GENETIC SUSCEPTIBILITY TO TYPE II DIABETES AND OBESITY:

THE ROLE OF UCP2, UCP3 AND CAPN10 GENES

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ABSTRACT

The global prevalence of type 2 diabetes (T2DM) and obesity is increasing, with obesity the most important predisposing factor contributing to the development of T2DM. Epidemiological and genetic evidence supports a major genetic component in both multifactorial and heterogeneous disorders. The identification of disease susceptibility genes in humans could greatly assist in the elucidation of underlying pathophysiological mechanisms and allow the development of more effective preventative and therapeutic strategies for these conditions.

Three candidate genes, uncoupling proteins 2 and 3 (UCP2; UCP3) and calpain 10 (CAPN10), are proposed and the rationale for their selection discussed. Gene variants were identified in UCP2 and UCP3. These variants were tested for association with T2DM, obesity and intermediate quantitative traits in a South Indian population and family collection, and also a cohort of British obese case/control subjects. No variant was associated with T2DM. However, investigations revealed positive associations with a UCP2 3'UTR 45bp Ins/Del and a novel UCP3 promoter variant (-55C/T) with variation in body mass (BMI) and fat distribution (WHR) respectively. The results support the view that uncoupling proteins may influence weight gain and hence progression to obesity/T2DM. A significant correlation with plasma leptin levels and the UCP2 Ins/Del variant might indicate one potential mechanism whereby weight could be modulated by uncoupling proteins.

A linkage study in affected sibling pairs of North European descent, was negative for the putative T2DM susceptibility gene region, *NIDDM1*. In contrast, haplotypes of four sequence variants of a T2DM susceptibility gene (CAPN10) identified in this region positively associated with T2DM in a South Indian population.

In conclusion, these investigations provide evidence that the three genes studied may contribute to susceptibility for development of T2DM or obesity. However, the findings are in agreement with the most likely genetic model for non-Mendelian complex diseases, that many genes are involved in determining susceptibility to disease with no single gene capable of determining the overall disease phenotype.

I wish to dedicate this thesis to my wife Brigitte

and children Josephine and Marlene

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BBS	Bardet biedle syndrome
BMI	body mass index
bp	base pairs
BSA	bovine serum albumen
CAPN10	calpain 10
cDNA	complementary DNA
CEPH	Centre d'Etude du Polymorphisme Humain
cM	centimorgans
DMSO	dimethylformamide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
DZ	dizygotic (twins)
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FM	fat mass
FFM	Fat Free mass; lean mass
HLA	human leucocyte antigen
HWE	Hardy-Weinberg equilibrium
IGT	impaired glucose tolerance
IFG	impaired fasting glucose
Kb	Kilobase(s); kilobase pairs
LADA	latent autoimmune diabetes in adults
LD	linkage disequilibrium
MELAS	mitchondrial encephalopathy with lactic acidosis and stroke like episodes

Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
MODY	maturity onset diabetes of the young
mRNA	messenger RNA
MZ	monozygotic (twins)
NaCl	sodium chloride
NaOH	sodium hydroxide
OGTT	oral glucose tolerance test
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIC	polymorphic information content
PPAR	Peroxisome proliferator-activated receptor
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
RQ	respiratory quotient
RMR	resting metabolic rate
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
ssDNA	single stranded DNA
T2DM	type 2 diabetes
TBE	tris borate EDTA buffer
TDT	transmission disequilibrium test
TE	tris EDTA buffer
TEMED	N,N, N,N,-tetramethylethylenediamine
UCP	uncoupling protein
UKPDS	UK Prospective Diabetes Study
(v/v)	volume: volume ratio
WHO	World Health Organisation
WHR	waist hip ratio
(w/v)	weight:volume ratio

CHAPTER 1:INTRODUCTION

1.1 GLOBAL IMPORTANCE OF TYPE 2 DIABETES AND OBESITY

Diabetes is a disease affecting between 100-150 million people world-wide. Type 2 diabetes mellitus (T2DM), also know as non-insulin dependent diabetes, is defined by the World Health Organisation as the predominant sub-type of diabetes accounting for 97% of diabetic cases [Alberti et al 1998a]. T2DM is not only an important cause of morbidity in respect of microvascular, macrovascular and neurological complications, but is also associated with an increase in mortality and a 2-3 fold increase in risk to cardiovascular disease. Although the global prevalence of T2DM is approximately 2.1% [Amos et al 1997], there is a broad spectrum of disease prevalence with frequencies ranging from 2% to 6% in Caucasian populations and exceeding 30% in some ethnic groups.

Global prevalence is also increasing and this has been mainly attributed to increasing obesity as a result of changing dietary intake and life style. The acquisition of a 'Westernised' life style with a more sedentary existence through a reduction in physical activity and combined with high calorie dietary intake have been implicated in accounting for the increased prevalence of obesity [Helmrich et al 1991; Eriksson et al 1996; Zimmet 1992; Aspray et al 2000]. This is clearly reflected in the migration from and urbanisation of populations in developing countries. Urbanized and migrated South Indian populations having an increased prevalence of T2DM compared to rural populations following more traditional lifestyles (figure 1.1.1) [Ramachandran et al 1997a; Mckeigue et al 1991].

T2DM has a close relationship with obesity, atherosclerosis and hypertension, with the severity of the complications being proportional to the degree of obesity. The inverse is also true with dramatic decreases in disease frequency on reduction of obesity (50-75%) and increased physical activity (30-50%) [Manson & Spelsberg 1994; Houmard et al 1994]. Obesity is also associated with an increased mortality, morbidity and decreased quality of life especially amongst middle-aged and older individuals. In the USA more than half the US population over 20 years old are overweight and nearly a quarter are clinically obese in terms of BMI [Kuczmarski et al 1994]. The excessive intake of calories in the USA is second only to smoking as the greatest cause of mortality [McGinnis et al 1993]. The modern medical case against obesity began in 1959 with the publication of the Metropolitan Life Insurance Company tables. These showed that the risk of premature death elevated steadily as weight increased above a BMI of 22.0 in males and BMI of 21.5 in females. Eighty percent of Americans now exceed these levels. Furthermore, a prospective study of

115,000 nurses found mortality rate increased with progressive increase in BMI with the risk of death increasing by more than 100% for BMI's over 29 [Manson & Spelsberg 1994]. The future gravity of the situation is further exemplified in that 25% of children in the USA are already overweight or obese [Kuczmarski 1993]. The economic costs of T2DM and obesity to countries for treatment in health care systems are enormous. In addition about $40billion /annum is spent in the USA on weight loss treatments, mostly in the form of diets with little or no success.

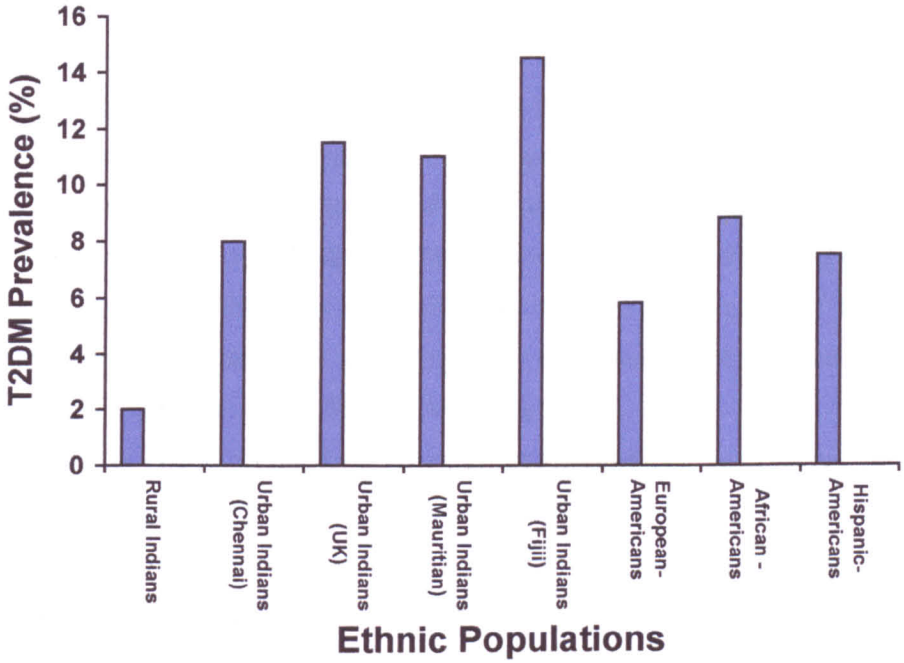

Figure 1.1.1: Urbanisation and migrational effects on the prevalence of T2DM

1.2 DISEASE PHENOTYPES

1.2.1 Type 2 diabetes mellitus

Diabetes mellitus is a complex metabolic disorder incorporating many syndromes. The phenotype is characterised by elevated glucose levels in the blood and urine. Glucose is excreted in the urine when the blood glucose levels exceed the re-absorptive capacity of the renal tubules. Water accompanies the excreted glucose, and so an untreated diabetic in the acute phase of the disease is hungry and thirsty. Diabetes mellitus was originally differentiated into two groups; those patients having autoimmune destruction of the insulin secreting pancreatic β-cells, and thus requiring an external supply of insulin, Insulin

Dependent Diabetes mellitus or type 1 diabetes (T1DM). Traditionally the remaining diabetic patients with no insulin requirement in the early stages of the disease, the non-insulin dependent diabetics were classified as T2DM.

T2DM patients are islet cell antibody negative and their hyperglycaemia may be initially controlled with diet and/or pharmaceutical hypoglycaemic agents but may eventually require insulin. Subjects tend to present in mid to old age (age 50-60 years), unless they are screened in pregnancy or present with another illness. T2DM patients may share a common final clinical phenotype of deteriorating glucose tolerance and associated diabetic complications, but this probably represents a heterogeneous group of diverse conditions. This view is supported by the identification of approximately 10% of T2DM patients with a primary autoimmune aetiology characterized by the presence of autoantibodies [Turner et al 1997]. In addition, other sub-groups exist where the diabetes is the result of single gene defects primarily affecting the β-cell, as with maturity onset diabetes of the young (MODY) [Froguel et al 1992; Yamagata et al 1996ab; Hattersley 2000].

Glucose tolerance is determined by the capacity of the pancreatic β-cell to secrete insulin in response to secretagogues and the ability of the secreted insulin to induce multiple actions in peripheral tissues, most importantly in skeletal muscle, liver and adipose tissue [Kahn & Folli 1993; Reaven 1993]. Although in MODY, a β-cell defect alone appears to be the primary cause [Taniguchi et al 1992; Arner et al 1991; O'Rahilly & Turner 1988b], patients with established T2DM display defects in both insulin secretion and insulin action [Taylor et al 1994]. Insulin resistance alone is not sufficient to cause diabetes and a complex relationship involving compensatory mechanisms exists between the two phenotypic characteristics [Bruning et al 1998; Garvey et al 1985; Lillioja et al 1993; O'Rahilly et al 1988a; Weigle & Goodner 1986]. As a consequence neither pathway can yet be defined as the primary defect of T2DM [Bergman 1997]. The heterogeneous nature of T2DM is possibly one of the reasons why the underlying physiological basis of T2DM still remains relatively unknown, with no biochemical markers presently capable of predicting future glucose intolerance.

1.2.2 Obesity

The measurement of Body Mass Index (BMI) is used clinically to determine normality of body weight and is calculated by dividing body weight by height squared (kg/m^2). Subjects are classified according to BMI as underweight <18.5; normal 18.5-24.9; overweight 25.0-

29.9; obesity ⩾30.0; morbidly obese ⩾40.0 [Katsilambros 2000]. The WHO has adopted 30kg/m^2 as the lowest limit for definition of obesity as the correlation between BMI and total body fat or percent body fat is only high in large and heterogeneous samples when BMI is above this level [Bouchard 1995].

However, BMI as a measure of body fat is not always entirely valid, as the measure of variance in percent body fat explained by BMI is only between 40% to 60% with a closer correlation at extremes of body fat content [Bouchard 1995]. Obesity is also a heterogeneous disease phenotype displaying a number of patterns of distribution of excess fat. The different phenotypes may be each modulated by a different set of causal factors. There is no unified classification of the individual obesity phenotypes based on heterogeneous clinical manifestations, although obesity can be sub-divided by body fat topography.

Patients can have peripheral obesity with anatomically excess fat deposited in the gluteal and femeral areas (lower body or gynoid- female type or subcuraneous-type). Alternatively they may have central obesity (upper body or male type or splanchnic or abdominal or intra-abdominal obesity) [Katsilambros 2000]. Endocrine abnormalities such as increased insulin levels, insulin resistance, cortisol levels, testosterone and androstenedione, and decreased growth hormone and progesterone are much more pronounced in the latter condition. However phenotypes are not fully independent as the level of covariation amongst the obesity types range from 30% to 50%. Fat distribution and age considerations are also of equal importance to overall increase in mass, this being particularly relative in ethnic groups where average body mass have not reached proportions seen in the westernised societies. The anthropomorphic measurement of waist-to-hip circumference ratio (WHR) positively correlates with abdominal visceral fat deposition, and upper body adiposity and is therefore a phenotypic indicator of regional fat distribution. However WHR is also confounded by a high degree of heterogeneity. Any studies both epidemiological and genetic are impeded by the limitations of the primary clinical diagnosis. Direct measurements of fat mass such as with bioelectrical impedience, rather than indirect/partial methods, allow indexes such as fat free mass (FFM) to be defined and allows a refinement in determining traits that influence body weight homeostasis.

1.2.3 Relationship between type 2 diabetes and obesity

Epidemiological evidence strongly supports a relationship between T2DM and obesity. The WHO in 1980 [WH0 expert committee 1980] cited obesity as the most important risk factor

in the development of T2DM with this association transcending ethnicity, differences in measurements of adiposity and criteria for diagnosis of T2DM [Prentice & Jebb 1995]. Progressively increasing body mass is positively correlated with both elevated fasting plasma insulin and impaired glucose tolerance (IGT), negatively correlated with whole body insulin sensitivity and therefore increases risk to T2DM [Kolterman et al 1980; Kissebah et al 1982]. This increased risk has been demonstrated in a number of ethnic groups including, Western Samoan [Collins et al 1994], Afro-Carribbean [McKeigue et al 1992], Asian Indian [Shelgikar et al 1991], Tanzanian [Aspray et al 2000] and South Indian populations [Ramachandran et al 1992].

However, other factors also contribute to the increased risk and modify the influence of adiposity, such as distribution of body fat, duration of obesity, fitness, ethnicity, and family history. Central adiposity is particularly associated with an increased risk to the development of both insulin resistance and diabetes [Bjorntorp1991]. Phenotypic patterns of fat distribution are determined by both ethnicity and gender and this may influence onset of disease. In South Indians fat distribution is far more important in influencing the prevalence of glucose intolerance than overall fatness and increased abdominal fat deposition found in females is associated with a lower efficiency in breaking down glucose [McKeigue et al 1992; Kissebah et al 1982].

Accordingly, weight loss markedly enhances metabolic control improving both insulin action in liver and muscle and restoring more efficient regulation of lipolysis, delaying the onset of diabetes [Viswanathan et al 1997; Reynisdottir et al 1995; Lonnqvist et al 1995].

Although a single metabolic aberration is unlikely to be sufficient to give rise to multiple functional defects that precede overt diabetes, the exposure of various organs to increased plasma lipids, specifically elevated free fatty acids (FFA) has been postulated to be a potential candidate [Reviewed by Bergman 2000]. The levels of FFA's are determined by the differences in the lipolytic activity of regional fat deposits with elevated FFA levels predictive for conversion from a state of impaired glucose tolerance to diabetes [Martin & Jensen 1991; Reynisdottir et al 1994; Coppack et al 1992; Charles et al 1997; Paolisso et al 1995; Krotkiewski et al 1983; Bjorntorp 1991]. The accumulation of fat deposits as a result of elevated plasma FFA in tissues such as muscle, liver and pancreatic β-cells may interfere with metabolic signalling pathways, including insulin, resulting in insulin resistance and impaired glucose utilisation [Nyholm et al 1999; Unger 1995; Campbell et al 1994; Peiris et al 1986; Baron 1994; Steinberg et al 1997 & 2000; Bergman 1997; Hamilton-Wessler et al

1999; Randle et al 1994; Baldeweg et al 2000; Boden 1997]. Although initially pancreatic β-cells could compensate for increased glucose levels by increasing insulin secretion, with acute exposure to elevated FFA levels stimulating insulin secretion [Steinberg et al 1997; Boden & Chen 1999]. Chronic hyperlidaemia can directly impair insulin secretion and the increasing levels of NEFA would further decrease glucose uptake by muscle, increase hepatic NEFA oxidation and stimulate gluconeogenesis eventually compromising β- cell function [Reaven 1995; Sako & Grill 1990; Unger 1995; Mason et al 1999].

However, alternative hypotheses have been postulated that implicate the disruption of the diurnal rhythm of cortisol by increased fatness in determining insulin resistance and increased risk to T2DM [Bjorntorp 1991].

Central obesity and associated risk factors such as hyperinsulaemia, hypertension and hypertriglyceridaemia, can also confer an increased population susceptibility to coronary heart disease [McKeigue et al 1992 & 1991; UKPDS1994]. The epidemiological observation of the frequent simultaneous occurrence of T2DM and obesity with a number of other associated phenotypes, including hyperinsulaemia, glucose intolerance, hypertension, dyslipidaemias and coronary heart disease suggests there is a relationship between these disorders [Reaven 1988]. This has given rise to the concept that these disorders constitute a single metabolic syndrome 'Syndrome X'. However, the evidence for this epidemiological association is not always consistent, [Jarrett 1992; Zimmet et al 1994] particularly between different ethnic groups [Saad et al 1991]. Finally, Neel et al has postulated that a BMI-hypertension interaction is more likely to drive this relationship rather than the existence of a Syndrome X [Neel et al 1998].

1.3 GENETIC EPIDEMIOLOGY

Evidence suggests that the development of T2DM is a result of both an interaction between diverse environmental factors on a background of heterogeneous genetic predisposition. Increasing body weight is the most important factor influencing progression to impaired glucose tolerance and T2DM, with body weight homeostasis and obesity both themselves determined by genetic and environmental components, some possibly the same as for diabetes. The complexity and mechanisms involved in the gene-environment interaction and the mode of inheritance of both T2DM and obesity are still largely unknown. The heterogeneity of the diseases at phenotypic and pathophysiological levels almost certainly

indicates that the genetic component is likely to be heterogeneous with no single locus accounting for the disease.

1.3.1 Segregation Analysis

Segregation analyses, the inference of susceptibility loci from the distribution of phenotypic variance in pedigrees, have been utilised to attempt to understand the genetic model and mode of inheritance of both T2DM and obesity. Early segregation analyses using simple correlation methods failed to take into account factors such as age related prevalence and environmental effects and in consequence a variety of genetic models were proposed. Later analyses utilising path or variance components have facilitated the generation of models that attempt to breakdown observed familial aggregation into separate genetic and environmental effects or heritabilities. Although relatively few formal segregation studies have been performed on T2DM, these studies have suggested the presence of major genes for quantitative traits related to diabetes. Several studies have proposed that an autosomal recessive or dominant transmission of a gene could account for approximately 30% of the variance of fasting plasma insulin levels in Caucasians and Mexican-Americans respectively [Schumacher et al 1992; Mitchell et al 1994]. Modelling has also provided evidence for a major T2DM susceptibility gene in Naurans [Serjeantson & Zimmet 1991] and a gene determining early onset of disease in both Pima Indians and Mexican-Americans [Hanson et al 1995; Stern et al 1996a].

Multifactorial studies in obesity have also shown significant familial aggregation of the disease. A number of adoption studies have supported a strong genetic component with major gene effects for both BMI and obesity suggested [Biron et al 1977; Stunkard et al 1986 & 1990; Price et al 1987,1990,1991; Vogler et al 1995; Ness et al 1991]. In contrast other studies have placed more importance on the familial environment [Khoury et al 1983; Annest et al 1983; Longini et al 1984]. Overall fatness and fat distribution have estimated heritabilities of between 29-69% depending on the ethnic background of the population [Tambs et al 1992; Longini et al 1984; Katzmarzyk et al 2000; McLaughlin 1991; Luke et al 2001].

However, a study on variance in glucose tolerance in European pedigrees with T2DM probands, concluded that the disease is unlikely to be due to a single autosomal dominant gene with high penetrance [Cook et al 1994]. Similar studies in obesity also found no evidence for a single major gene effect [Karlin et al 1981; Zonta et al 1987; Tiret et al 1992;

Rice et al 1993]. There may be several major genes each explaining a substantial proportion of the variability of disease susceptibility ('oligogenic' inheritance), which when combined explain a majority of the susceptibility. However there is insufficent evidence to exclude the possibility that potentially a large number of genes may be involved in susceptibility to the diseases, with each gene having only a minor contribution to the overall susceptibility. A further possibility also exists that overall susceptibility is due to a single or several oligogenes in combination with a background of numerous minor polygenic contributors or modifiers.

The genetic architecture of T2DM may be further complicated by the contribution of multi alleleic effects within genes, epistatic interactions between genes, and additive or multiplicative effects. However, complex segregation analysis in South Indian families [McCarthy et al 1994] which have a strong familial aggregation of diabetes [Viswanathan et al 1996; Ramachandran et al 1998] using the computer programmes which implement various genetic models that can account for environmental contributions (POINTER and COMDS) did not entirely reject a single major gene model but favoured other models in preference. Results from these studies tended to favour multifactorial and oligogenic models, and were inagreement with the best-fit model for T2DM proposed by Rich [1990], that susceptibility involved only a few genes with a moderate effect, superimposed on a polygenic background [McCarthy et al 1994].

1.3.2 Family Studies

The earliest family studies are generally unreliable as most predate a formal classification of diabetic subtypes and standardised methods to diagnose glucose intolerance. A German family study found that there were increased affection rates in siblings, parents and their offspring of 37.9%, 20.8% and 32.2% respectively, compared to the population disease prevalence [Köbberling & Tillil 1982]. Similarly in South Indian families where one and both parents were T2DM, a third and a half of offspring respectively were affected [Ramachandran et al 1988; Viswanathan et al 1985]. Moreover, in Pima Indians a parental history of disease associated with increased risk in siblings, independent of the increased risk associated with increasing obesity [Knowler et al 1990]. In this latter study an even greater risk to disease was conferred to offspring if there was a maternal family history or if either parents had an early-onset of disease. Data from the San Antonio Family Diabetes Study found family members had a varying degree of risk depending on their relationship

with the diabetic proband, with first-degree relatives having almost double the population risk [Mitchell et al 1994]. A generally accepted view is an estimated lifetime risk of a first degree relative is 35%, with a relative risk of 3 to 4 fold compared to the general population. The observation of familial correlations demonstrates an aggregation of T2DM in families, and this is generally assumed to support disease transmission via a genetic component. However, family members share both genes and environment (the latter especially in early life), and unless a formal segregation analysis is applied, such as a path type analysis with an 'environmental index', then the genetic and environmental components cannot be entirely separated or accounted for. The use of additional familial relationships, such as twins and adoptees, could also be used to circumvent this problem but these are also confounded by similar problems.

1.3.3 Studies in Twins.

Twin studies theoretically allow for the separation of the genetic component of variance, since monozygotic (MZ) twins share 100% of their genes, whilst non-identical dizygotic twins (DZ) only share half on average. Therefore observations of differences in the rates of concordance for diabetes between MZ and DZ twins have historically been interpreted as evidence that the disease has a genetic basis. Later studies that have endeavoured to avoid the selection bias present in the earlier studies, have found initial MZ concordance rates double that of DZ rates. A study of twins in Finland found rates of concordance of 34% and 16% for MZ and DZ twins respectively [Kaprio et al 1992], whereas in another study rates were 28.6% and 14.3%, for MZ and DZ twins respectively [Newman et al 1987]. In the latter study after a 10-year follow-up an overall concordance rate of 58% was observed in MZ twins.

