting that the major determinants are either hereditary or environmental factors not measured such as diet.

When using plasma markers of oxidative stress to study disease the difference between samples needs to be large to detect a difference as demonstrated by the failure to detect a difference in a small sample (chapter 5) or a weak stimulus (chapter 6). Once, the stimulus is increased to an extent that significant metabolic excursions are seen then plasma markers of oxidative stress could be useful in identifying small genetic differences (Chapter 6).

## 7.3 Limitations of Study

The aims of this thesis were to use a genetic strategy to study the role of uncoupling proteins 2 and 3 in the pathogenesis of type 2 diabetes. The demonstration of increased prospective risk of type 2 diabetes in association with the *UCP2-UCP3* gene cluster variants studied was performed in Caucasian men recruited from nine General Practices throughout the UK. A prospective study is a strong study design but the definition of type 2 diabetes has changed over time and the use of medical records to find cases may reduce the number of men identified but this is only likely to weaken the association found.

The data from the HIFMECH study was collected from across Europe and the Japanese American family study from the United States. The study of gene and gene-environment interactions is unlikely to be consistent across all these groups, and even within the two distinct North and South Europe groups there were differences in the impact of the genotype on traits and possibly on cardiovascular heart disease risk. The conclusions of the study are also based on the assumption that type 2 diabetes is a homogeneous disorder and that genetic causes across these populations are similar.

The baseline characteristics in each study were also different and not measured at a central laboratory. The normal ranges in each study are likely to be slightly different. The HIFMECH study had more information on metabolic markers but the population was less homogeneous. The cross sectional nature means that it is not possible to draw causative conclusions about associations. The Japanese American Family study was useful for investigating the heritability of plasma oxidative stress markers but had little environmental information on which to examine further the results identified. This study was not designed to examine genotype association with baseline characteristics and is underpowered. The power of the study is further weakened by the presence of related individuals.

Plasma markers of oxidative stress still offer a very poor reflection of the underlying redox state of the cell, and probably more importantly the mitochondria. The replication of findings using two different markers is a useful technique but also exposes the inadequacy of current assays. These data all use a manual method to determine plasma markers of oxidative stress and, although the intra-assay CV did improve with time, it was still significantly higher than that quoted in the product literature using automated techniques.

The *UCP2-UCP3* gene cluster has not yet been identified as associated with type 2 diabetes in any of the Genome wide scans yet performed. The failure to replicate these findings could suggest that the standard of statistical proof applied is not sufficient for the association to be regarded as proven. The association of the *UCP2-886G>A* variant with oxidative stress has recently been shown to exhibit a significant gene environment interaction [Stephens JW, 2007]. These variants may not be acting across a whole population but only in sub groups such as smokers and these studies would then not have the power to detect the increased risk. The failure to replicate these findings could also be based on population selection, as the *UCP2-UCP3* gene variants accelerated onset of type 2 diabetes, so an appropriate time frame is required to identify the effect.

## 7.4 Future Work

The structure and function of the *UCP2-UCP3* gene cluster is still not well understood. There are a number of paradoxes related to the function of UCP2 and the rates of transcription of both mRNA in different organs and under different stimuli needs to be better understood. *UCP2* transcription should be studied at baseline and under oxidative stress in different cell types and under different transcription factors. The possibility of such organ and transcription factor specific modification of *UCP3* transcription should also be investigated. This will enable a better understanding of the function of these genes and add to the understanding of the role of mitochondrial dysfunction in type 2 diabetes.

The gene variants could currently be used in a genetic testing model based on healthy middle aged white men. If genetic testing using these variants is to have a wider application, the role in women and in different ethnic groups also warrants further study to determine if is the same as healthy Caucasian men. The ability to predict development of type 2 diabetes at different ages, coupled with other variants also requires further study. Genetic tests to predict type 2 diabetes can only be useful if they give value above conventional risk factors. There is evidence that this can be the case for cardiovascular disease, another complex polygenic disease, although this required the use of 6 candidate genes (Humphries SE, 2007). The use of genetic testing for prediction of type 2 diabetes using three variants did improve prediction slightly [ROC 0.58] in a British Caucasian sample (Weedon MN, 2006). The value of the addition of *UCP2-UCP3* gene cluster variants to this group warrants further study.

The rate of oxidative damage is likely to be as a result of interaction between genes and the environment. The methods to assess oxidative stress burden such as plasma markers of oxidative stress require further study before being accepted as useful surrogate markers of risk of type 2 diabetes, including prospective predictive power. A study which collects data on lifestyle is also required to see what in the environment predicts plasma markers of oxidative stress.

The model developed offers a promising method of investigating not only the role of uncoupling protein genes in modification of oxidative stress burden but also a number of other genes and also possible interventions.

This study also raises the possibility that mild pharmacological uncoupling or potentiation of UCP3 function could act as drug targets for the prevention and development of type 2 diabetes.

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