Although concordance rates of MZ twins are consistently greater than DZ twins even under the assumption that both twin types have been subjected to a shared environment. However, the overall contribution of the genetic component is difficult to assess as other underlying factors such as non-additive genetic effects and the sharing of placental/neo-natal environment in MZ twins could also influence differences in concordance rates between twins (see 1.5 Epigenetic Effect). Two recent studies also identified differences in concordance rates between MZ and DZ twins in Danish [Poulsen et al 1999] and in British T2DM subjects [Medici et al 1999]. However, Poulsen et al observed that differences in concordance rates were significantly greater in abnormal glucose tolerant twins compared to

T2DM twins and proposed that genetic predisposition was more important for the development of abnormal glucose tolerance, and that non-genetic factors may predominate in controlling whether a genetically predisposed individual progresses to overt T2DM.
In respect of obesity an overview of fifty studies on the heritability of body fat/ obesity including both nuclear families and twin studies found heritability levels of 30-40% and 50-80% respectively [Bouchard & Perusse 1993]. However it has been suggested that only a true unbiased estimate of heritability (both additive and non-additive combined) can be achieved if the MZ twins were reared separately. Examples of these are rare, especially for T2DM, but strong correlations have been observed for obesity related phenotypes between MZ twins reared together and apart, suggesting the presence of strong shared genetic determinants [Stunkard et al 1990; Price et al 1991]. However, the magnitude of heritability levels for body fat in all adoption studies combined was calculated to be less than expected, at between 10% and 30% [Bouchard 1995].

1.3.4 Gene effects on prevalence

The marked difference in prevalence between racial groups in the USA [Harris et al 1998] and the effects of progressive admixture on relative risk to disease, usually as a result of migration, both provide supporting evidence that T2DM has a genetically determined component. Admixed populations comprising of both an ethnic group at extremely high risk to T2DM, such as Native American Indians and a low disease risk group eg. Caucasians, create an intermediate disease rate status [Brosseau et al 1979; Knowler et al 1990]. Under these circumstances it is hypothesised that the high-risk ethnic group have a high frequency of disease susceptibility genes and the rate of disease in the admixture population is proportional to the percentage of the gene pool derived from this ethnic population. In full-blood Nauruans the prevalence of diabetes after the age of 60 years is 83%, whereas it is 17% in those inhabitants who have a Caucasian ancestral gene lineage [Zimmet et al 1982].

1.3.5 Environmental-gene interaction on prevalence

In some isolated populations in which T2DM was previously virtually unknown, for example the Pima Indians of Arizona, the Nauruans of Micronesia, and Western Samoans, the prevalence of T2DM has dramatically increased to over 30% in the last 50 years [Zimmet 1992; Hodge et al 1994]. These increases have been associated with changes in lifestyle, from an active traditional rural hunter-gatherer way of life to the more sedentary

westernised existence (figure 1.1.1)[Hodge et al 1994; Taylor et al 1985]. Low levels of physical activity, modern comforts, combined with unlimited easily accessible supplies of highly palatable energy dense foods, leads to energy intake grossly exceeding energy expenditure pave the way to obesity. The overall effect of urbanisation varies depending on ethnicity of the populations, with genetic admixture and other environmental effects influencing susceptibility [Zimmet et al 1982; Hodge et al 1994].

The "thrifty genotype" hypothesis proposed by Neel [Neel JV 1962] endeavours to explain the increase in prevalence as a result of westernization due to an interaction of both genes and environment. Humans in the last 6-7 million years committed to their evolutionary survival, would have selected genes in the course of adaptation to a hunter-gatherer existence. Genes that allowed the development of physiological mechanisms to promote accumulation of energy stores when food was abundant so as to prevent depletion of body energy stores during periods of starvation or limited /seasonal food resources would have been advantageous. The change in lifestyle to one with a continual and abundant supply of high energy density food unaffected by seasonal availability, combined with improvements in health, hygiene and dietary intake, in human evolutional terms has been extremely recent. The "thrifty genotype" favours mechanisms that promote high efficiency in the storage of energy as fat and glycogen, yet under the recent major shift in human habits these genes could now have deleterious effects. Humans only have a limited capacity to adjust to these recent changes with a part of an individual's ability to adjust likely to be determined by genetic factors (figure 1.3.1).

1.3.6 Epigenetic Effects

The 'thrifty genotype' concept is considered highly contentious in some quarters, with an alternative hypothesis proposed that the transmission of diabetes to subsequent generations is the result of a 'thrifty phenotype'. Studies in rodents and humans show that the development of a foetus in an abnormal intrauterine environment can have major consequences on the metabolism of the offspring in later life, including the possible development of diabetes [Pettitt et al 1993; Barker et al 1993; Phillips 1998]. Furthermore, the offspring themselves later in adulthood can also develop abnormal intrauterine environments during gestation, providing a means of transmission of a diabetogenic effect to subsequent generations without genetic interference (Figure 1.3.1) [Aerts & Van Assche 1977; Aerts et al 1990; Holemans et al 1996]. Factors that alter intra-uterine nutrition, such

as starvation and protein deficient diets may affect the development of the foetal pancreas and so alter the adult pancreatic reserve [Hales et al 1991& 1992; Holemans et al 1996]. Low birth weight presumed to be the result of malnutrition or low protein diet during pregnancy also appears to predispose to the development of impaired glucose tolerance, insulin resistance, hypertension and obesity in later adult life [Phillips et al 1998 & 2000a]. Studies in the Pima Indians found that offspring had a greater risk of developing T2DM if the mother was diabetic, particularly if diabetic rather than prediabetic during pregnancy, possibly indicating an intrauterine effect [Pettitt et al 1993; Dorner et al 1987]. Studies in several other ethnic groups have also correlated a reduced foetal growth with loss of glucose tolerance in adults [Hales 1991 et al; Barker et al 1993; Phillips 1998; McCance et al 1994]. It has also been postulated that the increased concordance rates observed in MZ twins are attributable to differences in the intrauterine environment, as MZ twins more frequently share a chorion and amnion than DZ twins [Phillips et al 1998]. Size at birth may also dictate both plasma leptin and cortisol concentrations in adult life [Phillips et al 1999 & 2000b]. In a Danish study, the diabetic patients within both discordant MZ and DZ twins had a lower birthweight compared to their unaffected co-siblings providing further evidence that the development of diabetes could occur independently of a genetic cause [Poulsen et al 1997].

However it is debatable whether the individual phenotypic outcome of these epigenetic effects is not also the result of an interaction with genotype. Half of foetal birth-weight variation is due to the genotype of the foetus with 20% related to the parental genome. One alternative explanation for the 'foetal programming' observations is that genetically predetermined dysfunctional insulin secretion and insulin resistance results in impaired insulin-mediated growth in the foetus as well as insulin resistance and diabetes in adult life [Hattersley & Tooke 1999]. Insulin secreted by the foetal pancreas in response to maternal glucose concentrations is a key growth factor. Monogenic diabetic syndromes due to either decreased beta cell function or a defect in insulin action have also been found associated with impaired foetal growth [Hattersley & Tooke 1999]. Single gene defects that have been shown to detrimentally affect foetal growth include insulin growth factors (IGF) I & II genes, glucokinase (GCK), IGF I Receptor gene, and Insulin gene 5' VNTR [Woods et al 1997; Hattersley et al 1998; Johnston et al 1999 & 2000; Dunger 1998].

1.3.7 Non-genetic effects

Viral infections have also been implicated in both T2DM and obesity. The increased frequency of cytomegalovirus (CMV) in T2DM subjects compared to controls [Lohr & Oldstone 1990] has suggested that a low level chronic infection of β-cells could be responsible for the relative hyposecretion of insulin. However, other studies have failed to replicate these observations and there is suspicion that pancreatic sections were contaminated in the original study [Hattersley et al 1992a]. There are also four animal models of viral induced obesity, including canine distemper, Rous associated virus 7, Borna disease virus and a chicken adenovirus [Lyons et al 1982; Bernard et al 1988; Gosztonyi et al 1995; Dhurandhar et al 1992]. Recently a human adenovirus (AD36) was also implicated in the development of increased adipose tissue in a chicken model [Dhurandhar et al 2000].

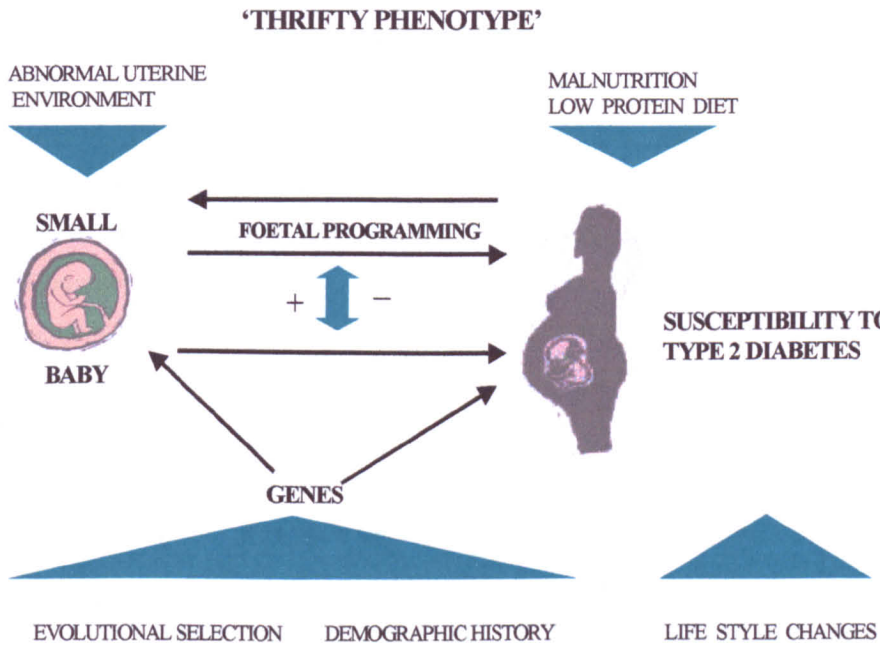

Figure 1.3.1: The 'Thrifty' hypotheses underlying a Genetic and/or Epigenetic intergenerational transmission of susceptibility to T2DM

1.4 GENETIC EVIDENCE FOR AN INHERITED SUSCEPTIBILITY

Epidemiological evidence supports the concept that T2DM and obesity are both heterogeneous and multifactorial disorders with a genetic predisposition. The plausibility of

a genetic contribution to T2DM has been further strengthened through the study of animal models and the identification of human monogenic syndromes of T2DM. However, as mentioned previously it is likely that in the case of the idiopathetic T2DM a number of genes, some with substantially more effect than others, and many with relatively minor roles will be responsible for an inherited susceptibility.

1.4.1 Animal Models of Type 2 Diabetes and Obesity.

Animal models can offer distinct advantages in understanding the causes, consequences and underlying physiological mechanisms involved in disease. Rodent models with single gene defects, Zucker, *ob/ob, db/db*, Yellow (A^y/a) agouti, tubby tb/tb, Cpe^{fat}/Cpe^{fat} and Fatty fa/fa rats and polygenic models, Goto-Kakizaki (GK) and Otsuka long Evans Tokushima fatty rats (OLETF), have provided evidence that genes can initiate biochemical abnormalities that can lead to both T2DM and obesity [Zhang et al 1994; Bultman et al 1992; Naggert et al 1995; Coleman & Eicher 1990; Kelyn et al 1996; Gauguier et al 1996].

At least seven loci controlling diabetes related sub-phenotypes have been identified in the GK rat [Gauguier et al 1996; Galli et al 1996] and gene knockout studies in rodents have lead to the identification of potential human T2DM susceptibility genes, an example being the transcription factor NeuroD/Beta 2 that modulates insulin gene transcription [Naya et al 1997; Malecki et al 1999].

However, the manifestation of different phenotypes between strains of the same species and between species with the same gene defects as a result of differing individual relationships between genes, gene modifiers, biochemical factors and interaction with environmental influences, overall emphasises the complexity of the physiological processes involved. Moreover, the relevance of findings from animal models when extrapolated to humans is uncertain. Currently animal models have not identified any genes that predispose to the common human forms of either T2DM or obesity.

1.4.2 Monogenic forms of T2DM

In humans a substantial part of the knowledge of the genetic component of diabetes has been based on rare conditions caused predominantly by single gene defects. These result in either β-cell dysfunction or less frequently insulin resistance. These sub-groups include only a minority (2-5%) of T2DM patients and comprise of two main genetic subtypes, MODY and maternally inherited diabetes and deafness (MIDD).

MODY: MODY is clinically defined as having an age of onset before the age of 25 years in at least two family members with an autosomal dominant mode of inheritance [Hattersley et al 2000]. MODY accounts for approximately 2-5% of T2DM in Caucasians and mutations have been identified in five or six different genes so far (table1.4.1) mainly consisting of transcription factors, in particular the hepatocyte nuclear factor family (HNF) (table 1.4.1). Mutations in genes leading to MODY might also predispose to the late onset diabetes. Homozygous mutations of the insulin promoter factor 1 gene (*IPF1*) give rise to the MODY4 phenotype, whereas heterozygous subjects may be more predisposed to a late-onset form of T2DM [Hani et al 1999; Macfarlane et al 2000; Hattersley et al 2000]. However, mutations in the genes for HNF4α, glucokinase (GCK), HNF1α, and HNF1β have not been implicated in T2DM in any ethnic population so far studied [Malecki et al 1998].

MODY Sub-group	GENE	CHROMOSOME	MODY-Frequency
MODY 1	HNF4α (*TCF1*)	20q	rare
MODY 2	GCK	7p	10-65%
MODY 3	HNF1α	12q	20-75%
MODY 4	IPF1	13q	rare
MODY 5	HNF1β (*TCF2*)	17q	very rare
MODY 6 ?	NeuroD/Beta2	2q32	rare

Table 1.4.1: Known MODY Sub-genotypes.

A novel basic helix-loop-helix transcription factor, neurogenic differentiation 1 (NeuroD/Beta2), involved in pancreatic endocrine cell differentiation and the tissue-specific regulation of the insulin gene [Naya et al 1995] has been proposed as a possible MODY 6 gene [Fajans et al 2001]. Two heterozygous mutations in NeuroD1 have been described which associate with the development of T2DM [Malecki et al 1999]. One variant (residue 206+C) gives rise to a truncated polypeptide lacking the C-terminal transactivation domain, a region that associates with the two coactivators, CBP and p300. The clinical profile of patients with the truncated NeuroD1 polypeptide was found to be more severe and suggestive of MODY rather than late onset T2DM [Malecki et al 1999].

MIDD: MIDD is another early onset form of T2DM, frequently characterised by progressive β-cell degeneration and deafness. Variants within the mitochondrial genome are associated with the disruption of oxidative phosphorylation processes consequently affecting intracellular calcium levels and exocytosis of insulin granules by depolarisation of the cell membrane. Mitochondrial DNA (mtDNA) is only maternally inherited because sperm

contribute almost no cytoplasm to the zygote [Giles et al 1980]. Consequently mtDNA associated diseases have a maternal dominant inheritance pattern with variable penetrance and clinical phenotypes due to heteroplasmy.

The most prevalent characterised mutation is at position 3243 tRNA (Leu, UUR) [van den Ouweland et al 1992]. This mutation is implicated in several disease phenotypes including the neurological disorder MELAS mitochondrial syndrome (myopathy, encephalopathy, lactic acidosis and stroke-like episodes), and both chronic progressive external opthalmogepia (CPEO) and Kearns-Sayre syndrome (KSS) [Goto et al 1990]. The 3243 mutation has a frequency of 1-3% in French and Japanese subjects with family histories of diabetes, but only occurs in 0.1-0.2% of British Caucasian T2DM patients and is virtually absent in South Indians [Saker et al 1997; McCarthy et al 1996; Smith et al 1997].

1.4.3 Monogenic forms of obesity

Obesity is also characteristic of a number of monogenic disorders in humans. Heterozygous mutations of the melanocortin 4 receptor gene (MCR4) are possibly responsible for the most common monogenic cause of human obesity, although no homozygous subjects have yet been identified [Farooqi et al 2000].

Bardet-Biedl syndrome (BBS) is a rare autosomal recessive disorder (< 1:100,000), with a broad spectrum of clinical phenotypic traits that include both obesity and diabetes. At least six different genetic loci have been linked to the disease (table 1.4.2) with causative mutations identified in a chaperonin gene responsible for McKusick-Kaufman syndrome (MKKS) [Mykytyn et al 2001; Slavotinek et al 2000; Katsanis et al 2000; Beales et al 2001; Nishimura et al 2001]. Recent studies on the BBS 6-MKKS gene suggests that phenotype is probably determined by interactions of more than one BBS locus, and that BBS may not be a 'simple' monogenic disease [Katsanis et al 2001]. It has also been hypothesised that heterozygous mutations of BBS genes may account for 1-2% of the severely obese patients and are therefore potential obesity candidate genes [Croft et al 1995].

The human obesity gene map for the year 2000 reported that forty-seven cases of obesity were caused by single gene mutations in six different genes. In addition, twenty-four Mendelian disorders have been mapped that exhibit obesity as one of their clinical manifestations and 115 different relevant quantitative loci in animal models have been identified [Perusse et al 2001b].

BBS Sub-group	GENE	CHROMOSOME
BBS 1	Not known	11q13
BBS 2	*BBS2*	16
BBS 3	Not known	3p13-p12
BBS 4	*BBS4*	15q22.3-q23
BBS 5	Not Known	2q31
BBS 6	MKKS	BBS6 20p12

Table 1.4.2: Known BBS sub-types.

1.4.4 Linkage Studies and Genome Scans

Classical parametric linkage analysis studies in simple Mendelian monogenic disorders have had sufficient power and have also been robust with low false-positive rates to enable identification of susceptibility loci in many chromosomal regions [Rao et al 1978; Risch 1990]. Examples of successfully positionally cloned disease genes include cystic fibrosis, familial polyposis, BBS6 and MODY [Kerem et al 1989; Groden et al 1991; Katsanis et al 2000; Hattersley et al 1992a].

However, classical linkage analysis methods using extended pedigrees can be problematic in complex diseases such as T2DM and obesity. Hence it has become commonplace to utilize a model free non-parametric approach using affected sibling pairs (ASP) in the search for diabetogenic genes. The principles and methodology of linkage analysis when applies to complex diseases are discussed in Chapter 3, section 3.8.1.

Genome scans for T2DM susceptibility loci have been performed on both ASP sets and extended families. Evidence of linkage has been found to a number of chromosomal regions in several diverse ethnic populations (table 1.4.3). In a number of chromosomal regions evidence of linkage has been replicated leading to the identification of regions harbouring potential disease susceptibility genes.

Potential candidate T2DM susceptibility genes that have been identified include the protein kinase C substrate (PEA 15) and potassium inwardly rectifying channel subfamily J member 9 gene (KCNJ9) to 1q21-24, insulin degrading enzyme (IDE) to 10q23-25, MODY 3 to 12q24, and calpain 10 to 2q37 [Wolford et al 2000; Wiltshire et al 2001; Ehm et al 2000; Horikawa et al 2000; Permutt & Hattersley 2000a].

Ethnic Population	Chromosomal region	Reference	Linkage
Mormon Caucasian USA	1q21-23	Elbein 1999	N
French Caucasian	1q21-24	Vionnet 2000	N
UK Caucasian	1q24.2	Wiltshire 2001	N
Pima Indian	1q25.3	Hanson 1998	S
Indigenous Australians	2q24.3	Busfield 2002	S
Mexican American	2q37 (*NIDDM1*)	Hanis 1996	S
French Caucasians	2q37	Hani 1997	N
Mexican American	3q27	Ehm 2000	S
French Caucasians	3q27	Vionnet 2000	S
Ashkenazi Jews	4q	Permutt 2001	N
Botnian Finnish	4q32-q33	Lindgren 2002	N
USA Europeans	5q13	Ehm 2000	N
UK Caucasian	5q13	Wiltshire 2001	N
UK Caucasian	5q32	Wiltshire 2001	N
French Caucasian	5q31-33	Vionnet 2000	N
UK Caucasian	8p21.3-22	Wiltshire 2001	S
Mormon Cauc USA	8p21.3	Elbein 1999	N
Indigenous Australians	8p22	Busfield 2002	N
Botnian Finns	9p13-q21	Lindgren 2002	S
UK Caucasian	10q23.3	Wiltshire 2001	N
Finnish (FUSION)	10q23.33-24.32	Ghosh 1999	S
French Caucasian	10q26.3	Vionnet 2000	N
Mexican American	10q26.12	Duggirala 1999	N
Mexican American	11p15.4	Stern 1996	S
Pima Indian	11q22-23	Hanson 1998	N
Caucasians	12q15	Bektas 1999	S
USA Europeans	12q15	Ehm 2000	N
Botnian Finnish	12q24 (*NIDDM2*)	Mahtani 1996	S
Botnian Finnish	12q24	Bowden 1997	N
Australians	12q24	Shaw 1998	S
USA Whites	12q24	Ehm 2000	N
USA Europeans	12q24	Lindgren 2002	N
Finnish (FUSION)	20p12	Ghosh 2000	N
USA Europeans	20q12-13 (*NIDDM3*)	Bowden 1997	N
USA Europeans	20q12-13	Ji 1997	S
Finnish	20q12-13	Ghosh 1999/ 2000	S
USA Europeans	20q12-13	Zouali 1997	N
Mormon Caucasian	20q12-13	Elbein 1995	N
French Caucasians	20q12-13	Hani 1997	N
Ashkenazi Jews	20q12-13	Permutt 2001	N
USA Europeans	20q13.1-13.2	Klupa 2000	S

Table 1.4.3: Autosomal Chromosomal regions with evidence of linkage for a T2DM susceptibility locus in humans. Loci with either suggestive linkage, replicated nominal evidence of linkage, or regions with maximum evidence for linkage within a given study have been included. S=LOD (or equivalent) >3.0; N=LOD (or equivalent) >1.0.

The replication of evidence for linkage to 20q12-13 in eight studies has supported the designation of this susceptibility region as *NIDDM3*. The most obvious candidate gene located in this interval was the MODY1-HNF4α gene. However, subsequent association studies on the gene did not support a role as a common determinant for susceptibility to

T2DM [Malecki et al 1998]. The *NIDDM3* interval contains at least twenty genes and one other candidate gene has been proposed, the melanocortin receptor 3 gene (MC3R) [Hani et al 2001]. A study in French Caucasians families identified two missense mutations that associated with insulin and glucose levels in normoglycemic subjects. However, neither MCR3 coding variant associated with overt T2DM or obesity [Hani et al 2001].

In respect of obesity there is also evidence of linkage to a number of chromosomal regions, including 12q24.3 in French Canadians, 10p in French and German Caucasians, 11q21-22 in Pima Indians, Xq24 and 18q21 in Finnish, and 3p in old order Amish [Perusse et al 2001a; Hager et al 1998; Norman et al 1998; Ohman et al 2000; Hsueh et al 2001]. Nevertheless at present there appears to be very little duplication of linkage results. The year 2000 human obesity gene map summarises that 113 loci have been linked to obesity indicators in genome scans, with putative loci found on all chromosomes except the Y chromosome, and in addition there are positive associations with 48 different candidate genes [Perusse et al 2001b].

1.4.5 Candidate disease genes

Besides the pinpointing of 'candidate' disease susceptibility loci through whole genome searches and linkage studies, candidate genes have been selected on the basis of a rational hypothesis. These have been examined by utilizing techniques that rely on linkage disequilibrium, this phenomenum and associated analytical methodologies are discussed in Chapter 3 (3.8.2).

Amongst the various candidate genes that have been selected include those genes that cause diabetes in animal models or are implicated in the human monogenic forms of the disease. Examples of some of the more promising candidate genes are discussed below. However, out of the numerous candidate genes studied so far only a few genes examined have been consistently culpable, and all so far only appear to play minor roles in common idiopathic T2DM.

A candidate gene for insulin resistance: The presence of insulin resistance in type 2 diabetics by inference has implicated the insulin receptor (IR), and other crucial factors involved in post-receptor signal transduction pathways such as insulin receptor substate-1 (IRS-1), IRS-2, and phosphatidylinositol-3-kinase (PI3-K). Homozygous disruption of either the IR or IRS-2 genes causes mice to become severely insulin resistant and die shortly after birth, whereas mice that have a combined heterozygous knockout of IR and IRS-1

progressively develop hyperinsulaemia and eventually diabetes [Bruning et al 1997; Withers et al 1998]. More than fifty coding polymorphisms have been identified in the human IR gene, some associating with familial hyperinsulimaemia. A common coding variant of IRS-1, Gly972Arg has been implicated in insulin-resistance in both human obesity and T2DM, possibly in combination with a variant of IRS-2 (Gly1057Asp) [Taylor 1992; Almind et al 1993; Hitman et al 1995]. However, associations have been inconsistent with a lack of replication and absence of evidence of linkage from genome scans for all three genes, suggesting that they are not major susceptibility loci for T2DM [Sesti et al 2001; Hitman et al 1995].

Candidate genes for insulin secretion: A consistently strong contender for an insulin secretory susceptibility locus is the sulphonylurea receptor-1 (SUR-1) [Inoue et al 1996; Hart et al 1999]. SUR-1 is a high affinity receptor for sulphonylureas that are expressed on pancreatic β-cells. SUR-1plays a crucial role in regulating glucose-induced insulin secretion by controlling K+-ATP channel activity of the β-cell membrane with a ATP-sensitive potassium channel Kir6.2. However, genome scans and specific regional linkage analysis have provided little evidence of linkage to 11p15.1 and the SUR1 gene [table 1.4.3; Stirling et al 1995]. In humans sequence variants within the cytoplasmic domain have been shown to disrupt the regulation of insulin secretion. A splice variant that results in protein truncation through removal of the second nucleotide-binding fold causes a rare autosomal recessive disorder familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [Thomas et al 1995]. Although gene variants are rare, often specific to one family, over 20 mutations have been identified in the SUR gene. Homozygotes for these variants invariably manifest clinically as hyperinsuliaemia. In addition, a common coding variant (exon 16-3t) appeard to be associated with T2DM in Dutch Caucasians but replication studies have been lacking [Nestorowicz et al 1998; Hart et al 1999].

Genes implicated in human monogenic T2DM are potential candidates for dysfunctional insulin secretion. Subjects who are heterozygous for variants of the IPF1gene (MODY4) do appear to be predisposed to late-onset T2DM [Hani et al 1999; Macfarlane et al 2000; Hattersley et al 2000]. Although there is little evidence of linkage to chromosome 13q in any human T2DM genome scans (table 1.4.3). Similarly, mice with a targeted disruption of the NeuroD1 gene have a striking reduction in pancreatic β-cells which have impaired cellular development resulting in severe diabetes and premature death [Naya et al 1997]. A variant in the human NeuroD1 gene predisposes to a clinical form of diabetes that resembles

MODY and another variant appears to be associated with T1DM in Danish subjects [Malecki et al 1999; Hansen et al 2000]. In contrast, a different missense variant (Arg111Leu) that disrupts the DNA-binding domain and abolishes the E-box binding activity of NeuroD1, was identified in patients who developed a form of diabetes more typical of T2DM, suggesting also a role in this disorder [Malecki et al 1999].

Positive linkage and association results for candidate susceptibility genes can provide substantial evidence that a gene may play a role in the disease aetiology. However, they cannot provide definitive proof for causality even if there is complete penetrance of the disease allele. Further, study designs that investigate function are required to provide scientific consensus that the aberrant gene/protein actually disrupts physiological pathways leading to the disease or to a related sub-phenotypic state.

CHAPTER 2:
CANDIDATE GENES STUDIED

2.1 UNCOUPLING PROTEINS

2.1.1 Cellular Respiration

Cellular energy is produced by the metabolism of foods and oxygen to carbon dioxide and water. ATP is generated by stepwise fuel oxidation via the electron transport processes of the respiratory chain in mitochondria. The increasing redox potential at complexes I, III and IV, pump H+ protons out of the matrix to the inner membrane mitochondrial space (Figure 2.1.1). This increases mitochondrial membrane potential by generating an electrochemical proton gradient ($\Delta\mu H^+$) across the inner membrane. The increased membrane potential drives protons back into the matrix via the ATPsynthase, in a reaction that is tightly linked to the coupling of fuel oxidation to energy production by the oxidative phosphorylation of ADP to ATP [Nicholls 1974].

A key challenge to organisms is maintaining efficient energy use by balancing rates of fuel oxidation and ATP production with rates of ATP utilisation. The chemiosmotic theory of Mitchell [1979] based on observations in isolated mitochondria, gave rise to the hypothesis that when cells are inactive and rates of ATP consumption are reduced, ADP should be low. As proton entry into the matrix via ATPsynthase requires ADP this flux will be also reduced. Continued electron transport however continues to increase $\Delta\mu H^+$ in the inter-membrane space, thus causing a "back pressure" on the proton pumps at complexes I, III, and IV and consequently fuel oxidation is inhibited. This theoretically links respiration to ADP availability, so fuel consumption only occurs when cells have been working ie. converting ATP to ADP.

2.1.2 Uncoupling Protein Activity

According to the Mitchell theory, protons are unable to enter into the matrix via ATPsynthase in the absence of ADP. However, mitochondria both isolated and *in situ,* continue to consume oxygen even in the absence of ADP phosphorylation ie. State IV respiration [Brown et al 1990]. Conclusions were made that mitochondrial coupling of respiration to ATP synthesis may only be partial in most cells and oxygen consumption may be controlled by an inner mitochondrial membrane permeability to protons [Nicholls 1974; Brand et al 1990 & 1999; Boss et al 1998b; Porter et al 1999]. Studies have since established

Brand et al 1990 & 1999; Boss et al 1998b; Porter et al 1999]. Studies have since established the existence of a basal proton conductance or proton leak across the inner membrane independent of the ATPsynthase pathway. The magnitude of the $\Delta\mu H^+$ drives both the proton leak and determines its rate by controlling inner membrane conductivity to protons [Brand 1994 & 1990; Porter et al 1995; Rolfe & Brand 1997; Rolfe & Brown 1997; Brown et al 1992; O'Shea & Chappell 1984].

Figure 2.1.1: Mitochondrial Energy Metabolism showing two proposed uncoupling mechanisms. Klingenberg (Green) and Garlid (Red). Diagram modified from Boss et al [2000]

The presence of an independent proton pathway does account for some of the flaws evident in Mitchell's theory where control of ATP production/utilisation is based on ADP availability. Additional mechanisms are necessary when there is a massive increase in ATP utilisation such as during muscle contraction with a 100-fold increase in energy demand. The rapid increase in ATP synthesis required could not be the result of a series of retrospective mass-action effects on metabolism, all initiated by an increase in ADP

at all under conditions when cellular respiration is significantly stimulated [Balaban 1990]. The rates of ATP production are also strongly controlled by factors other than ATP utilisation, eg. rates of fuel oxidation [Brand 1997] and can exert strong controlling effect on rates of ATP utilisation contrary to that predicted by the Mitchell theory [Ainscow & Brand 1999]. Rolfe and Brand [1997] proposed that the partial uncoupling observed in mitochondria could be a mechanism to increase the sensitivity and rate of response of oxidative phosphorylation to a variety of effectors.

Currently, two theoretical mechanisms have been proposed to account for an independent basal proton conductance [Ricquier & Bouillaud 2000; Stuart et al 2001]. Firstly, slippage where proton leakage is due to the modified properties of the respiratory chain, such as failure of proton pumps, or changes in the physical properties of phospholipid fatty acid bilayers or proteolipid bilayers juxataposed to the inner membrane [Brookes et al 1997a]. However, neither the composition of the inner membrane [Brookes et al 1998; Porter et al 1996], nor mediated changes via the proton-pumping respiratory complexes [Brown & Brand 1991] appears to account for differences in proton conductance.

Alternatively, proton transport across the mitochondrial inner membrane may be catalysed by one or more specific physical carriers. These carriers might cause dissipation of the transmembrane $\Delta\mu H^+$ through by-passing the ATPsynthase pathway and therefore uncoupling respiration from oxidative phosphorylation of ADP to ATP. The major consequence of uncoupling respiration by either mechanism would be the activation of increased substrate oxidation and the allowance of energy to be dissipated as heat at physiologically important levels ie Thermogenesis [Boss et al 1998b].

Basal conductance only accounts for approximately 5% of the proton leak flux in all mitochondria in rats [Brookes et al 1997b]. It has been demonstrated that heat production in rodent Brown Adipose Tissue (BAT) can also be the result of specific and regulated uncoupling processes in mitochondria [Nicholls 1979 & 1984]. Mitochondria in BAT are highly uncoupled, and uncoupled respiration was found to be both inducible, that is activated by non-estified fatty acids (NEFA) and also almost entirely inhibited by purine nucleotides, mainly GDP. Observations that uncoupled processes could be regulated supported the existence of an uncoupling protein. The exploitation of the high affinity of the purine nucleotide to binding sites on the inner membrane of BAT mitochondria, enabled the identification of a 32Kd protein [Heaton et al 1978; Klingenberg 1990]. This protein was originally named Thermogenin and later renamed uncoupling protein 1(UCP1) [Fleury et al

1997]. UCP1 has been characterised in a number of species [Lin et al 1980; Ricquier et al 1982; Jacobsson et al 1985; Bouillaud et al 1986; Aquila et al 1985].

UCP1 is a member of anion transport family of genes commonly having six transmembrane helices straddling the inner mitochondrial membrane with C-and N-termini protruding into the cytosolic site. Theoretically these proteins catalyse fatty acid dependent electrophoretic proton flux across the inner membrane. Two uncoupling mechanisms for UCP's have been proposed, both based on the absolute requirement of fatty acids for UCP H+ transport.

***Klingenberg Model*:** It is postulated that in a proton buffering models, protons could be conducted through a hydrophilic aqueous pathway within the UCP protein. This translocation channel would be lined with the fatty acid carboxyl head groups of the protein in conjunction with the resident H+ conducting amino acids such as histidines, and together they would buffer the protons across the inner membrane (figure 2.1.1) [Klingenberg & Huang 1999; Winkler & Klingenberg 1994].

***Garlid Model*:** In the fatty acid protonophore model proposed by Garlid et al [1996], UCP does not actually conduct protons but instead acts as an anion transporter. A variety of anions are transported, but the physiologically important substrates are fatty acid anions. The monovalent negatively charged fatty acid carboxylate head groups are translocated by UCP straddling the membrane from the matrix side across the inner membrane to the cytosolic side, driven by the high inside-negative mitochondrial membrane potential. Once the carboxylic anion head has crossed the membrane it picks up a proton from the cytosol. The protonated electro-neutral fatty acid then rapidly crosses the membrane bilayer to the matrix side by non-ionic diffusion independently of the UCP, hence delivering charged protons to the matrix. This 'Flip Flop' mechanism permits fatty acids to behave as regulated cycling protonophores, releasing H+ protons in the matrix independently of ATP synthesis, thus dissipating the proton gradient and generating heat, uncoupled thermogenesis (figure 2.1.1) [Garlid et al 1998; Skulachev et al 1991]. The Garlid model tends to be more favoured with the mechanistic integration of both proton and anion transport consistent with the anion transporting function of other members of the gene family.

UCP1 is exclusively expressed in BAT, a tissue primarily concerned with heat generation. A considerable number of studies using a variety of methods have conclusively confirmed that UCP1 catalyses proton conductance in rodents [Nicolls & Locke 1984; Klingenberg 1990,1999; Himms-Hagen 1990; Gonzalez-Barroso et al 1996; Fleury et al 1997; Brand et al 1999].

2.1.3 UCP2 and UCP3 homologues of UCP1

In 1997 two homologues of the UCP1 gene were discovered, cloned, sequenced and characterised namely UCP2 and UCP3 [Fleury et al 1997; Gimeno et al 1997; Boss et al 1997b & 1998d; Vidal-Puig et al 1997; Gong et al 1997; Argyropoulos et al 1998a; Tu et al 1999a; Pecqueur et al 1999; Liu et al 1998; Matsuda et al 1997; Solanes et al 1997]. In humans UCP2 and UCP3 both mapped to the distal segment of 11q13 (Figure 2.1.2) separated by 7kb (8kb in mice) [Pecqueur et al 1999; Surwit et al 1998; Liu et al 1998].

	UCP1	UCP2	UCP3 (L)
Amino Acid Homology		← 59%	
		←	57%
		← [1]73% →	
Amino acid number in monomer	306	308	311
[2]Six transmembrane domains	✓	✓	✓
[3]ALLBNB motif	✓	✓	✓
Gene Length (kb)	8.0	6.3	8.5
Number of exons	6	[4] 8	7
mRNA Splice variants	None	None	[5]Two
[6]Main Tissue Expression	BAT	Ubiquitous	Skeletal Muscle

Table2.1.1: Comparison of UCP homologues UCP1, UCP2 and UCP3

Additional information pertaining to table 2.1.1:

[1]UCP2 and UCP3 proteins are very similar with many of the non-identical residues in the UCP3 protein being conservative substitutions, which in most cases correspond to residues found in either UCP2 or UCP1 in various species [Boss et al 1997b; Vidal-Puig et al 1997; Gong et al 1997; Liu et al 1998; Matsuda et al 1997].

[2]All three UCP homologues share common features with members of the mitochondrial carrier protein superfamily, including possession of six transmembrane domains [Solanes et al 1997]. UCP proteins are homodimers with each monomer having three repeat units of approximately 100 amino acids long. Each unit contains two transmembrane domains with intervening amino acids looped into the membrane [Walker & Runswick 1993].

[3] Immediately following the first transmembrane domain of each repeat unit is a highly conserved basic motif ALLBNB (Acidic, Lipophilic, Basic, Neutral) [Klingenberg 1993; Liu et al 1998]. This motif is perfectly conserved in human UCP1, UCP2 and UCP3 and in the three murine UCP1, -2, -3 proteins.

[4] Human and mouse UCP2 genes both differ from UCP1 in having two additional non-coding untranslated exons in the 5' region.

[5] Human UCP3 gene is characterised by having two transcripts generated from a single gene by alternative splicing. One transcript produces a protein with 312 amino acids, UCP3L. The other short isoform, UCP3S, loses 37 C-terminal residues encoded by exon 7 producing a truncated protein of 275 amino acids lacking the COOH terminal region. Premature termination occurs in approximately 50% of the transcripts with intron 6 providing an alternative polyadenylation site AATAAA. The region extending to the other $AATAAA_L$ signal located approx 1.1 kb downstream of exon 7 in the long form (figure 4.4.1) is thought to be homologous to the domain in UCP1 and UCP2 that mediates inhibition of uncoupling activity by purine nucleotides and provides the sixth transmembrane domain [Boss et al 1998d; Solanes et al 1997].

[6] UCP1 is exclusively expressed in BAT, similarly UCP3 is almost exclusively restricted to skeletal muscle but at a level 2.5-5 fold greater in abundance than UCP2 [Boss et al 1997b]. UCP3 is also expressed in heart muscle but at less than 1% of the level found in skeletal muscle. In humans it is virtually absent from subcutaneous WAT but in mice expression is equally abundant in both skeletal muscle and BAT, and to a lesser extent in WAT and heart [Vidal-Puig et al 1997]. UCP2 is ubiquitously expressed in both humans and rodents. UCP2 expression is most abundant in WAT, BAT, but it is also expressed at high levels in heart, kidney and brain tissues with a relatively high level in the spinal cord and medulla, also in tissues throughout the immune system including spleen, thymus, bone marrow, trachea, leucocytes, macrophage and lymph nodes [Fleury et al 1997; Richard et al 1999]. It is found in less abundance in skeletal muscle, testis, Kupffer cells, stomach/gut cells and pancreatic islets [Larrouy et al 1997; Richard et al 1998; Ricquier 1997; Fleury et al 1997; Gimeno et al 1997; Chan et al 1999].

2.1.4 Uncoupling activity of UCP1 Homologues

Mitochondrial proton leaks have also been detected in a number of major oxygen consuming tissues other than BAT including WAT, liver, kidney, brain and skeletal muscle, with this latter tissue the major site of adaptive thermogenesis [Astrup et al 1985 & 1989; Zurlo et al 1990; Spraul et al 1993]. UCP2 and UCP3 are also predicted to have uncoupling activity this is summarised in table 2.1.2.

	EVIDENCE FOR UNCOUPLING ACTIVITY	UCP1	UCP2	UCP3 (L)	UCP3 (S)
	Structural Similarities	+	+	+	[1]-
Cellular Location	Immunohistochemically located to mitochondria	+	[2]-	[2]-	-
	[3]Immunological detection to mitochondrial fraction in Yeast	+	+	+	+
Uncoupling Activity	Proton transport activity demonstrated in liposomes	NK	+	+	+
	[4] Increase in respiratory rate and decreased respiratory control ratio in recombinant yeast	+	+	+	+
	[5]Decrease in mitochondrial membrane potential in recombinant yeast expressing gene	+	+	+	+
Factors Influencing activity	[6]Purine Inhibition of Uncoupling activity	+++	+	+	[7]-
	[6]Fatty Acid stimulation of Uncoupling Activity	++	+++	+++	[7]-
	[8]Retinoic Acid stimulation of Uncoupling Activity	+++	+++	NK	NK

Table 2.1.2: Evidence for UCP2 and UCP3 mitochondrial uncoupling activity. NK=Not known

Additional information pertaining to table 2.1.2:

[1]The region that is spliced out in the UCP3S is predicted to be critical for uncoupling activity, and provides the sixth transmembrane domain that might be crucial for insertion of the protein into the mitochondrial inner membrane. It has therefore been postulated that the activity of the UCP3S isoform is markedly different from the UCP3L isoform [Boss et al 1998d; Solanes et al 1997].

[2] Immunohistochemical techniques have been unable to locate either UCP2 or UCP3 proteins to mitochondria due to the lack of specific antibodies.

[3] [Larrouy et al 1997; Hinz et al 1999a]. In addition there is subcellular localisation of UCP3 in mitochondria in human breast carcinoma MCF7 cells [Boss et al 1999].

[4] Effects on growth rates of yeast are correlated with uncoupling activity [Gonzalez-Barroso et al 1996]. Expression of UCP1, -2 and -3 in heterologous yeast expression systems show similar quantitative effects consistent with the homologues having uncoupling activity [Fleury et al 1997; Bouillard et al 1994; Gimeno et al 1997; Liu Q et al 1998]. *In vitro* experiments that measure State IV respiration rate ie.respiration in the absence of ADP/ ATP synthesis, are purported to be controlled by both proton conductance and substrate oxidation [Brand 1990]

[5] *In vivo* experiments using flow cytometry with direct measurements of $\Delta\mu H^+$ by the uptake of mitochondrial membrane specific electrochemical potential-sensitive fluorescent dyes [Gimeno et al 1997; Fleury et al 1997; Gong et al 1997; Hinz et al 1999a; Hagen et al 1999; Zhang et al 1999; Jaburek et al 1999].

[6] Amino acid sequences located in the carboxy-terminal region, within and between the fifth and sixth transmembrane domains of UCP1 have been implicated not only in the activation of uncoupling activity by FFA but also in the purine nucleotide inhibition of activity [Gonzalez-Barroso et al 1996; Winkler & Klingenberg 1992; Bienengraeber et al 1998; Modriansky et al 1997]. Variation in residues in this region between UCP1, UCP2, and UCP3 and their different effects, suggests the biochemical regulation of the two homologues is distinct from UCP1 [Liu et al 1998]. Although the proton transport properties of UCP2 have been physiologically considered to be qualitatively identical to that of UCP1 [Rial et al 1999; Zhang et al 1999] studies have suggested that both UCP2 and UCP3L may have greater uncoupling activity than UCP1, with increased sensitivity to free fatty acids and decreased inhibition by purine nucleotides [Gonzalez-Barroso et al 1996 & 1998; Bouillaud et al 1994; Nicolls 1974 & 1984; Huang et al 1995a & 1995b; Hinz et al 1999; Jaburek et al 1999; Echtay et al 1997 &1998; Zhang et al 1999]. UCP3L and UCP3S isoforms were also found to have qualitatively similar biochemical activities, with some studies showing UCP3S having less activity than UCP3L [Hagen et al 1999] and other studies higher activity [Hinz et al 1999b]. Whilst in contrast some investigations have found also the magnitude of the uncoupling effects of UCP3L overall were greater than that of either UCP1 or UCP2 [Zhang et al 1999].

The acceptance of the theoretical models of either Klingenberg or Garlid assumes the obligatory involvement of fatty acids in influencing uncoupling activity [Klingenberg 1988;

Winkler & Klingenberg 1994; Bienengraeber et al 1998; Jezek et al 1994; Garlid et al 1996]. *In vitro* experiments on respiration in isolated BAT mitochondria have shown that free fatty acids (FFA), excluding those with esterified carboxyl groups, are prequisiste for UCP1 mediated H+ transport, with further supportive studies in mammalian BAT [Ricquier et al 1997]. It has been suggested that fatty acids stimulate protonophoric activity by acting as regulatory ligands with UCP1 [Bienengraeber et al 1998; Kopecky et al 1984].

However most of these studies showing the effects of FFA's on uncoupling activity are in yeast or isolated liposomes and it may be different in other organisms or tissues. In mammalian C_2C_{12} myoblasts, the uncoupling activity of UCP3 appears to be under much tighter control than in yeast [Brustovetsky et al 1992; Boss et al 1998c] suggesting that there may be tissue specific differences in factors controlling uncoupling activity and these may be specific for each UCP [Hagen et al 1999].

[7]The human UCP3S isoform lacks the C-terminal region but is still capable of inserting itself into the inner mitochondrial membrane. Therefore the putative purine nucleotide binding domain may not be obligatory for uncoupling activity and its absence may actually enhance uncoupling activity [Bouillaud et al 1994; Renold et al 2000].

[8] Although it is thought that increased UCP uncoupling activity and stimulation of transcription of the UCP gene may be mediated by cAMP induced increased intracellular FFA concentration and /or sympathetic nervous stimulation [Bukowiecki et al 1981] some studies suggest a role for either fatty acids or the inhibitory purines nucleotides on UCP uncoupling activity to be inconclusive [Gonzalez-Barrroso et al 1998; Rial et al 1983 & 1999; Zhang et al 1999; Baumruk et al 1999]. Alternative or additional factors may also be of equal importance although data is scarce. Retinoic acid appears also to be a strong promoter of uncoupling activity of both UCP1 and UCP2 in yeast expression vectors, with the latter positively regulated in a pH dependent manner. Furthermore, these experiments also suggested that retinoic acid was possibly more potent than fatty acids [Rial et al 1999]. In addition, to this extensive evidence supporting uncoupling activity for both UCP2 and UCP3, increased thermogenesis has also been recorded in yeast over-expressing UCP2 [Paulik et al 1998] and using direct microcalorimetric measurement of the thermogenic power of cells thermogenesis was increased in yeast transformed and overexpressing the UCP3L form when compared to controls [Hinz et al 1999a].

2.1.5 Physiological roles of UCP2 and UCP3

The potential biological roles for both UCP2 and UCP2 have initially been postulated based on their close structural and functional similarity to UCP1. However, accumulating data suggests they may have a number of physiological functions that differ from UCP1, although none to date has been entirely proven.

Thermogenic Role:

The known physological role of UCP1 could implicate both UCP homologues in obligatory or adaptive thermogenesis, and thus a role in mediating whole body energy homeostasis through the modulation of energy expenditure [Astrup et al 1985 & 1989; Zurlo et al 1990; Spraul et al 1993; Ravussin et al 1992; Vidal-Puig et al 1997; Boss et al 1997b]. Total energy expenditure results from a combination of three components [Ravussin 1995].

1.Basal or resting metabolic rate (RMR/BMR) is the obligatory energy expenditure essential for body cellular, biochemical, and physiological functions, accounting for approximately 60% of the total energy usage. Physiological processes include maintenance of ion gradients, protein synthesis, muscle activity and heat generation with consumption of energy largely dependent on adenosine triphosphate (ATP) production [Brand 1990].

2. Energy expenditure via thermogenesis induced by physical activity/exercise

3. Adaptive thermogenesis, the component of energy expenditure that changes in response to environmental stimuli such as cold induced shivering and non-shivering thermogenesis, chronic dietary excess or dietary imbalance, or microbial/viral infection-fever.

Obligatory Thermogenesis:

An obligatory cellular basal proton leak occurs in mitochondria of different tissues across many species and has been considered to contribute greatly to overall energy expenditure and metabolic rate (table 2.1.3) [Porter & Brand 1993; Rolfe et al 1994; Simonyan & Skulachev 1998]. This has been particularly reflected in rat hepatocyte and leg muscle studies in which basal proton conductance accounted for between 26% and 50% respectively of the total resting energy expenditure [Brand 1994; Rolfe et al 1999]. A two-fold stimulation of the respiration rate by induction of glucose/urea production and muscle contraction in these hepatocytes and muscles respectively, reduces this contribution to 22% and 34% respectively, commensurate with a compensatory basal proton conductance mechanism [Rolfe & Brand 1997a]. It has been postulated that in humans 18-22% of basal/resting metabolic rate could be attributed to a mitochondrial proton leak, with total

body adipocyte thermogenesis likely to contribute 5% (7% under stimulated conditions) and skeletal muscle approximately 14% [Rolfe et al 1996 & 1999].

SPECIES	PROTON LEAK--% RESPIRATION IN HEPATOCYTES
HORSE	16
PIG	15
SHEEP	27
FERRET	19
RAT	19-26
MOUSE	19
LIZARD	30
LAMPREY	40
FROG	25
SNAIL	25

Table 2.1.3: Cross species percentage contribution of basal mitochondrial proton leak to hepatocyte respiration

Variation in this obligatory energy cost resulting from the maintenance of physiological and cellular functions in humans appears to have a degree of familial heritability [Bogardus et al 1986; Bouchard et al 1989 & 1993]. There is considerable evidence to suggest that primary abnormalities in energy balance could contribute to the pathogenesis of both diabetes and obesity [Ravussin & Bogardus 1992]. Meta-analysis of eleven studies on energy expenditure and obesity, found resting energy expenditure in post obese subjects to be significantly lower than that in normal weight controls, although fat tissue mass is increased in obese subjects. It was postulated that the marked differences in BMI between subjects could be due to variation in energy expenditure over a long period [Ravussin et al 1988; Astrup et al 1996]. Accordingly, computer generated simulation studies suggest that only small reductions (20%) in RMR under conditions of constant energy intake could convert a lean person to an obese state [Weinsier et al 1993]. A genotypic variation that could cause a reduction in expression or diminish the thermogenic activity of UCP1, UCP2 and/or UCP3 could underlie an inherited propensity to weight gain. However, the situation may not be so clear cut in humans, as paradoxically a reduction in energy expenditure has also been observed accompanying weight loss [Leibel et al 1995].

Adaptive thermogenesis:

BAT is a specialised thermogenic tissue with diffuse deposition throughout the whole body of mammals, with increased deposition in certain mammals, such as rodents as an adaptive mechanism for survival. BAT owes its thermogenic capacity to an abundance of mitochondria, hence providing a high oxidative capacity, high content of cytochrome oxidase and low content of ATP synthetase. In addition there is an unique stoichiometry of protein complexes on the inner membrane including the exclusive expression of UCP1. In certain mammals BAT-UCP1 activity and the generation of body heat, the process of cold or diet induced non-shivering thermogenesis, is a crucial adaptation in newborn mammals, cold-adapted rodents and in animals emerging from hibernation. The heat generated is circulated throughout the body via arteriovenous anastomoses present in BAT to achieve an overall thermoregulation and hence this tissue has a major role in regulating whole body temperature and weight in certain animals [Stuart et al 2001].

The biological pathway involved in heat induction appears to be primarily controlled by the hypothalamus acting through thermoregulatory centres with direct sympathetic nerve system (SNS) innervation of brown adipocytes by cell surface β-adrenergic receptors [Rothwell & Stock 1987; Ricquier et al 1986]. Exposure to cold and β3 adrenergic receptor agonists upregulate UCP1 expression and increase the insertion of the protein into the mitochondrial inner membrane [Silva & Rabelo 1997; Nagase et al 1996; Rehnmark et al 1990; Trayhurn et al 1987; Geloen 1990]. Cold activation of the SNS causes a cAMP dependent stimulation of lypolysis, through the possible action of the β-adrenergic receptor agonist, noradrenaline resulting in increased release and oxidation of fatty acids [Himms-Hagen 1995]. Increased intracellular FFA levels may increase both H+ conductivity of UCP1 similar to that of catecholamines and also provide fuel for mitochondrial respiration [Prusiner et al 1968; Bukowiecki et al 1981; Reed 1968]. Transgenic mice that are UCP1 deficient develop hypothermia both under cold conditions and when lacking the stimulatory effects of both FFA and norepinephrine [Enerback et al 1997; Cannon et al 1999; Boss et al 2000].

It has been proposed that BAT could also play an essential role in thermoregulation in humans particularly after birth and during feeding in newborns [Himms-Hagen 1995]. Furthermore, it has been shown that BAT has a wide spread representation in the adult human body, although only in small deposits. Therefore a relationship between BAT and energy balance or body weight regulation in adult humans cannot be completely excluded [Garruti & Ricquier 1992]. It has been suggested that BAT could be responsible for up to 1-2% of energy expenditure in humans, and that if there was an uncompensated defect in this

pathway, hypothetically there could be a 1 to 2kg gain in weight per annum [Lean 1989]. Therefore theoretically a dysfunctional UCP1 gene could lower the rate of energy expenditure resulting in small calorific inequalities over an entire life span, leading to an increase in fat storage and predispose an individual to the development of obesity [Ravussin et al 1988; Griffiths et al 1990]. Although depressed thermogenic responses have been observed in some subgroups of obese patients [Jequier & Schutz 1985] in thermo-neutral humans and large mammals there is less deposition of BAT except in neonates and it is unlikely that UCP1-BAT in adult humans has a major role in whole body energy homeostasis as found in rodents and hibernating mammals. [Lean et al 1986].

The idenfication of other uncoupling proteins expressed in tissues other than BAT has led to the concept that other cell types may also have important thermogenic properties [Harper & Brand 1995; Brand 1997]. UCP2 is expressed in a number of tissues implicated in the regulation of energy balance, including BAT, WAT and skeletal muscle. Although white adipose tissue (WAT) shares several characteristics with BAT such as the ability to synthesis and store triglycerides and release NEFA's. WAT is primarily concerned with storage and possesses relatively few mitochondria and is therefore unlikely to have a similar thermogenic activity to BAT. In contrast skeletal muscle, in which both UCP2 and to a greater extent UCP3 is selectively expressed, is quantitatively the major site of adaptive thermogenesis and energy expenditure, with fat-free mass mainly comprised of skeletal muscle accounting for 80% of the variation of resting energy expenditure between individuals [Ravussin 1992; Zurlo et al 1990].

In rodent BAT both UCP2 and UCP3 have been induced by cold exposure, although the response varies between species and strain and appeared to be dependent on the temperature and length of exposure [Himms-Hagen 1990; Denjean et al 1999; Larkin et al 1997; Boss et al 1997a, 1997b & 2000; Carmona et al 1998; Emilsson et al 1998; Yoshitomi et al 1998a; Enerback et al 1997]. However, evidence is inconsistent and scant in rodents and humans that tissues other than BAT, and the uncoupling protein homologues are involved in cold adaptation mechanisms (table 2.1.4) [Fleury et al 1997; Boss et al 1997a & 1998c; Larkin et al 1997; Carmona et al 1998]. Nevertheless, UCP2 expression has been observed to be up-regulated in specific tissues (table 2.1.4) in response to cold apparrently as part of the recovery of body temperature following endotoxin-mediated hypothermia in mice. It has been proposed that localised thermogenic effects may exist to maintain function, or protect

essential tissues from damage under conditions of extreme cold [Adams 2000; Simonyan et al 1998; Skulachev et al 1998; Richard et al 1998].

The regulation of energy homeostasis / body fat equilibrium in mammals is dependent on a balance between diet, regulated by energy input through appetite control, and energy expenditure. It has been proposed that there is operative in rodents an adaptative thermogenic mechanism provided by BAT and mediated by UCP1 activity that controls dietary imbalance. Facultative diet induced thermogenesis (DIT) in BAT could be responsible for preventing obesity in rodents by buffering excess calorific intake by regulating energy expenditure, and body fat levels [Rothwell & Stock 1979 & 1981; Segal et al 1992; Himms-Hagen 1990].

It has been observed that high fat feeding induces non-shivering thermogenesis in rodents with a concomitant increase in BAT-UCP1 [Rothwell & Stock 1979]. Furthermore, evidence suggests that rodent strains which are prone to obesity may have a reduced thermogenic activity possibly as a result of defective BAT thermogenesis or reduced tissue deposits, compared to those that are relatively resistant following a high fat diet [Schemmel et al 1970; Hamann et al 1996; Himms-Hagen 1990; Seydoux et al 1982]. Studies that involved the ablation of BAT (60-95%) through the expression of a dipteria toxin gene with a UCP1 promoter (UCP-DTA) found a reduction (50%) in BAT thermogenesis that augmented obesity, hyperphagia and insulin resistance in the transgenic mice [Lowell et al 1993]. Furthermore, a reciprocal experiment in which gene constructs (aP2-*UCP*) in mice up-regulate UCP1 expression in both BAT and ectopically in WAT, adiposity was reduced and transgenic mice became resistant to high fat diet induced obesity [Kopecky et al 1995 & 1996; Baumruk et al 1999]. The UCP1-DTA transgenic mice were also leptin resistant suggesting that leptin may a play a role in thermogenic pathways, with possible direct effects on UCP1 expression or via sympathetic outflow [Mantzoros et al 1998; Elmquist et al 1998; Haynes et al 1997; Haque et al 1999; Satoh et al 1999].

Overall the evidence above suggests a role for UCP1 in body weight regulation in rodents. However, UCP1-DTA mice raised at thermoneutrality do not become hyperphagic or obese and UCP1 knockout mice also do not become obese suggesting the existence of other compensatory adaptative mechanisms [Melnyk et al 1997; Enerback et al 1997]. Regulated energy expenditure is controlled largely by adrenergic stimulation with skeletal muscle the most important site of catecholamine and diet induced thermogenesis in both rats and humans [Thurbly & Trayhurn 1979; Astrup et al 1986; Simonsen et al 1993]. Therefore

following the discovery of UCP2 and UCP3 many investigations have been carried out to determine whether these uncoupling proteins in other tissues also play a role in energy homeostasis.

In rodents the regulation of UCP2 or UCP3 mRNA expression appears to influenced by many different dietary and related hormonal factors including thyroid hormone, leptin, insulin, β3-adrenergic stimulation, high fat diet, plasma FFA, fasting and exercise (table 2.1.4)[Gong et al 1997; Larkin et al 1997; Astrup et al 1985 & 1989; Spraul et al 1993; Boss et al 1998c & 1999; Millet et al 1997; Weigle et al 1998; Cortez-Pinto et al 1999; Savontaus et al 1998]. Some inducers such as thyroid hormones are established effectors of thermogenesis and metabolic rate and correlate positively with mitochondrial uncoupling activity in both liver and skeletal muscle, and may have direct action on mitochondria [Harper & Brand 1995; Lanni et al 1999; Goglia et al 1999; Jekabsons et al 1999]. The thyroid hormones L-thyroxine (T4) and triiodo-L-thyronine (T3) both have marked thermogenic effects and promote both UCP2 and UCP3 mRNA expression in certain tissues, independently of the effects of leptin [Goglia et al 1999]. Furthermore, in a variety of rodent tissues the expression of UCP2 increases and decreases in response to hyperthyroid and hypothyroid states respectively [Lanni et al 1997; Masaki et al 1997].

Plasma leptin also increases energy expenditure, suppresses apetite, and induces a concommitant up-regulation of BAT UCP1 expression in rodents [Mantzoros et al 1998; Scarpace et al 1997]. In the leptin deficient obese *ob/ob* mice, the effect of infusion of leptin on these pathways leads to a significant reduction in body weight. Leptin also regulates UCP2 expression and FFA metabolism in adipocytes (table 2.1.4)[Zhou et al 1997; Halaas et al 1995; Hwa et al 1996 & 1997]. However, the mechanisms are not entirely elucidated as contrary to what would be expected leptin appears to suppress UCP2 expression in both rodent WAT and hepatocytes, although increased uncoupling activity is detected in both these tissues [Gimeno et al 1997; Larrouy et al 1997; Chavin et al 1999; Melia et al 1999]. In contrast to the effects on UCP2, transfected *ob/ob* mice expressing leptin UCP3 expression skeletal muscle was increased. Supporting this observation, in *fa/fa* rats that are leptin receptor deficient, UCP3 mRNA levels were decreased [Boss et al 1998c; Liu et al 1998; Gong 1997]. Moreover, intra-cerebroventricular infusion of leptin during food restriction prevented both an expected decrease in UCP3 expression in muscle and increased UCP3 mRNA levels in BAT [Cusin et al 1998].

Studies in rodents that are prone and resistant to obesity, show that high fat diet in addition to induction of UCP1 in BAT also up-regulated UCP2 expression in WAT, suggesting a role for UCP2 in mediating energy expenditure, regulation of body and the delay of onset of obesity in resistant strains [Fleury et al 1997; Gong et al 1999; Matsuda et al 1997; Surwit et al 1998, Rippe et al 2000]. Furthermore, UCP2 may have played a compensatory role in stemming the progression to obesity in mice where UCP1was knocked out [Enerback et al 1997]. However, high levels of UCP2 mRNA are maintained in tissues under conditions of suppressed thermogenesis, and observed increases in UCP2 expression in WAT in response to high fat diet are actually very modest [Rippe et al 2000; Gong et al 1999]. This suggests that unlike UCP1, modulation of UCP2 alone in rodents would be insufficient to prevent obesity. Furthermore, the relationship of UCP2 with dietary intake is not entirely clear as UCP2 expression responds variably to different dietary components in different tissues, with expression being downregulated in some mouse tissues, such as stomach and intestines in response to high fat diet (table 2.1.4) [Tsuboyama-Kasaoka et al 1999; Rippe et al 2000].

UCP3 expression in rodent skeletal muscle was also induced by high fat diet, although observations have been inconsistent with strain and/or species dependent effects [Gong et al 1999; Matsuda et al 1997; Tsuboyama-kasaoka et al 1999; Surwit et al 1998; Fleury et al 1997]. However, on comparison of lean rats to an obese rodent model (Zucker *fa/fa* rats) a decreased thermogenic capacity was detected that correlated not only with decreased expression of UCP1 in BAT, but also with down-regulation (40%) of UCP3 mRNA expression in both BAT and soleus muscle [Himms-Hagen 1990; Boss et al 1998c]. Studies with transgenic mice over-expressing human UCP3 protein in skeletal muscle, the mice weighed less than their wild type litter-mates despite being hyperphagic [Clapham et al 2000]. This study suggested that UCP3 could be a determinant of human energy expenditure and metabolic efficiency and hence contributing to body weight regulation.

Variation in rodent experimental data for UCP2 and UCP3 could be due to the genotype of the animals influencing mRNA levels. However, changes in UCP2 or UCP3 mRNA levels may not be primary determinants of body fat content but a reflection of metabolic responses to changes in fat intake and body composition. The magnitude of diet induced increased expression of both UCP2 and UCP3 reported were also considered to be only modest when compared to other effects such as starvation, exercise and thyroid hormone.

Experimental studies in starvation have shown that in response to a severe energy deficit, energy expenditure falls by a magnitude greater than can be accounted for by the loss of

metabolically active tissues [Gaesser & Brooks 1984]. It is generally conceptualised that this disproportionate reduction in metabolic rate results from the autoregulatory suppression of basal thermogenesis, as a means to increase metabolic efficiency and provide a buffer against the energy deficit. Experiments involving diet restriction in rodents show differences in thermogenic activity in response to diet, possibly as a result of defects in the mechanisms necessary for the activation of thermogenesis [Himms-Hagen 1995; Champigny & Ricquier 1990]. Most studies in rodents have found acute calorific restriction causes a down-regulation of UCP1, UCP2 and UCP3 mRNA expression in BAT, consistent for true thermogenic roles for these proteins [Trayhurn et al 1987; Boss et al 1998c]. However, paradoxically under the same acute fasting conditions UCP3 expression was unexpectedly increased in skeletal muscle and UCP2 expression increased in skeletal muscle, WAT and pancreatic islets in rodents (table 2.1.4) [Bao et al 1998; Boss et al 1997a, 1998b & 1998c; Aubert et al 1997; Camirand et al 1998; Samec et al 1998a; Matsuda et al 1997 & 1998]. In contrast chronic long-term diet restriction results in decreased expression of UCP's (table 2.1.4)[Cusin et al 1998; Samec 1998; Cortright et al 1998; Esterbauer et al 1999; Boss et al 1998c].

Parallel differences in the regulation of UCP3 expression have also been observed during exercise in rodents (table 2.1.4)[Cortright et al 1999; Tsuboyama-Kasaoka et al 1998]. Acute short-term exercise, a one hour long bout, causes a transient increase of muscle UCP3 mRNA levels with expression peaking at 3 hours after the end of exercise, and completely disappearing after 22 hours. This is accompanied by acute metabolic and hormonal response similar to that observed with short-term calorific restriction [Tsuboyama-Kasaoka et al 1998]. However, following endurance training UCP3 expression was down-regulated [Boss 2000]. The observed differences in uncoupling protein expression are almost certainly due to the influence of other fluctuating factors determined by the feeding or exercise states. It is postulated that fasting increases muscle sympathetic nervous system tone, circulating catecholamines, and also induces adipose tissue lipolysis and lipoprotein lipase activity causing the release of fatty acid stores and hence elevating circulating FFA levels [Stich et al 1997]. The increased expression of UCP2/UCP3 under fasting conditions could be strongly under the influence of circulating plasma FFA levels and FFA fluxes towards tissues [Boss et al 1998b]. The thermogenic role of both UCP2 and UCP3 in rodents still remains highly contentious, but evidence from rodents does suggest an involvement in metabolic pathways concerned with dietary intake and energy balance.

INDUCER	SPECIES AND TISSUES								
	*RODENT BAT		*RODENT WAT	HUMAN WAT	*RODENT SKELETAL MUSCLE		HUMAN SKELETAL MUSCLE		*RODENT OTHER TISSUES
	UCP2	UCP3	UCP2	UCP2	UCP2	UCP3	UCP2	UCP3	UCP2
[1]COLD	↑+ *m*	↑ ↔	↑ ↔		↑+*m* ↔	↑ ↔	↑		↑*m* liver ↑*m* brain ↑*m* heart
β3-adreno-receptor agonists	↑*m* ↔*Zle/fa +m(ob)*	↑*Zfa*	↑*r/Zle/+m(ob)* ↔*Zfa+m(6J+AJ) m*CL		↔*Zfat* ↓ *Zle/+m*	↔*Zfat* sol			
Denervation	↔				↕ *m*	↑ gsn ↓ *m* gsn			
T3 thyroid	↑↔		↑		↑↔	↑*mCL*			↔liver
High fat diet	↑ +*m* ↔ +*m6J/ AJ*	↓	↑ +*mAJ/6J* ↔+*mAJ/6J* ↓		↑ qdr ↔ gsn/sol	↑ qdr/gsn ↔qdr			↑ liver ↓ +*m*Gut
Acute diet deprivation (fasting)	↔ ↓	↓	↑	↑L+ O	↑ *m* ↔	↑	↑L/O ↔OD	↑L/O ↔OD	↑pancreatic islets
Chronic food deprivation (Starvation)	↓		↓	(+UCP3 in L) ↓ O	↑ ↔gsn/ta ↓ sol		↓ O	↓ O	↓Liver
Exercise short bout					↑ *m* gsn ↔ gsn	↑gsn(w) ↔gsn(r) +*m* gsn			
Exercise Endurance	↔+*m*		↔+*m*		↑ *m* gsn ↓ ta/sol	↔ gsn ↓			
Increased Fat Mass	↑*m (ob)* ↓*Zfat*	↓*Zfat*	↑*W +m (ob/db)* ↔*Zfat* ↓ *Zfat*	↑SC ↓IA	↔*Zfat* ↓ *Zfat*	↓*Zfat* sol	↑ ↔ ↓ ab	↔ ↓	↑ liver *m (ob)* ↔liver *Zfat*
Diabetes	↓	↓	↑	↑	↔	↑	↑O ↔	↑O ↔	

Table 2.1.4: Factors that potentially regulate UCP2 and UCP3 mRNA transcription

	*RODENT BAT		*RODENT WAT	HUMAN WAT	*RODENT SKELETAL MUSCLE		HUMAN SKELETAL MUSCLE		*RODENT OTHER TISSUES
	UCP2	UCP3	UCP2	UCP2	UCP2	UCP3	UCP2	UCP3	UCP2
Leptin	↑*r/SD* ↔ r/*Z/SD*	↑	↑*r/Z/SD* ↔ *Z+m(ob)* ↓*m (6J)*	↔	↔*m (6J)* qdr	↑*m (ob)* ↓ *fa/fa*	↔	↑	↔liver *Z+m* ↓liver
PPARα agonists	↔		↔ *r*CL		↑*m (db/Ks)*	↑*m*CL ↔*m*CL			
PPARβ				↑		↑*m*CL			
PPARγ agonists	↑ *r/SD* +*m*CL ↔ *m(db)*		↑*m*CL ↔ *r/SD/W* +*m*CL		↑*m (KK)* sol ↔ *Z/SD*	↑*m*CL ↔*m*CL			
PPAR δ			↑*m*CL			↑*m*CL			
Retinoids	↑*m*CL		↑*m*CL			↑*m*CL			
TNF	↑		↑+*m*		↑+*m*				↑liver+*m*
GH				↔			↔	↔	
Insulin				↔O/L	↑	↑	↔O/L	↔O/L	
Streptozotocin	↓		↔		↑ gsn				↔liver

Table 2.1.4: Continued.

KEY: BAT=Brown adipose tissue; WAT=white adipose tissue; *RODENT, refers to studies in rats (strains included where known, see below) unless marked; *m (strain)*= mouse study or *+m (strain)*=additionally in mice.

mRNA expression denoted as:↓ decreased; ↑ increased; and ↔ unchanged in rat (*r*) mouse (*m*) tissues or cell lines (rat =*r*CL; mouse *m*CL).Rat strains: *r*=unspecified; *SD*=Sprague-Dawley; *W*=Wistar; *Z*=Zucker *Zle*=zucker lean, *Zfat*=Zucker fatty-obese strain. Mouse strains: *m*=unspecified; *6J*=C57BL/6J Diabetes/Obesity susceptible; *AJ*=A/J obesity resistant strain; *Ks*=C57B1/Ks; *db*= db/db diabetic Ks; *KK*=hyperglycaemic/ hyperinsulaemic strain; *ob*=ob/ob obese leptin KO.

Skeletal Muscle Subtypes: Sol=soleus; qdr= quadriceps; gsn= gastrocnemius (w)=white,(r)=red; ta=tibialis anterior; ab=abdominal :

Adipose depots; SC=Subcutaneous; IA=Intra-abdominal. Phenotype L=lean; O=obese; D=T2DM; OD= obese/T2DM

All data derived from references quoted in text in sections 2.1.5 and 2.1.6.

Thermogenic role in humans:

The implication that uncoupling proteins other than UCP1 can modulate proton leaks in other tissues and hence energy expenditure in rodents has led to many studies trying to correlate UCP transcriptional and/or thermogenic activity with overall weight gain or loss in humans. In humans UCP2 expresses at its highest level in adipose tissue and decreased thermogenesis has coincidentially been observed in WAT of obese individuals [Gimeno et al 1997; Bottcher & Furst 1997]. However studies in humans have found paradoxically that UCP2 mRNA levels positively correlated with increased fat mass and BMI, and so consequently there was no relative difference between lean and obese subjects (table 2.1.4) [Millet et al 1997]. Therefore, it appears that there is no UCP2 related reduction in thermogenic capacity in obese subjects. A similar strong positive correlation with UCP2 expression with subcutaneous adipose mass was also reported in premenopausal obese women on a very low calorie diet [Barbe et al 1998], however in another study in the same tissue there was no correlation [Millet et al 1998]. In contrast, examination of visceral (intraperitoneal) and extraperitoneal adipose tissues, found decreased and increased UCP2 expression respectively in morbidly obese subjects compared to lean subjects (table 2.1.4)[Oberkofler et al 1998]. It is apparent that overall, correlations with basal energy expenditure, obesity and UCP2 expression in human adipose tissue have been inconsistent. Nevertheless, some of these differences may reflect differential tissue specific expression of UCP2 in regional adipose deposits. Interestingly, higher levels of UCP2 mRNA were also observed in WAT in both *ob/ob* and *db/db* obese mouse models compared to their equivalent lean littermates, although these observations may be influenced by the lack of a complete leptin signalling pathway in both models [Gimeno et al 1997].

Correlations of UCP2 mRNA expression in skeletal muscle have also been inconsistent, with positive correlations with BMI, percentage of body fat and several other indices of human obesity in some studies [Bao et al 1998] and no variation of UCP2 mRNA levels in obese subjects in other studies (table 2.1.4) [Millet et al 1997]. The differences in expression of muscle UCP2 could also be due to muscle types, as contrary to the other studies decreased expression of UCP2 (28%) was observed in the abdominal muscle of obese subjects [Nordfors et al 1998]. Similarly, expression patterns of UCP3 in skeletal muscle and obesity in humans are also inconsistent with no correlation between BMI/obesity and UCP3 mRNA levels in muscle reported in several studies [Millet et al 1998; Nordfors et al 1998]. However, in Pima Indians expression of both the UCP3L and UCP3S isoforms did

negatively correlate with BMI, with increased expression associated with a reduction in body mass [Schrauwen et al 1999a]. Furthermore, there was a positive correlation between UCP3L expression and sleeping metabolic rate [Schrauwen et al 1999a].

There have been very few studies examining the effect of dietary intake on uncoupling protein expression in humans (table 2.1.4). However, observations that infusion of a triglyceride emulsion into both rats and humans elevated circulating levels of both FFA's and skeletal muscle UCP3 expression, but without a similar effect on UCP2 expression in either skeletal muscle or adipose tissue, suggested a role for UCP3 cannot be completely dismissed [Weigle et al 1998; Khalfallah et al 2000]. FFA's up-regulated uncoupling protein expression, with high levels of plasma NEFA correlated with increased UCP3 expression in muscle of obese subjects might also suggest a role in a compensatory mechanism to prevent weight gain [Boss et al 1998a].

Studies in humans on the effects of food deprivation on uncoupling protein expression have found effects similar to that observed in rodents (table 2.1.4). Similarly there appeared to be a biphasic effect with acute calorific restriction up-regulating UCP3 expression in skeletal muscle, and UCP2 expression in both adipose tissue and skeletal muscle in both lean and obese subjects (table 2.1.4) [Millet et al 1997 & 1998]. In contrast, expression of UCP3 and UCP2 in skeletal muscle and WAT respectively are decreased in both non-diabetic and T2DM obese subjects following a prolonged and stable weight reduction by a very low calorific diet (table 2.1.4) [Vidal-Puig et al 1999; Schrauwen et al 2000]. Similar effects have also been observed on uncoupling protein expression following gastric banding surgery in humans and in mouse tibialis anterior muscle when animals are maintained in a thermoneutral environment [Esterbauer et al 1999; Boss et al 1998c].

The differences in expression observed under varying conditions of dietrary deprivation in humans may be related to fluctuating levels of plasma FFA'. Increased FFA levels have been correlated positively with the up-regulation of UCP3 expression in both rodents and humans in various tissues including WAT, BAT, muscle and pancreatic islets [Boss et al 1997b & 1998a; Gong et al 1997; Gimeno et al 1997; Hidaka et al 1999; Weigle et al 1998; Millet et al 1997; Khalfallah et al 2000; Matsuda et al 1997 & 1998; Warden et al 1993; Surwit et al 1998; Fleury et al 1997; Carmona et al 1998b; Hwang et al 1999; Shimabukuro et al 1997; Vigueriie-Bascands et al 1999]. Similarly, acute exercise induced UCP3 up-regulation is accompanied by raised plasma FFA levels [Cortright et al 1999; Tsuboyama-Kasaoka et al 1998]. In contrast to acute fasting, during long stable food restriction plasma

FFA levels remained unchanged suggesting that under these conditions modulation of UCP3 expression appears to be independent of FFA levels. However, chronic dietary effects and correlations of plasma FFA levels with induction of UCP2 expression have been less consistent in both rodents and humans [Boss et al 1998c; Vidal et al 1999; Barbe et al 1998; Khalfallah et al 2000].

FFA's may have a direct action on UCP expression being controlled by the intracellular metabolism and oxidation of lipids. Alternatively the recruitment of intermediary nuclear receptors may be involved in mediating expression. Factors that have been implicated are members of peroxisome proliferator-activated receptor (PPAR) family. PPAR's are involved in adipogenesis energy homeostasis and transcriptional activation of lipid-regulated genes [Brun et al 1996; Willson et al 1996]. The activity PPAR isoforms in response to changes in plasma levels and muscle uptake of specific fatty acids could have a direct action on UCP expression, without the involvement of lipid oxidation [Schoonjans et al 1996]. In human skeletal muscle two PPAR subtypes predominant, PPARα and PPARβ, with the former expressed at high levels [Auboeuf et al 1997; Costet 1998]. PPARα, PPARß and PPARγ have all been implicated in the regulation of UCP1, -2 and –3 mRNA (table 2.1.4) [Brun et al 1999b; Kelly et al 1998; Aubert et al 1997; Camirand et al 1998; Viguerie-Bascands et al 1999]. In addition peroxisome proliferator-activated receptor response elements (PPRE) including RXR/PPAR hetereodimer sites have been identified in the promoters of UCP genes [Solanes et al 2001; Tu et al 1999b & 2000]. In *in vitro* studies, administration of a retinoid receptor RXR agonist LGD1069 has an additive effect on UCP expression [Kliewer et al 1997; Carmona et al 1998a; Solanes et al 2001; Tu et al 2000]. The observations that a deficiency in PPARα could to lead to obesity might indicate possible interactions between PPAR's, uncoupling proteins and weight gain [Su 1998; Costet 1998].

There is considerable evidence implicating FFA's in modulating UCP expression in certain tissues under different dietary conditions. Plasma glucocorticoids levels can also have similar effects to plasma FFA levels during acute fasting and long-term food restriction [Ohno et al 1990; Guezennec et al 1988]. Furthermore, factors such as leptin and insulin may also be involved in metabolic pathways modulating UCP expression under different dietary conditions. The results of one study inferred that up-regulation of UCP2 by leptin in adipocytes may contribute to the stimulation of acetate and fatty acid degradation by leptin [Ceddia et al 2000]. A reduction in FFA levels could consequently also down regulate UCP expression, suggesting the possible existence of a feedback loop. In rodents insulin is also

positively correlated with UCP2/UCP3 expression in skeletal muscle and WAT [Pederson et al 2000; Savontaus et al 1998] and pancreatic islet insulin status in rats has been correlated with UCP2 mRNA levels [Kassis et al 2000]. However in humans the infusion of insulin during calorific restriction appears not to modify UCP2/UCP3 mRNA levels [Millet et al 1997].

The inconsistent and unexpected effects of diet and dietary restriction on proteins that presumably have thermogenic activity could be due to a number of reasons. The suppression of uncoupling protein expression following long term chronic food restriction and gradual weight loss may be part of a mechanism to assist rapid recovery in body weight by reducing whole body energy dissipation and decreasing energy expenditure.this would be consistent with a thermogenic role for UCP2 and UCP3 [Leibel 1995]. A role for UCP3 was further supported in that it is downregulated following endurance training and associated with a decreased diet-induced thermogenesis in rats [Boss et al 2000; LeBlanc et al 1982]. The observations under conditions of acute starvation are contrary to a thermogenic role. However, studies using direct microcalorimetric measurements in mice suggest that up-regulation of UCP3 expression during fasting is not necessarily reflected in changes in muscle basal heat production rate, which was found to remain unchanged [Boss et al 1998c]. This concurrs with findings that decreased expression in skeletal muscle of UCP3 mRNA is not reflected in a reduced cell uncoupling protein levels [Schrauwen et al 2000]. Furthermore, following induction of uncoupling using a chemical uncoupler, FCCP (carbonyl-cyanide p- trifluoromethoxyphenylhydrazone), muscle heat production rates are diminished (31%) in fasted mice compared to mice fed *ad libitum.* Thermogenesis by anabolic-catabolic pathways may therefore be reduced during fasting and it is possible that up-regulation of UCP3 is a compensatory mechanism to maintain normal heat production under these conditions [Boss et al 1998c]. In addition some of the inconsistencies observed with both UCP2 and UCP3 expression in skektal muscle may be due to specific effects in muscle types. Fasting in rats only increased UCP2 mRNA in soleus muscle and not in other muscle types [Samec et al 1998a] and studies on UCP3 expression in both fasted rats and humans found induction of expression was dependent primarily on the constitution of muscle as either type I fibres or type II fibres [Samec et al 1998b & 1999; Vidal et al 1999]. In addition completely opposite effects on UCP3 expression were observed within the same muscle tissue (gastrocnemius) following denervation between rodent species (rat and mouse) (table 2.1.4) [Cortright et al 1999].

Finally the inconsistent observations on UCP mRNA expression between all these studies could be the result of differences in the relative stage of progression to the disease state [Simoneau et al 1998]. Alternatively the expressed levels of mRNA do not truly reflect cellular uncoupling protein content and are not necessarily indicative of the quantity of 'active' uncoupling protein that is actually inserted into the mitochondrial inner membrane. Indeed, in one study in human skeletal muscle it was demonstrated that cellular levels of UCP3L mRNA were not equivalent to active UCP3 protein content [Schrauwen et al 2000]. Alternatively UCP2 and UCP3 may not be involved in obligatory or adaptive thermogenesis and other roles have been proposed below.

Fuel Utilisation:

Observations that there were concomitant increased uptake and utilisation of lipids as fuel substrates by skeletal muscle with UCP expression has been interpreted as a possible alternative functional role for uncoupling proteins [Boss et al 1998b; Samec et al 1998a & 1998b]. Mixed muscle tissue consisting of both type I and II fibres have a high capacity to switch between glucose and lipids as fuel substrates under fasting conditions. Studies investigating the effects of starvation on UCP2/UCP3 expression in this type of muscle in humans and rats suggested that effects on UCP expression correlated with fuel utilisation [Millet et al 1997 & 1998; Samec et 1998]. Re-feeding following starvation, down-regulated expression of UCP's that correlated with a reduced requirement for lipids as fuel substrates. This mechanism could also be equally applicable to the observations following gradual weight loss of decreased UCP3 expression, as in one particular study there was no actual decrease in UCP3 protein levels in muscle, but expression was correlated with levels of fatty-acid binding protein (FABP) content [Schrauwen et al 2000]. Changes in skeletal muscle cytosolic-FABP is related to uptake and oxidation of FFA by skeletal muscle [Veerkamp & van Moerkerk 1993], and maintaining oxidation levels following weight loss would facilitate further weight loss, suggesting also a role for UCP3 in the handling of lipids as fuels [Schrauwen et al 2000]. Furthermore, it has been proposed that the occurrence of a higher content of UCP2 in skeletal muscle in obese humans [Simoneau et al 1998], is in accord with a reduced postabsorptive lipid utilisation by muscles and consistent with a role in the regulation of lipids as fuel substrates [Boss et al 1998a & 1998c; Samec et al 1998a]. In humans heterozygote carriers of an exon 6 splice donor mutation in the UCP3 gene, which produces a protein identical to hUCP3S, were associated with a reduction (50%) in

fat oxidation capacity and an elevated non-protein respiratory quotient [Argyropoulos et al 1998b]. Coincidentally the mutation had double the allele frequency in obese subjects compared to lean controls in the African-American group studied. Finally, further evidence in favour of a role in body weight regulation through fuel partitioning and insulin sensitivity was provided by measurements of UCP2 and UCP3 mRNA levels in muscle biopsies. Levels correlated with the promotion of carbohydrate oxidation and insulin-stimulated glucose disposal rates, rather than influencing basal energy expenditure or insulin-induced thermogenesis in both T2DM and non-T2DM subjects [Willi et al 1998].

Control of Reactive Oxygen Species (ROS):

It has been postulated that uncoupling proteins may also have physiological roles in antioxidant activity by the maintaining low levels of the reactive oxygen species (ROS) that could cause oxidative damage to cells [Skulachev et al 1998]. The high expression of UCP2 in cells of the immune system suggested a putative role in immunity and /or thermoregulatory response to infection ie.fever [Fleury et al 1997]. Subsequent studies relying on the sensitivity of UCP2 to GDP in immune system cells, proposed that UCP2 could be involved in the control of hydrogen peroxide production [Negre-Salvayre et al 1997]. Elevated UCP2 expression in *ob/ob* obese mouse hepatocytes in addition to helping to balance ATP supply may also protect the liver from increased ROS generation as a result of increased substrate supply and fat oxidation [Chavin et al 1999]. Studies using UCP2 [Arsenijevic et al 2000] and UCP3 [Vidal-Puig et al 2000; Gong et al 2000] knockout mice have also provided further evidence that the control of activity and /or expression of UCP's may modulate levels of ROS in tissues. Reduced UCP2 levels in macrophages increases ROS production and this could act as a bactericidal agent during infection, whereas increasing UCP levels in other tissues in response to infection, food restriction, or exercise might prevent the effects of excessive oxidative stress. Finally, cytokines involved in inflammation processes such as tumour necrosis factor (TNFα) also appear to regulate expression of uncoupling proteins [Masaki et al 1999].

2.1.6 Potential role of UCP2-UCP3 inT2DM

Normoglycaemic women at increased risk of future diabetes display defective post-prandial thermogenesis [Robinson et al 1992 & 1994] and there is evidence of linkage to UCP/ UCP3 chromosomal region with post-glucose load insulin levels in Pima Indians [Pratley et al

1998]. Uncoupling proteins have been implicated in glucose utilisation, insulin resistance and insulin secretion. It has been postulated that UCP's may provide not only a defense mechanism against high fat induced obesity but also against impairment of glucose metabolism [Matsuda et al 1997]. Studies with transgenic mice over-expressing the human UCP3 gene in skeletal muscle found that there was not only a reduction in weight, despite being hyperphagic, but mice also exhibited lower fasting plasma glucose, lower insulin levels and increased glucose clearance rates [Clapham et al 2000].

Studies of UCP2 or UCP3 expression in human skeletal muscle found no difference in induction between obese and lean subjects under conditions of food restriction [Millet et al 1997; Boivin et al 2000]. In contrast studies in matched obese T2DM and non-T2DM subjects under a 5-day calorific restriction found skeletal muscle UCP2 and UCP3 expression was only induced in the non-diabetic subjects, despite diabetics having relatively elevated skeletal muscle UCP2 and UCP3 mRNA levels compared to non-diabetics under normal dietary conditions [Vidal et al 1999]. Vidal et al proposed that the lack of up-regulation of expression in the diabetics could be due to both the absence of increased plasma NEFA levels and the lack of up-regulation of expression of adipose tissue hormone sensitive lipase (HSL), which are both normally elevated by acute calorific restriction [Vidal et al 1999]. HSL is a rate-limiting enzyme of lipolysis that promotes the hydrolysis of triglyceride stores in the adipose tissue and hence increases the release of fatty acids. Obesity and T2DM are both pathologically characterised by abnormalities of lipid metabolism, with reduced lipid utilization and oxidation associated with diminished plasma fatty acid uptake by skeletal muscle resulting in increased plasma NEFA levels and fuel partitioning associated with insulin resistance [Kelley & Simoneau 1994; Blaak et al 1999]. If lipid metabolism and uncoupling expression are closely linked in muscle, an altered expression/regulation could occur in diabetic muscle. Further analysis of the relative mRNA levels of the two splice variants of UCP3 in skeletal muscle found that whereas in the non-T2DM obese subjects levels of transcripts were similar, in the diabetics the UCP3S isoform was 2 fold (7fold more than non diabetics) more abundant than the L isoform (3fold greater than in non-diabetics). This resulted in the UCP3S isoform accounting for two-thirds of the total UCP3 mRNA in diabetics [Vidal et al 1999]. Overall, under normal dietary conditions UCP3 mRNA levels were higher in diabetics, however the increased ratio of the UCP3S isoform might result in a possible impairment of function. The exon 6 splice variant mutation has been implicated in diminished fatty acid uptake and fat oxidation capacity

[Argyropoulos et al 1998b]. Acute calorific restriction only increased UCP3 expression in the non-diabetics and not diabetics, but this was entirely the due to the up-regulation of the UCP3L isoform, which was also positively correlated with a concomitant increase in NEFA and HSL mRNA levels [Vidal et al 1999]. A similar increased ratio of UCP3S to UCP3L isoforms in diabetics has been observed in another study, although in contrast to the Vidal et al study, steady state UCP3 muscle mRNA levels were significantly lower in the diabetics compared to controls [Bao et al 1998]. A significant reduction in UCP3 muscle mRNA levels in diabetics was also reported in another study, although the ratio of splice isoforms were not analysed [Krook et al 1998].

In respect of muscle UCP2 levels these have been recorded as being either increased or no different in diabetics compared to non-diabetics [Vidal et al 1999; Bao et al 1998; Krook et al 1998]. However, elevated levels of muscle UCP2 mRNA have been positively correlated with insulin stimulated glucose uptake and GLUT4 levels, suggesting a link with glucose utilisation in muscle [Tsuboyama-Kasaoka et al 1998; Cortright et al 1999; Boss et al 1998e]. This is further supported by the demonstration in rats that thiazolidinediones increased glucose catabolism purportedly by up-regulating skeletal muscle UCP2 expression possibly through intermediatory PPAR activity [Shimokawa et al 1998].

UCP2 is also expressed at high levels in rat pancreatic islets. Over-expression of the UCP2 gene in rat islets normalised the diminished glucose stimulated insulin secretion present in the Zucker diabetic fatty rat [Wang et al 1999] and inhibited it in normal rats [Chan et al 1999]. These findings implicate UCP2 in modulating β-cell function and insulin secretion possibly by controlling fluctuations in cellular ATP content. Up-regulation of UCP2 expression by factors such as increased plasma FFA levels, in the pre-diabetic state could contribute to the loss of glucose responsiveness in the pancreatic islets [Chan et al 2001].

2.1.7 Linkage and association studies in UCP homologues

Genetic studies have reported associations of human UCP1 variants, primarily the A3826G variant, with effects on body composition and obesity. Positive associations have been found with; fat gain over time [Oppert et al 1994; Cassard-Doulcier et al 1996]; synergistic effect of the Trp64Arg mutation of the β3 adrenergic receptor gene with weight gain in morbid obesity [Clement et al 1996]; resistance to weight loss in obese subjected to a low calorie diet [Fumeron et al 1996] and effects on RMR in obese Finns [Valve et al 1998]. However, these studies have been inconsistent and inconclusive, with equally as many negative

association studies in various ethnic groups [Urhammer et al 1997a; Gagnon et al 1998; Schaffler et al 1999; Hamann et al 1998]. However, differences in the expression of UCP1 has been shown in intraperitoneal tissue between obese and lean subjects [Oberkofler et al 1997] with common sequence variation of UCP1 gene accounting for variability of UCP1 mRNA abundance [Esterbauer et al 1998].

Evidence of linkage to quantitative trait loci (QTL) relevant to obesity and T2DM have been found in humans between markers mapped to the chromosomal region encompassing the human UCP2/UCP3 gene cluster on 11q13. Studies in a French Canadian population has revealed highly significant linkage with resting metabolic rate (RMR) with two microsatellite markers D11S911 $p<0.000002$ and D11S916 $p=0.006$ (figure 2.2) [Bouchard et al 1997] and suggestive linkage ($p=0.02$-0.04) with D11S1321 with quantitative traits of percentage fat and fat mass. D11S911 and D11S916 closely flank the UCP2-UCP3 cluster and also have strong evidence for linkage with the eating disorder anorexia nervosa at significance levels of $p=0.0002$ and $p=0.09$ respectively [Campbell et al 1999]. Evidence for suggestive linkage has also been found to markers (D11S2000 and D11S1975) in close proximity to human UCP2/UCP3 region with a prediabetic phenotype, (2hr OGTT insulin concentrations) in Pima Indians [Pratley et al 1998]. A putative type I diabetic gene, IDDM4 is also mapped to D11S1917 within this same 30cM chromosomal region [Hashimoto et al 1994]. Suggestive linkage (MLS of 2.6) was also found at D11S1337 and it has been postulated that there could be a susceptibility locus within 15cM of this marker. Interestingly D11S916 and D11S911 are mapped to within 5 to 10 cM of this marker.

Murine UCP2 is mapped to a region on chromosome 7 that is syntenic to the human chromosome 11q13 [Boss et al 1998d]. There has been several quantitative trait loci (QTL) associated with obesity and diabetes linked to this mouse region [Yoshioka et al 1997; Surwit et al 1998]. The region is also co-insides with a QTL's for obesity in three independent mouse models and one congenic strain [Warden et al 1993 & 1995; Seldin et al 1994; Taylor et al 1996]. Furthermore, the region harbours QTL's for both diet induced obesity and diabetes in the C57BL/6J (B6) mouse [Surwit et al 1988]. QTL's for plasma hyperinsulaemia and glucose are tightly linked to the tubby mutation also found in this region, but are thought to be independent of the tubby mutation [Pecqueur et al 1999]. In rats the UCP2 and UCP3 genes have been localised to a region of chromosome 1 which has been linked to glucose intolerance and adiposity in the Goto-Kakizaki (GK) type II diabetic rat model [Kaisaki et al 1998].

A common human variant identified in exon 4 (C164T) of UCP2 causes an alanine to valine (A55V) amino acid change has been associated with variation in sleeping metabolic rate in Pima Indians [Walder et al 1998]. In addition, two further independent studies in Danish Caucasians found subjects homozygous for the valine allele had a lower 24 hour resting energy expenditure, enhanced metabolic efficiency, and lower fat oxidation [Astrup et al 1999] and with heterozygous subjects associated with lower BMI [Urhammer et al 1997b]. These findings suggest that variants of UCP2 could influence body weight regulation, progressive weight gain and hence an increased risk to obesity and/or T2DM in humans.
At the inception of this thesis no UCP2/UCP3 gene variants had been identified nor had any association studies been published.

2.1.8 Aim of studies on the UCP2 and UCP3 genes

The aim of this section of the thesis was initially to identify polymorphic variation within the UCP2 and UCP3 genes in two different ethnic groups. Common variants would then be further investigated to determine whether genetic variation in either gene is associated with susceptibility to obesity, T2DM or intermediate quantitative traits that predispose to either disease. A cohort of British obese and lean subjects would be used to analyse relationships with overt obesity and with traits such as an underlying indices of energy expenditure. Secondly a complementary family and population based South Indian study groups were to be utilised to analyse association of uncoupling protein variants with T2DM and related intermediate quantitative traits. Hypothetically a South Indian study group not fully exposed to obesity promoting environmental factors should offer certain advantages [Ramachandran et al 1992]. In Caucasian populations of European or North American extraction the majority of the recent rise in the prevalence of obesity can be explained by modern lifestyle. The effect of genotype on a phenotypic trait might be more detectable in a population not subjected to the same extent the compounding effects of 'westernisation'.

2.2 CALPAIN 10

2.2.1 Linkage to *NIDDM1*

The eventual positional cloning of the first potential T2DM gene, calpain 10 (CAPN10) to the *NIDDM1* region at the distal long arm of chromosome 2 was achieved as a direct result of the information gained from the first genome wide scan for T2DM [Hanis et al 1996; Horikawa et al 2000]. The original genome scan was a biphasic study of two Mexican American (MA) groups, with a primary study group consisting of 330 affected pairs from 170 sibships and 408 individuals, and a second phase group of 110 Mexican American affected sibpairs both from Starr County Texas. The MA population represented had a high frequency of T2DM approximately three times that of the general US population. The prevalence of disease in Native Amerindians is ten times higher than the general US population [Knowler et al 1990; King & Rewers 1993; Gardner etal 1984; Stern et al 1990]. Genes predisposing MA to T2DM are derived from their Native Amerindian ancestors, with 31% of their contemporary gene pool from this ancestry. Therefore the risk of acquiring these genes is roughly proportional to the percent of the gene pool derived from this ancestral source.

The primary scan utilising 490 markers found 19 markers with nominal evidence for linkage (MLS >0.74, $p<0.05$). One region on Chromosome 2q (D2S125) showed the overall strongest evidence of suggestive linkage with a MLS of 3.2 ($p<0.0005$) and combined with the second phase generated a combined MLS of 4.1. Marker D2S126, 28cM proximal to D2S125 had the second highest MLS with an MLS of 2.58. Multipoint analysis was carried out at three different values of λ_s [Risch 1990] with an upper limit set at 2.8 reflecting the observed ratio of risk for siblings in MA families with T2DM. Multipoint analysis at a λ_s of 1.6 produced a maximum multipoint lodscore of 3.05 for D2S125, providing evidence of linkage with susceptibility with T2DM with an estimated sibling relative risk for T2DM accounting for between 21% and 30% of the familial clustering of T2DM in this MA study group [Hanis et al 1996]. Furthermore, at this λ_s level exclusion of 71% of the genome was possible including the majority of loci that had nominal evidence in the two-point analysis, with only two other regions other than *NIDDM1* on chromosomes 3 and 15, having appreciable positive multipoint lodscores.

2.2.2 Positional Cloning of CAPN10 gene

Further genotyping and refinement of genetic maps placed *NIDDM1* near D2S140, with a LOD score interval spanning 12cM at 2q37. Analysis for epistasis (ie. evidence for statistical interactions between unlinked regions that allows multi-point allele-sharing analysis to take the evidence for linkage at one region into account in assessing the evidence for linkage over the rest of the genome) with other loci demonstrating nominal evidence of linkage, found an interaction of genes on chromosomes 2 *(NIDDM1)* and 15 (near *CYP19*). The statistical weighting derived from this interaction increased the LOD score for D2S125 from 4.1 to 7.3. This reduced the putative susceptibility region for NIDDM1 to 7cM between map positions 259-266 cM [Cox et al 1999]. In this chromosomal sub-region (2q37.3) 1cM equates to approximately 240kb so the physical distance confronted was surprisingly less than the 7Mb normally expected (1cM-1Mb). There were no obvious candidate genes in this interval, but with the utilization of many SNP's identified in this region, several genes were localised by LD mapping.

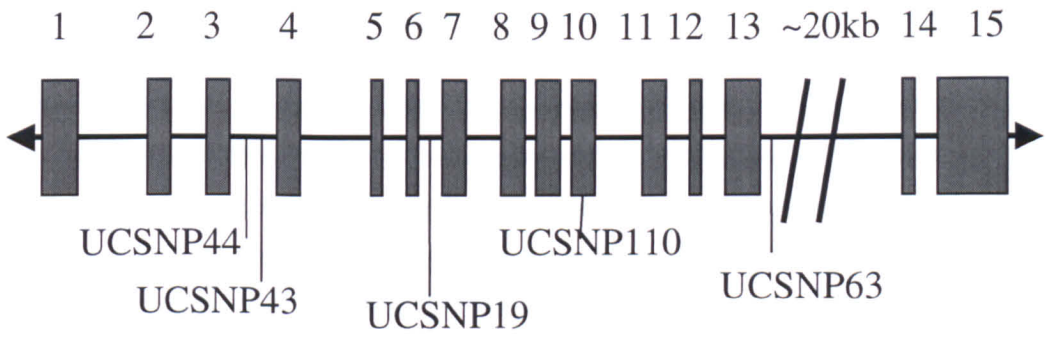

Figure 2.2.1 CAPN10 gene coding region with T2DM associated SNP's; 15 exons spanning 31kb

The selection of those sibships that were concordant for the presence of the 'at risk' alleles from the linkage data (with 'evidence of linkage') allowed further association studies with a subset of the polymorphism's. This focused the sequencing to a 66kb span in which three genes were subsequently identified. These were a G-protein coupled receptor 35(GPR35), a gene encoding a protein with homology to aminopeptidase B (RNPEPL1), and Calpain10 (CAPN10) a calpain-like protein.

2.2.3 Association of CAPN10 with T2DM

Two SNPs, UCSNP43, and UCSNP 22 were the only polymorphisms to have a significant association and ' evidence for linkage' with T2DM [Horikawa et al 2000]. The application of simulation experiments calculated that UCSNP43 was the only SNP to have a highly significant association, with some other SNP's, UCSNP19, -25 and –22 nominally associated. UCSNP19 localises to the intron between exons 6 and 7 of CAPN10, and UCSNP22 and –25 both localise to a region inter-genically 3' to CAPN10 and 5' to GPR35. The common G allele (allele 1) of UCSNP43 (G/A) located in intron 3 of CAPN10 was significantly increased in MA T2DM subjects compared to random controls, although homozygosity of the G/G allele was not associated with any additional risk. Mobility shift assays using an intronic fragment encompassing UCSNP43 and a second SNP 11bp upstream, UCSNP44 (T/C), identified differences in binding affinity of nuclear proteins [Horikawa et al 2000]. Furthermore, transcription studies suggested that these polymorphisms may play a role in the regulation of expression of CAPN10 or another gene located nearby and the G allele of UCSNP43 was associated with increased promoter activity [Baier et al 2000]. In Botnian Finns the frequency of the G allele (allele1) of UCSNP43 was also significantly associated, with 77% in T2DM subjects compared to 67% in controls (p=0.0049). In Botnian Finns the uncommon T allele (allele 20 of another intronic variant UCSNP63 (C/T) was also increased in 7% T2DM vs 3% in controls (p=0.017) but not in MA or German populations. The modest association of UCSNP43 in MA, could not alone account for the original linkage data, so haplotypes were tested with combinations of other SNP's within CAPN10 [Horikawa et al 2000]. Two common haplotypes were identified, both with the G allele (allele1) of UCSNP43, and with a combination of two other SNPs, UCSNP19 (32bp Insertion/deletion), a SNP in near perfect LD with a group of four CAPN10 intronic SNPS, UCSNP56, -59, -30, and –65 and one coding mutation in exon 11, UCSNP48 (silent mutation at codon A620) and the Finnish associated UCSNP63 variant. The greatest risk to T2DM in MA was defined by the heterozygous haplotype 112 and 121 combination (order UCSNP43, -19, -63) and was designated an 'at risk' to T2DM haplotype combination. This 'at risk' haplotype combination conferred an overall 2.8 fold increased risk to T2DM diabetes in MA, and was also associated with a 2.55 and 4.97 fold increase in risk in Botnian Finns and Germans respectively [Horikawa et al 2000].

2.2.4 Functional role of CAPN10 in T2DM

The CAPN10 gene is ubiquitously expressed in humans, including skeletal muscle, liver, and pancreas, with a complex pattern of post trancription splicing producing at least eight transcripts (calpain10a to -10h) [Horikawa et al 2000]. Although nothing is presently known of the function of the CAPN10 protein, calpains are a superfamily of calcium-activated/dependent nonlysosomal neutral cysteine processing proteases. These proteases function intracellularly cleaving specific substrates at a limited number of sites causing the activation and inactivation of the function of other specific proteins [Sorimachi et al 1997; Saido et al 1994; Carafoli & Molinari 1998]. Calpains have been implicated in the regulation of a variety of cellular roles, including influencing intracellular signalling pathways including controlling proliferation and differentiation of preadipocytes [Patel & Lane 1999], myoblasts [Ueda et al 1998], osteoblasts [Murray et al 1997] and chondrocytes [Yasuda et al 1995]. Calpains have also been implicated in apoptosis, ischemia and inflammation with possible pathogenic roles in Alzheimers disease, and limb-girdle muscular dystrophy type 2A. The latter disorder may result as a consequence of a mutation in the CAPN3 gene [Lonjou et al 1998; Permutt et al 2000b]. Functional calpains generally have four domains forming a large variable catalytic subunit (80kDa). This subunit assembles with a common small subunit (30kDA) to form heterodimers. CAPN10 lacks the usual IV domain, the calmodulin-like Ca^{2+} binding domain, which may mean CAPN10 lacks Ca^{2+} modulated activity [Dear et al 1997]. However, other studies suggest that the III domain may also be important in activation by calcium [Tompa et al 2001].

Insulin Action and Secretion: Inhibition of all calpain protease activity results in reduced insulin-mediated glucose transport and diminished incorporation of glucose into glycogen in isolated rat muscle [Sreenan et al 2001]. Calpains also appear to influence insulin-induced downregulation of insulin receptor substrate–1, a key mediator in insulin action [Smith et al 1996]. In humans, studies in Pima Indians found no association with the UCSNP43 and T2DM. However, investigations in normoglycaemic subjects revealed that homozygotes for the G allele of UCSNP43 had reduced (58%) skeletal muscle CAPN10 mRNA levels compared to heterozygotes, with subjects homozygous for the A allele having the highest mean levels [Baier et al 2000]. Furthermore, genotype at this locus was correlated with several metabolic indices with the G/G homozygotes having reduced glucose oxidation rates, lower sleeping metabolic rates and a preference to oxidise lipids rather than protein or carbohydrates, when these are supplied exogenously in a respiratory chamber. This latter

finding may indicate that differences in nutrient partitioning between subjects may be partly dependent on CAPN10 genotype [Baier et al 2000].

Studies using cysteine protease inhibitors have implicated members of the calpain family, although not specifically CAPN10, with either the promotion [Croce et al 1999] or inhibition [Yamazaki et al 1997] of protein secretion depending on cell type. Tissue specific expression of at least one transcript has been observed in pancreatic islets [Horikawa et al 2000]. Studies using cell permeable cysteine protease/calpain inhibitors in mouse pancreatic islets have been used to elucidate the role of calpains in glucose metabolism and secretion. However these studies have produced conflicting results. Short-term exposure (4 hours) of the islets to inhibitors leads to doubling of the insulin secretory response due to accelerated exocytosis of insulin granules by [Sreenan et al 2001; Zhou et al 2000]. However a 60% decrease in response was observed when the same inhibitors were applied for 48 hours. Morphologically the mouse islets contained enlarged vesicles containing insulin granules with additionally abnormally distended mitochondria and lysosomes [Sreenan et al 2001; Zhou et al 2000]. It was therefore suggested by Sreenan et al that the reduced insulin secretion may be caused by altered glucose metabolism in the mitochondria as the response of intracellular free calcium and rate of glucose metabolism was also diminished with calpain inhibitors.

Concurrent to the studies in this thesis a number of other groups have looked for association between CAPN10 and T2DM. These contemporary studies are described in the discussion of chapter 7. However, the overall evidence suggests that variation in CAPN10 activity could play a pathophysiological role in both insulin action and insulin secretion. Variation in activity of the protein might be genetically determined, through mutations influencing, splicing, regulation of transcription rates and mRNA stability.

2.2.5 Aims of the CAPN10 study

Aims of this study were firstly to replicate the linkage findings for *NIDDM1* on Chromosome 2q, with T2DM in a family collection drawn from the UK and Ireland. Secondly, following the positional cloning of the CAPN10 gene to *NIDDM1,* variants of CAPN10 UCSNP44 UCSNP43, UCSNP19, UCSNP63 and UCSNP110 were investigated individually, and as haplotypes/haplotype combinations to explore their contribution to the susceptibility to T2DM or related intermediate traits in a South Indian population.

CHAPTER 3:
SUBJECTS AND METHODS
3.1 SUBJECTS STUDIED
3.1.1 Introduction

The subjects studied in this thesis comprised of members of two distinct ethnic populations and several clinical collections. These included family samples recruited via a T2DM proband from both a North European collection and from South India. In addition a random population sample were available from the same South Indian Dravidian ethnic group. Finally a case/control study group comprising of unrelated British Caucasian obese and lean subjects were also utilized. Informed consent was obtained from all subjects involved in the studies. This section outlines the selection criteria for these study groups.

3.1.2 British Diabetic Association Warren 2 Repository

The British Diabetic Association (Diabetes UK) Warren 2 is a T2DM repository of British and Irish pedigrees collected nationally by six centers with The Royal London Hospital a participant. The repository comprises of T2DM affected sibpairs and their immediate relatives. Overall the sibpairs had a mean (SD) age of diagnosis of 55.6 ± 8.6, mean (SD) BMI 28.7±5.4 kg/m^2, median waist hip ratio 0.92 (IQR 0.11) and 48% of probands were female. Treatment was 20% diet, 64% therapy with oral hypoglycaemic agents (OHA), and 16% with insulin.

3.1.3 Ascertainment criteria for pedigrees.

Principle recruitment criteria were:

1. Affected sibpair with T2DM diagnosed between the 35 and 70 years of age.
2. All four grandparents known to be of British origin. Families are excluded if both parents and a high proportion of sibships display T1DM.

Pedigree members were considered affected if they:

1. Diagnosed having T2DM by WHO criteria, and were on therapy with oral hypoglycaemic agents, insulin or on specific diets.
2. Fasting plasma glucose concentration of >6 mmol/l, that is >3 standard deviations above the mean of a normal population.
3. Plasma glucose concentration of >9.3 mmol/l, after one hour continuous infusion of glucose at 5mg/kg weight per minute.

Pedigree members were considered unaffected if they had a fasting plasma glucose <6mmol/l or had a plasma glucose <9.3 mmol/l, one hour after a continuous infusion of glucose 5 mg/kg ideal weight per minute. As this disease has a late age of onset the diagnostic certainty of a normal test increases with age and this was taken into account in the computation of recombination frequencies.

3.1.4 Pedigree selection criteria.

1. Inclusion of families segregating immune-mediated diabetes (i.e. T1DM, LADA) was kept to a minimum: by exclusion of sibships with first and second degree relatives with T1DM; requiring at least one year interval between diagnosis and commencement of insulin; exclusion of subjects with a history of ketoacidosis and those subjects who were GAD positive.
2. Clinical exclusion of MODY (see 3.1.5) and mitochondrial diabetes was made on the basis of personal and family history.
3. Paternity / family relationships were verified by typing 136 independent markers and unsubstantiated families were excluded.

3.1.5 Criteria for exclusion of pedigrees with MODY

1. Age at diagnosis of at least two affected members of the family <25 years
2. Diabetes could be treated for at least 2 years without insulin.
3. The occurrence of diabetes in a family was suggestive of an autosomal dominant mode of inheritance (at least three generations of diabetes, and/or second cousins with T2DM before the age of 25 years).

3.1.6 South Indian Pedigrees

South Indian subjects, all Tamil –Dravidian in origin, were recruited via the M.V. Hospital for Diabetes-Diabetes Research Centre (DRC) in Chennai (formerly Madras), Tamil Nadu, India. Dravidians are considered to be Caucasian on the basis of genetic marker studies and anthropological parameters eg language [Cavalli-Sforza] and are distinct from other Indo-European speaking South Indian populations. Families were ascertained through probands clinically diagnosed with T2DM at the MVH-DRC. Families were recruited if both parents were alive and had at least one other sibling. All possible siblings were collected when available. T2DM was defined by an insidious onset of disease without ketosis and follow-up

of > 2 years since diagnosis, and if patients were on insulin this had not commenced within a year of diagnosis. All other participating members of the families were submitted to 75g OGTT if glucose tolerance was unknown. Glucose measurements were performed on venous plasma using the glucose oxidase peroxidase method (Roche Mannheim)

In this population the incidence of, T1DM, MODY and Fibrocalculous pancreatic diabetes (FCPD) are comparatively minor cause of diabetes [Mohan et al 1988; Ramachandran et al 1988]. All diabetic subjects were considered to be T2DM and were included with the pedigree unless excluded by the following criteria:

1. T1DM was excluded on clinical grounds. T1DM diagnosed on the basis of acute onset of symptoms before the age of 35 years, a history of ketoacidosis or ketonuria, weight loss prior to diagnosis and total dependence on exogenously administered insulin from time of diagnosis. None of the diabetics in the family had a family history of T1DM and if the proband was insulin treated then this did not commence within a year of diagnosis.
2. FCPD diagnosed on the basis of clinical history, suspected subjects were investigated for the presence of pancreatic calcification and/or ductal dilatation (Kambo et al 1989; Mohan et al 1988, WHO 1985) using abdominal x-ray and pancreatic ultrasound.
3. MODY was excluded essentially the same as for Warren 2 (3.1.5). Additionally available data on glucokinase mutations in these subjects also allowed exclusion of the MODY 2 locus [McCarthy et al 1994].
4. Mitochondrial Diabetes. None of the diabetic probands carry the MELAS mt3243 mutation, and it is not a common cause of diabetes in this ethnic group [McCarthy et al 1996]
5. Exclusion from non-paternity. Families had been previously typed with 5 multi-allelic markers from two chromosomes and 11 biallelic loci from four different chromosomes [McCarthy et al 1996 &1994]. Families suspected of having an ex-paternity member were tested with the paternity/forensic testing kit, PowerPlex 16 (Promega Inc, Madison WI USA). This kit uses a panel of 15 short tandem repeat markers (STR) and Amelogenin for gender specification, and then typed using a ABI 310 Genetic Analyser (ABI, Foster City, CA, USA). This assay was carried out by the Department of Haematology, the Royal London Hospital.

3.1.7 Anthropometric details of South Indian Trios

Probands as an entire group had a mean age of onset of T2DM of 33.8 ± 9.9 years, with mean BMI 26.6 ± 4.7 kg/m^2 and 36% were female. The treatment of the probands was 6% diet, 80% oral hypoglycaemics and 14% insulin. Sixty-three percent of probands were male and clinical details by gender are provided in Table 3.1.1. The mean (SD) age of fathers (n=95) was 65.4 (± 7.6) years, and mean BMI 25.3 (± 9.6) kg/m^2 and mean WHR of 0.96 (± 0.05); the corresponding figures in mothers (n=95) were 58.5 (± 7.1) years, and mean BMI 25.7 (± 4.3) kg/m^2 and mean WHR 0.88(± 0.07). In the parental group 69.6% had T2DM, as were 21% of co-siblings. None of the probands carried the mt3243 mutation [McCarthy et al 1996].

	South Indian T2DM probands	
	Male probands	Female probands
Number	60	35
Age at diagnosis (y)	34 (29-38)	32 (23-37)
Treatment: Diet only n(%) Oral agents n(%) Insulin n(%)	 7% 85% 7.4%	 6% 71% 23%
BMI (kg/m^2)	26.5 (± 4.5)	27.8 (± 4.6)
Waist hip ratio	0.94 (± 0.05)	0.86 (± 0.07)

Table 3.1.1: Clinical Characteristics of South Indian T2DM probands. Age at diagnosis given as median (range), BMI and WHR as mean (SD).

3.1.8 South Indian Population Survey

DNA was extracted from four hundred and sixty-eight South Indian subjects. These subjects were originally collected using a cluster analysis design across all socio-economic groups to investigate the prevalence of T2DM and associated risk factors in an urban community. The

subjects were > 20 years old and were randomly recruited by door to door survey in Chennai. This group had the same Dravidian ethnicity as the families as determined by; surname, residence, mother language, and when available ancestral history [Ramachamdran et al 1992].

Participants fasting capillary blood glucose was measured using glucose oxidase test strips and 2 hour, 75g OGTT and a Reflolux II Analyser (Boeringer mannheim-Roche). Those identified as T2DM underwent OGTT. Anthropometric measurements recorded included weight, height, waist, hips and skinfold thickness.

3.1.9 Anthropometric details of Urban Survey

The mean age of the subjects were 45 (± 12) years, mean BMI 22.5 ± 4.2 and 48.6% were female. Clinical details are provided in table 3.1.2.

	South Indian Urban Survey	
	Male	Female
Number	241	227
Mean Age (years)	47 (40-55)	42 (34-53)
BMI (kg/m^2)	22.0 (± 3.7)	23.1 (± 4.6)
Waist hip ratio	0.91 (± 0.06)	0.85 (± 0.06)
Diagnosis		
Normoglycaemic	323 (69.1%)	
IGT/IFG	60 (12.8%)	
T2DM	85 (18.1%)	

Table 3.1.2; Clinical Characteristics of South Indian Urban Survey. Mean Age at time of recruitment is given as median (range), BMI and WHR as mean (SD).

In the urban survey for T2DM, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), glucose tolerance was defined by the most recent WHO criteria [Alberti & Zimmet 1998a].

IFG/IGT subjects had a mean age of recruitment in males of 52 (±15.3) years, mean BMI 22.5 (±3.3) and WHR of 0.92 (±0.05); the corresponding figures in females were 44.9 (±12.5), BMI 24.4 (±4.9) and WHR 0.86 (±0.06). T2DM subjects were recruited at a mean age in males of 55yrs (±10.8) with mean BMI 23.4 (±3.3) and a WHR of 0.94 (±0.05) the corresponding figures in females were 50.7 (±10.4), BMI 24.1 (±4.1), and WHR 0.87 (±0.04). Thirty-eight percent of the T2DM were newly diagnosed and 62% had established diabetes at the time of the survey. In the latter group, 15% were on diet, 83% on OHA's and 2% were on insulin.

3.1.10 Unrelated UK Caucasian Obese\Lean Subjects

Blood samples were obtained from a cohort of one hundred and fifty-six predominantly female, unrelated European (UK) Caucasoid pre-menopausal morbidly obese patients, defined by a BMI of greater than 30 kg/m^2. These patients were serially recruited from either the Obesity Clinic at The Royal London Hospital (n = 116) or recruited as a result of a local advertisement (n = 40). The mean BMI (SD) was 40.3 ± 6.4 kg/m^2 (range 30 – 67), mean age was 44.0 ± 11.5 and 86% were female. A dietary review confirmed that no obese subject was following a very restrictive diet at the time of venesection and blood samples were taken following overnight fast. The majority of the obese subjects had a family history of obesity and all had a BMI > 30 kg/m^2 preferably over BMI 45 kg/m^2. Subjects were otherwise healthy having no family history of diabetes, and were not diagnosed diabetic following measurement of fasting insulins and OGTT.

Fasting serum leptin protein levels was analysed in a subset of 83 female obese subjects. Plasma leptin levels were determined by radioimmunoassay using a commercially available kit (Linco Research, St Louis, MO, USA) carried out by the Department of Clinical Biochemistry, The Royal London Hospital. The kit uses a polyclonal antibody against highly purified human leptin and both the calibrators and ^{125} I-labelled tracer are prepared with recombinant human leptin. The intra- and the inter-assay coefficients of variation were 3.4% and 3.6% respectively at a leptin concentration of 25.6 µg/l.

Indirect calorimetry was performed in a second subset of obese patients by staff at the Obesity Clinic, The Royal London Hospital. Energy expenditure was measured as the rate at which heat is produced by the body using a Deltatrac II metabolic monitor, and resting metabolic rate (RMR) was calculated using de Weir's equation [Weir 1949]. In the same subset, bioelectrical impedance, using a Bodystat 1500 monitoring unit (Bodystat Ltd,

Douglas, IoM, UK), measured tissue resistance to an electrical current and allowed estimation of body composition, ie.fat-free mass (FFM) and fat mass (FM) were calculated using predictive algorithms based on regression equations [Foster& Lukaski ,1996].

DNA was extracted from blood samples obtained from one hundred and six slim normoglycaemic volunteer controls (males BMI < 27 and females BMI < 25) recruited from European Caucasian volunteers amongst staff at The Royal London Hospital, and were therefore considered to be from the same geographical area as the obese subjects. Ideal weight individuals were classified as non diabetic on the basis of a negative family history, normal weight and some random plasma glucose< 6.0mmol/l and a limited number of OGTT's were performed. Their mean BMI was 21.6 ± 2.0 kg/m^2 (range 17.3 - 26.3), mean age 31.9 ± 9.5 years and 71% were female.

3.2 GENOMIC DNA FROM PERIPHERAL WHOLE BLOOD.

3.2.1 DNA extraction from large blood volumes using NUCLEON ™ kit

High blood volume extraction method (Gibco)

1. Whole blood (5-10 ml) was poured into a 50 ml Falcon tube, an equivolume of reagent A added, mixed and allowed to stand for 3-4 min.
2. Tube was spun 2300 rpm for 4 min, and supernatant discarded.
3. Pellet was re-suspended in 40 ml reagent A, mixed thoroughly, stood for 4 min and step 2 repeated.
4. Pellet was re-suspended in 2.5 ml of reagent B and placed in a 5 ml tube.
5. Sodium perchlorate (5M) 700ul was added and rotary mixed at RT for 15 min.
6. Mixture was incubated for 25 min @ 65°C, inverting twice in duration.
7. Chloroform was added -2.5 ml (kept @ -20°C), rotary mixed for 10 min, and then centrifuged at 2,500 rpm for 1 min.
8. 400µl of nucleon silica suspension was added slowly, and not mixed, then centrifuged at 4,000 rpm for 3 min.
9. The upper layer was decanted to a fresh tube, and centrifugation repeated.
10. The solution was removed to a fresh 50 ml Falcon tube and two volumes of cold absolute ethanol added and left at -20°C for 15 min. Precipitated DNA was spooled out using a modified glass Pasteur pipette. DNA was re-suspended in 200µl sterile distilled water overnight.

11. The quantity and purity of DNA was determined by a UV spectrophotometer. (Appendix II).

3.2.2 High-through put DNA extraction using QIAamp ™ 96 kit

QIAamp 96 DNA Spin Blood kit (Qiagen UK Ltd, Crawley ,Sussex) high-through-put method simultaneously extracting two 96 well plates of small peripheral blood volumes in 2 hours . All centifugation steps were carried out on a Sigma 6K-15 centrifuge (Sigma Laborzentrifugen GmbH)

1. 25µl of Qiagen Protease stock solution was added to each of the 96 wells of round-well block.
2. A 200µl aliquot of each peripheral blood sample was added to each well
3. 200µl of chaotropic solution AL (Qiagen) was added to each well. Wells were capped and the block shaken vigorously for 15 seconds.
4. The 96 well block was centrifuged briefly at 3000rpm.
5. The block was incubated at 70°C for 10 minutes in an oven and then spun briefly at 3000 rpm to collect any lysate on the caps.
6. The caps were removed and 210µl of 100% ethanol was added to each well. The block was resealed and shaken vigorously for 15 seconds and then spun briefly at 3000 rpm.
7. A QIAamp 96 column plate (96 tubes with DNA binding silica gel membranes) was prepared by placing it on top of a 96 well receptacle.
8. The 96 blood and reagent mixtures (now 635µl/well) were applied to the QIAamp 96 column plate . Plate was sealed with an adhesive strip and centrifuged for 6000 rpm for 4 minutes.
9. 500µl of buffer AW (Qiagen) was added to each of the 96 columns. The plate was resealed and centrifuged at 6000rpm for 2 minutes.
10. Step 9 was repeated again except, centrifuged at 6000rpm for 4 minutes.
11. The QIAamp 96 column plate was removed from the waste receptacle block and and placed on a 96 microtube collecting rack and incubated in an oven at 70°C for 10 minutes to dry the silica membrane.
12. 200µl of Buffer AE (Qiagen) preheated to 70°C in a Dri-Block was added to 96 column membranes and then sealed and incubated at RT for 5 minutes.

13. DNA was eluted from column membranes into the microtubes after centrifugation at 6000rpm for 4 minutes. This step was repeated with a further 100 µl of preheated AE Buffer.

14. Random samples from the eluted DNA were electrophoresed on an agarose gel to assess integrity, uniformity of concentration across plate and to approximate concentration from know quantity DNA MW markers. Yields were usually estimated to be approximately 30ng-40ng/µl.

15. Aliquots of DNA were diluted to 5-10ng/µl in 96 -Deep well plates.

3.3 THE POLYMERASE CHAIN REACTION (PCR)

3.3.1 Introduction

The Polymerase Chain Reaction (PCR) is an *in vitro* method of nucleic acid synthesis by which a specific segment of DNA can be replicated exponentially [Saiki et al, 1985; Mullis and Faloona, 1987]. The reaction requires a template, usually double-stranded genomic DNA, cDNA or mRNA. Synthetic oligonucleotide primers (amplimers), designed to anneal to opposite strands of the double stranded DNA and flanking the desired target segment, prime the reaction and dictate the direction of synthesis. A DNA polymerase drives the synthesis in a reaction buffer with deoxynucleotide triphosphates providing nucleotides and magnesium ions providing essential monovalent cations for the enzyme to function. The primers guide the polymerisation to produce copies of the two complimentary strands of the target segment from one double-stranded DNA template. The resultant copies are also capable of binding the primers, and act as a template for further amplification. Thermally stable DNA polymerases allow repeated cycles of denaturation of the double-stranded DNA, annealing of the primers to their complimentary sequences, and extension of the annealed primers. Thus successive cycles will theoretically double the amount of target DNA synthesised in the previous cycle. The target DNA segment is exponentially amplified at a rate equivalent to approximately 2^n, where n is the number of cycles of amplification completed.

3.3.2 Standard PCR conditions

PCR reaction conditions were standardized, although some target DNA segments required optimisation reflecting the length of the target sequence, the magnesium ion concentration, and primer annealing characteristics. Molecular applications downstream of the PCR also

influenced adjustments in the reaction profile, cycle profile and choice of thermostable DNA polymerase used.

Standard PCR Reaction mix:

PCR Reaction BUFFER 10x	$1/10^{th}$ vol	2.0µl
Magnesium Chloride	1.0-5.0mM	variable
dNTPs 1.0mM mix	200uM	0.2µl
Primer 100pmole/ul Forward	5-20pmoles	0.1µl
Primer 100pmole/ul Reverse	5-20pmoles	0.1µl
DNA polymerase 5units/ul	1-5units	0.2µl
Template DNA	10-50ng	5.0µl
Contaminant free water	to volume	20.0µl reaction vol

All reaction mixes were made as a single master mix for all samples.

Standard Cycle Profile:

94°C for 3minutes

25-40 cycles of:

94°C	for 20-40 seconds (30 seconds) denaturing
50-68°C	for 20-40 seconds (30 seconds) annealing
72°C	for 30-60 seconds extension

Followed by 10 minute final extension at 72°C, and then held at 4°C.

Adjustments to PCR reaction mix:

Reaction Buffer: Appendix I. Additionally a buffer containing $(NH_4)_2SO_4$ provide stringent primer-annealing conditions over a wider range of annealing temperatures and Mg^{2+} concentrations.

Mg2+: Monovalent cations are essential for the enzymatic action of DNA polymerases. Concentration of MgCl in the buffer was optimised for each primer set by titrating final concentrations of between 0.5mM to 3.0mM, (the concentration window most reactions tended to work at). Buffers based on $(NH_4)_2SO_4$ and containing 1.5mM MgCl, required little to no adjustment.

dNTPs: (1.0mM each dCTP,dTTP,dATP,dGTP). These were mixed at an equimolar concentration and used in sufficient quantity so as not to be rate limiting but without excess. Excess increased the amount of non-specific fragments and could compromise downstream applications.

Primers (amplimers): Apart from primer design (3.3.3), a minimalist approach was adopted. Primary reactions establishing PCR conditions contained excess of each primer, 20-50 pmoles/20µl reaction subsequently the amount necessary was titrated, usually less than 5pmoles/reaction of each primer did not work efficiently.

Thermostable DNA polymerases: The standard thermostable DNA polymerase isolated from Thermus aquaticus (Taq Dna polymerase Promega UK), and recombinants of this (HotstarTaq, Qiagen), were used for most PCR experiments. Thermostable DNA polymerase with increased thermostability, from Thermus littoralis (Dynazyme-Flowgen) was used in some applications. Taq DNA polymerases randomly mis-incorporates dNTPs at a level of 5% in the extension phase. Later PCR reactions with downstream DNA sequencing applications, it was preferable to use an enzyme combination with a proof-reading thermostable DNA polymerase at a ratio of 9:1 (AB Technologies) to rectify mismatched bases.

Adjustments to Cycle Profile:

Denaturation: The temperature and time in for this most instances remained constant. Increase in temperature and time would only be necessary for very long target DNA fragments (>2Kb), or difficult sequences (long tracts of GC repeats). Prolonged higher temperatures (>94°C) are detrimental to processivity. The half-life of Taq DNA Polymerase is 60 minutes at 94°C, but only 10 minutes at 97°C.

Annealing: A melting temperature (Tm) based on base composition for each primer was either calculated by the design programme (see **3.3.3**) or available with the primer database. PCR reactions were initially performed at sub-optimal annealing temperatures, 5-10°C below primers Tm. The temperature was then sequentially increased initially by increments of 5°C and then fine tuned by 1°C increments, until the specificity required was achieved. An annealing time of 20-30 seconds was adequate for all templates less than 1Kb.

Extension: 72°c is the optimal extension rate for thermostable DNA polymerases, most processing 2-4Kb per minute. Target DNA segments used were usually less than 1000bp, so a 30 second extension time was more than adequate.

Cycle number: The cycle number was adjusted in response to the PCR downstream application and the efficiency of the primers/PCR reaction combination. Cycle numbers above 27 can generate 'junk ' non-specific DNA fragments so the fewer cycles the better. PCR amplification of fluorescent micosatellite markers required fewer cycles (20-25 cycles) as sensitivity of detection was not a limitation and reduced problems of background

interference. PCR-RFLP applications required sufficient cycles to generate enough DNA (usually 30-40 cycles), so that following restriction endonuclease digestion all resulting fragments could be visualised with ethidium bromide staining.

PCR machines used included: MWG Biotech, MJR, Perkin Elmer

Quality Control of PCR:

PCR is an extremely sensitive technique with the potential to produce 1 x 10^6 copies from a single copy of target DNA. There was always the possibility of contamination producing false-positive amplification results. Contamination from airborne human biological debris, such as skin cells or hair roots (>60% content of dust) was avoided by using good aseptic techniques in a designated work area. Materials such as plastics, tubes, microtitre plates, were virgin and as an extra precaution, exposed to 15 minutes of 254nM UV irradiation to degrade any contaminating DNA. HPLC pure water also subjected to UV irradiation before use was a preferred source as distilled water was a common source of contamination.

Contamination was also controlled by using aerosol resistant pipette tips (filter tips) and where possible in association with multi-channel pipettes. PCR assay contamination controls were included in every assay, using water as a substitute for the DNA template.

3.3.3 Design of amplimers

Specific amplification of a DNA fragment (amplicon) by definition requires a unique set of primers (amplimers). In some instances, such as with all the microsatellite markers used, primer sets have already been designed, tried and tested and information found in databases (see **3.4.1**). Unique pairs of amplimers were designed using software such as *PRIMER v3* at the Human Genome Mapping Project web-site (www.hgmp.mrc.ac.uk). Primer criteria were as follows:

1. Optimum primer size 20 bases (min 18bases,max 27 bases).
2. Optimum Tm annealing temperature 60°C (min.57°C, max 63°C)
3. Oligonucleotide melting temperature formula [Rychlik et al 1990]
4. Maximum Tm difference between primers, preferably 0°C
5. GC content optimally >50% (20%-80%)
6. Maximum complementarity, default 8 bases, maximum allowable local self-complementarity and between forward and reverse primers, and maximum 3' complementarity, default 3 bases, the most 3' part of primer most important for annealing specificity.

Amplimer pairs identified were checked for cross homology with all other known human DNA sequences using the *BLASTn* search at HGMP resource website.

3.3.4 Touchdown PCR

Sometimes PCR reactions under standard conditions do not produce the ideal results. Mispriming by one or both primers either internally or externally to the target template can produce spurious 'shadow ' bands, and primer-dimers. These shorter fragments frequently dominate possibly due to the repetitive stochastic advantage they have over the longer correct products during the reaction cycling. This problem becomes greater after increasing number of cycles and when the target template is in small amounts. Adjustment of the PCR parameters such as Mg++ and annealing temperatures often solved these problems but in some situations the spurious interactions are as stable as the desired ones. Touchdown PCR [Don et al 1991] was developed circumvent this problem by exploiting the exponential nature of PCR reactions and reduces the need to optimise the reaction conditions.

Principally:

1.The initial annealing temperature is set above the calculated annealing Tm .eg for an annealing Tm 55C a starting point of 65C.

2. The annealing temperature is decreased 1C every second cycle until 'touchdown' at eg 55C then 10 cycles are carried out.

3.3.5 Hotstart PCR

This is a very simple modification to the standard PCR that was employed in most PCR reactions. A considerable amount of mispriming occurs at low reaction temperatures, when the completed reaction mix is ramped from room temperature to its first denaturing step of the first cycle. Non-specific PCR products were avoided by the addition of one of the reaction components (thermostable polymerase, dNTPS, Mg++) just prior to cycling by holding the reaction mix at 85°C. The introduction of heat sealed 96 multiwell plate PCR reactions prevents this, and there are numerous 'Hotstart" systems (HotStarTaq ™ Qiagen UK Ltd) commercially available that employ the use of a heat labile blocking component to inactivate the thermostable polymerase until required.

Reaction Mix for 20µl reaction volume:

10x PCR Buffer (Tris.Cl, KCL,$(NH_4)_2SO_4$,15mM $MgCl_2$ pH 8.7)	2.0 µl
dNTPs x4 ,1mm each, final concentration 100µM	0.2µl

100pmole/ul Forward Primer	0.1 µl (-0.3µl)
100pmole/ul Reverse Primer	0.1 µl (-0.3µl)
HotStarTaq polymerase 5units/µl	0.1µl
Genomic DNA 10ng/µl	2.0-5.0µl

UV irradiated HPLC H_20 to final 20.0µl volume.

Reaction mixture is preheated to 95^{0}C for 15 minutes to activate the thermostable polymerase prior to first cycle of standardised PCR profile above (3.4.2).

3.3.7 Visualisation of PCR product.

In all experiments following completion of PCR, the product was run out on a 10cm x 10cm x 0.6cm thick 2.0% agarose gel to ensure that amplification of the target

1. 100ml gel was prepared with 2g agarose (Flowgen) in 1 x TBE buffer in a polypropylene conical flask.
2. Agarose and buffer solution and mixed by swirling of the flask.
3. It was then heated to boiling point in a microwave oven with intermittant mixing. The gel was slightly cooled and ethidium bromide added to a concentration of 0.5µg/ml.
4. The gel was then poured into a horizontal gel mould, castellated combs inserted, and allowed to set.
5. The gel was subsequently submerged in a horizontal electrophoresis tank filled with 1 x TBE buffer.
6. 1.0µl of 10x Ficoll Bromophenol blue loading dye was pipetted into a 5.0µl PCR sample and loaded into wells of gel.
7. The gel was run at 90V (6V per cm) for 30 minutes.
8. The DNA fragments were then visualised on a UV transilluminator and Alpha Imager (Innotec/Flowgen) and sized using a 100bp ladder (Promega UK Ltd) and sizing software Permanent records were kept either by a thermal print, or storage as TIFF or BMP file.

3.4 MICROSATELLITE MARKERS.

3.4.1 Introduction.

Microsatellites are short tandem repeats (STRs) of between 10 and 50 copies of simple sequence and occur abundantly and randomly throughout most eukaryotic genomes. These most common form are dinucleotide repeats such as CA, and GA, trinucleotide repeats and

tetranucleotide repeats, but can be up to six base pairs. They may be in perfect tandem repetition or imperfect, interrupted, or compound repeats. The tandem repeats exhibit a high level of length polymorphism, and hence allelic variation. CAn dinucleotide repeats occur every 30Kbp, distributed equally in 5' and 3' UTR's and introns, and are the most commonly used microsatellites. They are also usually smaller than 100 base pairs and ideally suited for PCR.

The mutation rate of microsatellites is estimated at $5x10^{-4}$-10^{-5} which is sufficiently low enough to allow their use in linkage studies, as probes for linkage disequilibrium, and with disease mutations in population association studies [Hearne et al 1992]. The disease contribution of the test locus, its recombination distance from the marker and the markers polymorphic information content (PIC) [Botstein et al 1980] all affect the power to obtain significant evidence of linkage. The PIC value of a marker is calculated from the allele frequencies in the population and is related to mean repeat length [Weber 1990] and is a useful index of informativeness for genetic mapping, as a high PIC value in pedigrees will mean parents are often heterozygous at the micosatellite locus and the segregation of its alleles can be observed unambiguously in the progeny. Microsatellites markers in the candidate regions of interest used in this thesis were identified from genetic maps at a number of websites such as the Genome Database (GDB), Centre d'Etude Polymorphisme Humaine (CEPH) and Cooperative Human Linkage Center (CHLC) which provide database access to a large number of chromosomally mapped markers with information held on PCR primers and conditions (Weber & May 1989; Litt & Luty 1989]. Markers were selected on the basis of map position, PIC values higher than 0.7, and ability to unambiguously amplify by PCR and allow multiplexing of amplicons.

3.4.2 Genotyping using Fluorescent Microsatellite Markers

The ABI 373 Sequencer (Applied Biosystems Incorporated ABI) system uses four distinct laser sensitive fluorescent dyes that can be distinguished by wavelength of fluorescence (HEX, TET, FAM 540nm, TAMRA580nm). One primer for each amplicon was labelled with one of three dyes at the 5' end during oligonucleotide synthesis and the fourth dye was used for an internal lane size standard (ABI GS 500 TAMRA size range 30-500bp). Dye-labelled primers were purified at source by HPLC to remove unincorporated dye. Manipulation of dye and amplicon sizes allowed simultaneous running of a number of microsatellites in a single gel (multiplexing).

1. Screening was carried out on a 96-well microtitre plate format with 2ng of subject genomic DNA pipetted to each well. The plate was divided into three sections for gel loading purposes, each gel/section of 32 wells, with 30 wells, for study subjects and 2 wells with control (CEPH) DNA, the same two consistently used throughout the study to control for inter-gel variation.
2. Each plate of DNA of the study set was used to amplify a single fluorescently labelled microsatellite marker using either standard or 'Touchdown' PCR.
3. PCR conditions were optimised for each microsatellite to achieve optimal specificity, clarity and signal intensity.
4. Aliquots of each PCR product from the same study subject but from each micosatellite marker plate were combined into a single 'multiplex' 96-well microtitre plate to a maximum volume of 4µl. The ratio of amount of each marker product pooled was dependent on the previously assessed signal intensity of each marker dye/PCR product combination. Generally 500-1000 fluorescent units was sufficient for each marker analysis.
5. Multiplexed labelled PCR products were size separated utilising vertical denaturing PAGE on the ABI 373 Sequencer.
6. 0.2 µl of the ready labelled size standard (GeneScan2500 or GS 500 ABI Inc, Foster City US) and 1.0 ul of Formamide/dextran blue loading buffer was added to each sample well. Samples were then denatured by heating to 94 C for 2 minutes and then placed on ice just prior to loading the gel.
7. The 373 Sequencer Glass plates (12cm well-to-read) were carefully cleaned to manufacturers specification. Essentially using non-abrasive, non-dye containing detergents (Alconox- Sigma Aldrich), distilled water and then air dried.
8. A 0.4mm thick 6% acrylamide/bis acylamide 19:1, 8M Urea denaturing polyacrylamide gels was made using a two-part ready mix gel, (Sequa 6 gel National Diagnostics Inc). 40ml of Acylamide/Bisacrylamide mix was combined with 10 ml Buffer/TEMED mix in a 250ml spouted plastic beaker. Then 200µl of freshly made 10% (W/V) Ammonium persulphate (Kodak Chemicals) was added and gently mixed avoiding introduction of air bubbles. The gel mix was poured between the glass plates followed by insertion of a 36 well castellated comb/well former. The gel/glass cassette was laid horizontally and allowed to polymerise for at least 2 hours.

9. After installation of the gel cassette onto the sequencing machine, 5.0µl aliquot of the denatured samples from the multiplex plate were loaded using 0.4mm thick 'Duck Bill' tips (Anachem) to each well sequentially. One third of a multiplex plate was loaded per gel, representing 30 individual subjects, 2 inter-gel controls and as many marker loci combined for multiplexing.
10. Gels were electrophoresed for 4 hours at a constant wattage of 40 watts (1500 volts). PCR products are detected using a fixed distance scanning argon ion laser. Fluorescence data was digitised and transmitted to a Macintosh Centris computer (Apple Computers System 6.0) Data Collection was by GENESCAN ABI 672 software (*v* 1.1).

3.4.3 Allele Scoring Software

The digitised information represents the PCR products/ microsatellite alleles as fluorescent peaks (Electrophoretograms). The GENESCAN software automatically calculated microsatellite allele sizes by using the Local Southern Method [Southern]. This method interpolates allele sizes using the internal lane molecular weight standards flanking the microsatellite products to estimate parameters for a model that assumes the mobility of the DNA fragments in a gel to be inversely proportional to size in base pairs. This method has been previously found to be the most precise method available for allele sizing within this system and circumvents problems of inter lane variation in a gel [Ghosh et al 1997].

Data from the GENESCAN software was imported into a second software package GENOTYPER (ABI *v*1.02) for allele calling. A category for an individual microsatellite marker was generated by superimposing all allele peaks from several gels. Categorisation allowed the establishment of the limits of a marker allele size range and the creation of individual allele/peak bins within this range.

Allele bins were derived from the means of the common allele sizes (not necessarily the real PCR product sizes) both within gels and between gels. The acceptable base pair deviation within an allele bin was determined to allow for the common artefacts including non-template polyadenylation, i.e. addition of extra adenosine triphosphate base at the 3' end of the amplicon that increases size of all fragments randomly by one base pair. A maximum deviation for dinucleotide repeats of 0.9 base pairs greater and smaller than each mean allele size was set to prevent overlap of bins.

Filtering of the peaks using bin/category information and the setting of an adjustable minimum peak height /signal intensity eradicated most of the spurious background peaks,

promoting scoring of only the major allele peaks. Inter-gel differences were detected with the CEPH DNA controls and further external adjustments were made accordingly. Alleles correctly scored within a bin were rounded off to the nearest whole number and given an integer label equal to that mean size in base pairs and an allele bin letter code.
Finally, all sample and allele scores culminated into an Excel spreadsheets for databasing and subsequent statistical analysis.

3.5. DI-ALLELIC MARKER ANALYSIS.

3.51. Introduction

Di-allelic (or bi-allelic) polymorphisms by definition have only two alleles. Single nucleotide polymorphisms (SNP) are the most abundant type of di-allelic variants usually with the less frequent allele greater than 1%, less common are the insertion/deletion variants, and rare tri- and tetra-allelic polymorphism. Di-allelic SNP density in the human genome is estimated to be on average 1 per 1,000 bases, not accounting for regional differences in density that can be up to 100 fold. Four types of di-allelic single nucleotide polymorphism exist, primarily as transitions, pyramidine base to pyramidine base, C <> T and in the second complementary stand purine to purine G <> A. The other three types are transversions, pyramidine to purine and the reverse, C<>A (G<>T); C<>G (G<>C); and T<>A (A<>T); complementary strands in parentheses. Two-thirds of all SNP's are transitions, transversions accounting for the remainder in approximately equal amounts. The genetic determinants of disease are frequently SNP's, and they can be therefore causal in nature. The majority of SNP's are neutral and their usefulness stems from being proximal to a disease causing mutation. The consequence of only having two alleles makes them less informative than microsatellites markers, but their increased frequency and mutational stability makes them useful for both family or case/control association studies. Approximately eighty-five percent of SNP's are common to all human populations albeit with differing allele frequencies, and are also potentially useful in mapping diseasecausing mutations common to a number of ethnic populations.

3.5.2 PCR-RFLP

When polymorphisms disrupted a restriction endonuclease (RE) recognition sequence this was exploited to screen for the variants. The target region was PCR amplified from genomic

DNA using specific primers, and then the product digested with the appropriate restriction enzymes.

Standard Restriction mix total 25µl/sample:

Sterile, deionised water	Xµl
RE 10x Buffer	2.5µl
Acetylated BSA (10ug/ul)	
or spermidine (100mM)	0.25µl
RE 10units/ul	0.75µl
PCR product.	5.0-10.0µl

Fragments were digested by incubation of between 5-8 hours at optimal temperature for RE either in a heated water bath or dri-block.

All restriction fragments were size separated, visualised and scored on standard agarose gels or metaphor gels, with adjustment of agarose percentage according to size of digested fragments. Specific PCR-RFLP assays have been described in the individual chapters.

3.5.3 PCR-generated RFLP

Frequently a SNP will not naturally disrupt a RE recognition site, so by modification of usually a single base in one of the amplimers a recognition site was generated in the PCR product that could be disrupted by polymorphism. Where possible the amplification target sequence included a second non-polymorphic cutting site for the RE to facilitate as an internal restriction control. When this was not possible it was preferable that the wild-type allele of the variant allowed restriction by the restriction endonuclease.

3.5.4 Mutagenically Separated-PCR

Mutagenically Separated PCR (MS-PCR) is a single PCR reaction with competition between two primers both extending in the same direction on the same template DNA region. A constant third primer completes the PCR reaction by extending in the opposite direction. One of the competing primers is longer, and each competing primer differs at the 3' end by one base that represents each allele of the SNP. Nucleotides in each competing primer are also sometimes mismatched to the template to improve the allele specificity [Newton 1989]. The longer primer is used at a lower concentration than the shorter primer to compensate as the shorter one has a lower annealing temperature. The ratio of long to short primer is normally between 1:5 to 1:15, determined empirically with the best PCR annealing

temperature by observation of a known heterozygote, and homozygotes positive controls (genotyped by sequencing), until conditions were such that the heterozygotes has two bands of equal intensity, and the homozygotes were unambiguous. The common primer is used at the same concentration as the short primer so as not to be limiting in the reaction. False heterozygotes can be observed, especially if the PCR annealing temperature is too low, allowing non-specific annealing of the long primer. The longer primer appears to have a greater affinity for the DNA template when PCR conditions are less than ideal. Known homozygotes and heterozygotes were included as positive controls in all assays.

3.5.5 Insertion /Deletion analysis

Insertion/deletion polymorphisms were analysed by PCR amplification followed by electrophoretic size separation on agarose gels. Gel conditions, percentage and type of agarose and dependent on the resolution required to differentiate allele size unambiguously and were empirically determined.

3.5.6 PCR Product/Allele Separation and Visualisation

Separation of PCR products only requiring a low level of resolution were prepared essentially as in Section **3.3.7** with adjustments made to percentage of agarose where necessary. PCR fragments requiring a higher resolution separation, i.e. fragment size differences of less than 10%, specialist agaroses such as MetaPhor ™ and Nusieve ™ Agaroses (FMC Bioproducts USA/Flowgen UK) were used. These agaroses could resolve size differences as low as 2% when in a 200-800bp range (minimum of 4 bp difference). A rapid gel format employed improved resolution to approximately 1%.

Gel separation protocol:

1. A 100ml gel was prepared using 2-4g (2-4%) MetaPhor agarose (FMC-Flowgen) in 1x TBE buffer
2. Agarose and TBE solution were mixed by swirling of the flask and left for at least one hour at RT to allow for rehydration.
3. Mixture was heated to pre-boiled state in a microwave oven, stood for 15 minutes at RT and reheated to boiling point.
4. The gel mixture was allowed to cool sufficiently before adding ethidium bromide to a concentration of 0.5µg/ml and then poured into a horizontal electrophoresis gel mould. Gels were set at 4°C to increase the rigidity.

5. The gel was then submerged in an electrophoresis tank filled with 1 x TBE buffer or 0.5x TBE for Rapid Horizontal Gel format.
6. 1.0ul of 10x Ficoll Bromophenol blue loading dye was pipetted into a 5.0µl PCR sample and loaded into each well of gel.
7. Gels were run at 90Volts (6V per cm) for 2.5 hours or Rapid format gels were run at 250 volts (17volts/cm) for less than 1 hour using buffer at 15°C, cooled and circulated by a pumped refrigeration unit.
8. Separated DNA fragments were visualised on a UV transilluminator, and alleles scored accordingly after sizing using a combination of 100bp MW ladder (Promega UK Ltd) and sizing software using an Alpha IS1000 Digital Imaging System (Alpha Innotec/Flowgen, Lichfield, UK). Permanent records were kept as thermal prints, or stored as a TIFF or BMP files.

3.6 FLUORESCENT CYCLE SEQUENCING OF PCR TEMPLATES:

3.6.1 Introduction

Direct sequencing of double stranded PCR generated templates were carried out using an ABI 373 Automated Sequencer. The cycle sequencing method used was quintessentially the chain termination method of Sanger and Coulson with modification. Typically this DNA sequencing uses a modified thermolabile T7 DNA polymerase to extend from a primer annealed to a DNA/RNA template. The substitution of this polymerase with a thermostable polymerase introduces an amplifying thermal cycling stage in the sequencing reaction. This offers a number of advantages over conventional sequencing.

1. A single oligonucleotide primer in the reaction results in the linear amplification of one strand of the double stranded template, reducing template requirement

2. High denaturation temperature during cycling circumvents the problems of rapid re-annealing of linear ds DNA templates.

3. High annealing temperatures during cycling increase the stringency of primer hybridisation.

4. High polymerisation temperature decreases the secondary structure of DNA templates allowing polymerisation through highly structured regions.

The ABI 373 Automated Sequencer sequencing technology is based on the sensitive laser detection of four different fluorescent dyes. The ABI Prism ™ DyeDeoxy ™ Terminator Cycle Sequencing Kits uses each chain terminating dideoxyribonucleotide triphosphates,

ddGTP, ddATP, ddTTP, and ddCTP labelled with a different wavelength permiting a single reaction tube single gel lane.

3.6.2 Template Considerations

The double stranded DNA template was generated by PCR. The length and quality of the sequence was determined by a number of factors:

1.The ABI 373 Automated Sequencer, Well to Read gel plates of 36cm has an ability to sequence a maximum of 400-500 bases.

2. The terminal sequences at both ends of the PCR template were sometimes difficult to interpret, often due to excess unincorporated ddNTPS. This was generally overcome by amplifying a region in excess of the target sequence, and by overlapping contiguous templates.

3. In some circumstances the PCR Primers were inadequate for sequencing so 'nested' primers were utilised internal to the original PCR primers.

4. PCR product template had to be of sufficient quality and quantity, other methods were employed (see next sections) if the amplicons did not meet the necessary requirements.

3.6.3 PCR template purification by Spin Dialysis

When required templates were concentrated and excess primers and dNTPS were removed using spin dialysis columns (Microcon microconcentrators; Amicon-Millipore Ltd). The Microcon 100 was used with a membrane molecular weight cut of 125 bp for dsDNA i.e the filtrate includes dsDNA smaller than 125bp (and ssDNA < 300 bases) passes through the dialysis membrane.

1. The membrane cartridge device was assembled with a 1.5ml tube receptacle.
2. The completed PCR reactions with the identical template DNA were combined and made up to a volume of 500µl with sterile water.
3. The 500µl mix was added to the top of the membrane cartridge, capped, and centrifuged in a (fixed angled rotor) microfuged at 2000xg for 5 minutes at RT. The device was removed from centrifuge and the filtrate discarded from collection receptacle.
4. The device was re-assembled and a further 500µl of water was added to the top, and step 3 repeated.

5. The membrane cartridge was removed from the waste receptacle, inverted and place in to a new tube. The assembled device was centrifuged at 500xg for 1 min, and retentate with PCR product collected in the new tube.
6. A 500µl loading normally resulted in a 5-20µl retentate.
7. A diluted sample of the retentate was run on an agarose gel to estimate quantity using a pre-quantified DNA MW marker. PCR templates were diluted to a concentration of 10-30ng/µl.

3.6.4 PCR template purification by gel excision

When multiple non-specific PCR products were unavoidably present, the amplified fragments were purified by separation on an agarose gel (1-2%), and the desired template band cut out and extracted using Agarose Gel DNA Extraction Kit (Boehringer Mannheim GmbH).

1. Following separation on an agarose gel in 1xTBE buffer, the excised band was placed in a pre-weighed 1.5ml tube.
2. For every 100mg of gel, 300µl of agarose solubilisation buffer (unknown chaotropic agent) was added.
3. The silica suspension provided was resuspended and 10µl added to the gel fragment solution and mixed by vortexing to bind the DNA.
4. The mix was incubated for 10 minutes at 60°C and vortexed every 2-3 minutes.
5. The sample was then microfuged at 16,000rpm for 30 seconds, and the supernatant discarded.
6. The silica/DNA matrix pellet was then resuspended in 500µl nucleic acid binding buffer (formula unknown) using a vortex. Microfuged as in Step 5 and the supernatant discarded
7. The pellet was then washed in 500µl of Washing Buffer (formula unknown), centrifuged and discarded as in step5, and then repeated.
8. Final supernatant was carefully removed, tube inverted on tissue and the pellet allowed to air dry at RT for 15 minutes, or until bright white indicating fully dry.
9. Add 25µl of deionised sterile water (pH8-8.5) to the pellet to elute the DNA. Sample was vortexed and incubated at 60°C for 10 min, with a further vortex every 2 minutes. The sample was microfuged for 30 seconds at 16,000rpm, and supernatant containing the DNA template transferred to another tube.

10. Step 9 was repeated with another 25µl of water ,and combined with the first elution to increase recovery yield.

3.6.5 Dye Deoxy Terminator Sequencing reaction

1.Reaction mix components were added and mixed in a 0.5ml thin walled PCR tube. Primers were pre-diluted 1:100 from 100pmole/µl to 1pmole/µl in water.

Terminator Ready Reaction Mix (ABI)	8.0µl
PCR template 10-30ng/µl	5.0µl
Primer (Forward OR reverse) 3.2pmole	3.2µl
HPLC water	3.8µl
Total Reaction Volume	20.0µl

Reaction was overlayed with a drop of mineral oil.

2.The reaction was Cycle sequenced on a DNA Thermal Cycler (TC1 or Model 480 –Perkin Elmer). Programme profile was:

Rapid thermal ramp to 96°C; 96°c for 30 seconds; Rapid thermal ramp to 50°c

50°C for 15 seconds; Rapid thermal ramp to 60°C; 60°C for 4 minutes

This was repeated for 25 cycles, and then cooled and held at 4°C.

3. Early sequencing kits (ABI) required more thorough removal of excess dye terminators, later kits reduced amounts in reaction so only an ethanol precipitation was necessary see Step 10 for alternative purification.

4. 80µl of water was gently added and mixed with the 20µl reaction volume underneath the mineral oil layer. The 100µl reaction was removed to a 1.5ml microfuge tube, avoiding transfer/contamination with mineral oil.

5. 100µl of phenol:H_2O: chloroform mix at RT (68:18:14 ABI) was added and then vortexed to extract terminators. The tube was then microfuged at 16,000rpm for 2 minutes.

6. The lower organic phase was removed from the reaction extraction, 100µl of phenol:H_2O: chloroform mix added again, vortexed and microfuged as in step 5.

7. The upper aqueous phase was removed to new microfuge tube. Extension products were precipitated by addition of 15µl 2M sodium acetate pH 4.5 and 300 µl of 100% ethanol.

8. The mixture was microfuged for 15 min at 16,000rpm to pellet products. The ethanol was carefully aspirated with a micropipettor to leave pellet intact.

9. The pellet was washed carefully (without disturbing the pellet) with 250µl of 70% ethanol, and then aspirated off. Remaining ethanol was wiped out with clean tissue paper and the pellet dried in a vacuum centrifuge.

10. Alternative method for later sequencing kits; 2.0µl of 3M Sodium acetate. PH4.6 and 50µl of 95% ethanol was added and mixed with the 20µl reaction volume. This was incubated for 15 minutes on ice and then microfuged at 16,000rpm, at 4°C, for 30 minutes. The pellet was then treated as in step 9.

11.To the pellets 5µl of loading buffer (Appendix I) was added, vortexed vigourously, and microfuged.

12. Shortly before loading of gel, the reaction was denatured by heating for 2 minutes at 90°C in a heated block and then placed on ice.

3.6.6 Polyacrylamide Sequencing gel

A 0.4mm thick 6% acrylamide/bis acylamide 19:1, 8M Urea denaturing PAGE (Sequa 6 Gel, National Diagnostics/Flowgen) and glass plate preparation for the 373 Automated Sequencer was essentially as in section **3.4.4** from Step 7.

Except:

1. A 24cm well-to-read glass plates were used and therefore gel volume was 100ml, 80ml of Acylamide/Bisacrylamide mix was combined with 20 ml Buffer/TEMED mixed and 400ul of freshly made 10% (W/V) Ammonium persulphate (Kodak Chemicals) was added to catalyse polymerisation of gel.
2. Gels were run for 12 hours at 40 Watts constant power at 30C.

3.6.7 Sequence Analysis Computer Software

Following visual inspection of the digitised raw sequence data and the attainment of correct and optimal base spacing (between 9-12 scans), sample files were imported into the Sequence Navigator Software (ABI Inc.)

A reference sequence was created from a database sequence (GDB) of the target template, if available. The sequence was converted into a sequence string imported into the Sequence Navigator program.

Multiple alignment comparisons were carried out including both forward and reverse complementary sequences against the reference sequence using the *CLUSTAL* software program. Mismatched sequence was examined by eye on the electrophoretograms. Putative

polymorphisms were confirmed by repeating the PCR-sequencing reactions, as false positives could be due to random non-template incorporation of dNTPs by Taq polymerase during the PCR reaction.

3.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY

3.7.1 Generation of ds DNA probes

Double stranded DNA test fragments were generated either by PCR, using primers designed to encompass the region of interest. Alternatively single stranded oligonucleotides were synthesised for controls and region of interest, with one oligonucleotide complementary to the other. Complementary strands were annealed together to form a double stranded fragment. Allele variation was incorporated into each matched pair to allow investigation of binding effects of mutations and competition experiments.

PCR reaction for fragment generation:

50µl 10x PCR Buffer

5µl dntps

3µl F primer

3µl R primer

5µl QiagHotStar TAq

66µl Sterile water to final 100 µl volume

Hotstart PCR with 50 °C annealing. All reactions were combined and lyophilised to reduce volume to facilitate loading on a 1% agarose gel. Bands were cut out and put in pre-weighed 1.5ml tubes and DNA extracted using Agarose extraction system (3.6.4) and eluted in 50µl (20µl + 30 µl) ddH2O.

2.0µl was run on agarose gel to check integrity and estimate quantity, diluted to a concentration of approximately 20ng/µl, use 100ng in DIG labelling reaction,

Single Stranded oligonucleotides were annealed as follows:

To generate double-stranded DNA probes by annealing synthesised oligonucleotides.

Oligonucleotides were mixed in an equimolar ratio 1:1 in TEN buffer (appendix I) and incubated for 10 minutes at 95°C. Reactions were cooled slowly to room temperature and then diluted with TEN to a concentration of 3-4 pmol/µl

1.Mix single stranded oligos in an equimolar ratio 1:1 in TEN.

2. Incubate for 10 minutes at 95C

3. Cool slowly to room temperature

4. Dilute with TEN buffer to 3-4pmol/ul

3.7.2 Digoxigenin-11-ddUTP (DIG) end-labelling of probes

Reactions of 20µl, with either 5µl of PCR Probe (20ng/µl) or 1µl of annealed oligonucleotide probe or Roche kit control.

4µl labelling buffer (5x conc Roche-appendix I)

4µl CoCl2 solution

5µl of ds PCR Probe

1µl DIG-11-ddUTP solution (Roche)

1µl terminal transferase

Xµl ddH2O to final volume

1. Reactions were mixed on ice and then incubated for 15 minutes at 37°C and then placed back on ice.

2. The probe was then precipitated with 2µl of 4M LiCl, and 60µl of ice cold ETOH followed by incubation at –70°C for 30 minutes.

3. The precipitate was pelleted in a micro-centrifuge for 15 min at 4°C, 12,000 RPM.

4. The supernatant was removed and the pellet washed 3x with 500µl of 70% chilled ETOH and finally vacuumed dried.

5. The labelled probe pellet was dissolved in TEN to obtain a concentration of 4ng/µl or 0.155pmol/µl.

3.7.3 Test for DIG labelling efficiency

To test for both DIG labelling and chemiluminescent detection efficiency.

1.Dilute DIG labelled EMSA probe and kiy labeled control in TEN buffer (appendix I) by serial 10 fold dilutions 1:10 to 1:100,000.

2. Spot 1µl (4ng) of undiluted kit labelled control and each serial dilution of control and labelled EMSA probe onto dry positively charged nylon membrane (Boehringer Mannheim 1209 299). DNA was bonded to membrane by baking at 120°C for 30 minutes.

3. Spot 1µl of kit ready DIG labelled DS contol oligonucleotide (0.4ng/µl) as a chemiluminescence detection control.

4. Chemiluminescent detection was performed as per section 3.7.6. A spot should be detectable in the 1:1000 dilution (4pg) of the labelled control DNA and EMSA probe fragment.

3.7.4 Gel Shift Assay

For EMSA assay's, reactions were carried out as follows in a final volume of 10µl:

1. All reactions were mixed and incubated on ice for 10-15 minutes, then 20 minutes at RT, and then placed back on ice. Reaction component volumes were as table 3.7.1:
2. A control labeled EMSA probe fragment with a known a *trans*-acting binding site (eg. OCT2A) was utilized in preliminary experiments for optimisation of assay and gel conditions with the HeLa Nuclear Extract (HNE).
3. Optimal binding was initially determined for both control and novel EMSA probe fragments, by using duplicate sets with either Poly AT or Poly IC which can markedly reduce non-specific binding of nuclear extract proteins.
4. Principally an experiment consisted as follows with reference to table 3.7.1:

A. DIG labeled control

B. DIG labeled control + specific *trans*-acting binding factor or HNE

C. DIG labeled control + specific *trans*-acting binding factor or HNE + unlabelled control fragment as a specific competitor

D. DIG labeled novel probe

E. DIG labeled novel probe + HNE

F. DIG labeled novel probe+ HNE + unlabelled novel probe at a 100 fold excess as a specific competitor

G. DIG labeled novel probe + HNE + unlabelled non-specific competitor (eg control probe)

5. Following incubation, 2.5µl of loading buffer (appendix I) without without BPB was added per 10µl GS reaction, and then loaded immediately on a pre- electrophoresed 10cmx 10cm PAGE. One lane of gel was loaded with running buffer plus BPB as a tracking lane.
6. 4.1% non-denaturing polyacrylamide gels (40:1 in 0.5 xTBE) were pre-electrophoresed for 1-2 hours before sample loading and then and ran at 80 volts (constant voltage) until

tracking lane BPB dye was ¾ way down gel (MINI protean apparatus Biorad). Running buffer (0.5X TBE) and hence gels were kept at 4 °C.

	A	B	C	D	E	F	G
Binding Buffer (Roche)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Poly d(AT) or d(IC)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Poly L-lysine	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DIG labeled -novel probe or control probe	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Unlabelled novel probe 20ng/ul	0	0	0	0	0	3.0	0
Unlabeled Control probe	0	0	1.0	0	0	0	1.0
HNE or specific factor	0	2.0	2.0	0	2.0	2.0	2.0
DdH20 to 10ul	5.0	3.0	2.0	5.0	3.0	0	2.0

Table 3.7.1: Reaction volumes for EMSA assay

3.7.5 Capillary transfer from PAGE:

DNA and DNA/protein complexes were transferred to positively charged nylon membrane (Boerhringer Mannheim GmBH) using a positive pressure apparatus, (POSIBLOT Stratagene Ltd) overnight in 0.25xTBE buffer. Following transfer nylon membranes were rinsed in 10x SSC and baked at 120°C for 15-30 minutes to bond DNA and DNA/protein complexes to membrane.

3.7.6 Chemiluminescent Detection (Boehringer-Mannheim).

1. Membranes were rinsed briefly (1-5min) in Washing Buffer
2. Membranes were incubated for 30 minutes in 100ml of Buffer 2
3. Antibody conjugate solution was diluted to 75mU/ml (1:10,000 anti-digoxigenin –AP, Fab fragments) in Buffer 2.
4. Membrane was incubated for 30 minutes in 20ml of antibody conjugate solution.

5. Unbound conjugate was removed by washing 2x for 15 minutes in 100ml Washing Buffer.

6. Membranes were equilibrated for 2-5 minutes in 20ml of Buffer 3.

7. Membranes were then incubated, DNA side face down, in 10ml CPSD™ substrate solution for 5minutes.

8. Excess substrate solution was drained off, and without allowing membrane to dry, placed in plastic wallet and heat sealed whilst avoiding air bubbles.

9. The sealed membrane was incubated at 37°C for 15 min in an oven and then exposed to X-ray film (Roche) in a light proof cassette for between 15minutes and 1 hour at RT. The film was then developed using commercial photographic developer and fixer reagents.

3.8 STATISTICAL ANALYSES

3.8.1 Non-Parametric Linkage Analysis

Genetic distance (centimorgans cM), and hence the rate, is determined by the probability of a meiotic recombination event (recombination fraction θ) occurring between the loci. An allele at one locus on one chromosome independently segregates with a given allele at another locus on another chromosome with a 50% probability, so for two loci to be considered linked or in genetic linkage on the same chromosome the recombination fraction must be less than 50%, with distances between loci decreasing with declining recombination fraction. Linkage analysis estimates the recombination fraction from counts of the recombinants and non-recombinants within pedigrees with a large number of informative meioses, and if this is less than 50%, then tests if the observed deviation is statistically significant. Typically linkage analysis is used to determine the positions of disease loci relative to genetic markers, but is also used to map marker positions relative to each other. Linkage analysis generally involves the calculation of the logarithm of odds ratio (LOD scores) [Morton 1996]. A lod score of 3.0 for a given recombination fraction means that the chances are greater than 1000:1 that the loci are linked, and by a widely accepted convention this is the level at which there is significant evidence for linkage for a simple mendelian autosomal disorder [Landers & Kruglyak 1995; Morton 1998a]. A lod score of 2.0 (100:1) is generally assumed to represent a level in which there is only suggestive evidence of linkage. Data can also be interpreted inversely to exclude regions of the genome with lod score of

less than –2, providing evidence of exclusion of linkage for a given region at a particular recombination fraction (θ).

There are theoretical difficulties in the application of Lod score methods to complex diseases. Problems stem mainly from that the calculation of classical lod scores requires the specification of various aspects of the disease model including the full specification of mode of transmission of all the loci involved, combined with allele frequencies and penetrance values. This is normally possible when mapping simple Mendelian disease genes and markers, however in complex (non-mendelian) diseases it is difficult to estimate penetrance values, including phenocopy risks, and also the allele frequency of the disease mutation. It is possible that mutations at different loci have different kinds of effect on susceptibility, either major or minor, and dominant or recessive. In complex disorders individual families may have several different modes of transmission operative and also different loci may interact within the same family. Therefore no single model of transmission may be an appropriate mode of inheritance.

In addition, the late age of onset of T2DM and the increased disease-related mortality hampers the collection of large multi-generation pedigrees [Panzram 1987]. This is further hindered as T2DM patients diagnosed early (between 25-40 years old) have a large proportion (83%) of both parents affected and these families are therefore less informative [O'Rahilly & Turner 1988b].

Therefore a model free non-parametric linkage analysis approaches have been widely used in the search for diabetogenic genes. However, compared to conventional linkage analysis these non-parametric methods require a large number of affected sibling pairs (ASP) to achieve sufficient power to detect disease susceptibility loci.

The affected sib pair analysis relies on the principle that if a marker is linked to a disease locus, consequently the same marker allele will be inherited by both affected siblings more often than expected by chance. Methods have been developed that can use parental genotype data when available; Identity by Descent (IBD) and without access to parental data; Identity by State (IBS) [Risch 1990]. In the context of complex traits with no single major gene, the power of a study is a particularly important aspect. Mathematical modeling has determined that the likelihood of detecting linkage is a function of the parameter λ_R or λs, the affected relative risk ratio or sibling relative risk ratio in ASP studies [Risch 1990]. This parameter (λs) is the ratio between the risk to an individual developing the disease (co-sibling) if a family member is already affected (Ks) versus the risk to a member of the general

population (K) calculated by Ks/K. The higher values of λs indicate a fewer number of causal genes [Risch 1990]. The number of sib-pairs required to detect linkage is both a function of λ, and density of chromosomal markers and therefore the potential power of a study can be assessed [Risch & Merikangas 1996].

GENEHUNTER is a nonparametric linkage program in which a mode of inheritance does not need to be specified, and can be equally applied to pedigrees and nuclear families and affected sibling pairs. This program calculates a non-parametric statistic, measuring the sharing among affected individuals, called the NPL (Non-Parametric Linkage) Score, and generates an associated p value. A graphical representation can be generated from the NPL scores across a map using *npl_plot.ps*. NPL statistics were plotted on the map, at appropriate cM distance increments, regardless of marker positions in the map.

Linkage analysis has proven to be a very powerful tool in localising disease susceptibility loci to chromosomal regions, however it relies on differences in allele sharing of affected sibling pairs, in which a quarter will share two alleles purely by chance. Therefore, the determination of the map positions of loci based on recombination events in a family could be subject to error. Furthermore, siblings are only separated from their parents by one meiotic event, hence shared chromosomal regions span over many centimorgans, representing millions of base pairs of DNA. The lack of sufficient genetic resolution with linkage alone suggests that it may be less than ideal for fine gene mapping purposes and final localization of susceptibility loci [Risch 2000].

3.8.2 Linkage Disequilibrium and Association Studies

It has been observed that in randomly mating populations, loci in close proximity with each other on the same chromosome remain 'linked' over many generations. This occurs as a result of an insufficient number of meiotic recombination events to separate loci, thus the loci have not yet reached 'linkage equilibrium'. This phenomenon is referred to as linkage disequilibrium (LD). The extent of LD in proximity of any given loci in a specific population is a function of the age of that population. In relatively recent (2000 years) populations, such as found in Finland, individuals are only separated by as little as 80 meioses and hence there is extensive LD [de la Chapelle 1993]. However, the degree of regional LD is also a consequence of the specific demographic history of the population with genetic drift, bottlenecks and cycles of expansion and contraction also having major influences [Ott 2000].

Analyses that utilize LD, unlike linkage analysis, do not assess recombination just in the observed data, but exploit the consequence of many recombinations between loci over a large number of generations. Moreover, the expectation that LD is only observable between loci that are tightly linked over short genetic distances suggests that it is a powerful tool that can be exploited for fine mapping and disease susceptibility gene discovery purposes [Risch & Merikangas 1996]. If there exists sufficient LD between a specific ancestral disease causing mutation that has arisen in a given population and a genetic marker in close proximity then this can be used to identify disease susceptibility genes.

Case-Control Association Studies:

The underlying premise for these studies is that polymorphic sites within a candidate gene or in close proximity are either causative of disease or act as markers for the disease by being in linkage disequilibrium with the causative pathogenic mutation. Association studies investigate whether there are statistically significant differences in allele/genotype frequencies of a polymorphic site or series of variants (haplotypes) between unrelated ethnically matched unaffected (controls) and affected subjects (cases) within a population. If a marker loci and pathogenic mutation are tightly linked ie. in strong LD, then an increase in frequency of a marker variant allele in the affected group is indicative of an association with disease. Genetic markers can comprise of any type of polymorphisms including microsatellites, minisatellites (VNTR's), insertion/deletions, and the more abundant and widely spread single nucleotide polymorphisms (SNP).

Although population association studies can be particularly useful in genetic studies where the late onset of disease can impede the collection of multi-generational families, such as in T2DM. Case-control type studies are based on non-random statistical associations, which may be non-genetic in origin and are prone to Type I errors, ie false-positive associations [Ott 1999]. For example high levels of undetected consanguinity, where samples are not truly unrelated and epistatic gene-gene interactions can lead to artefactual associations. In addition statistical consensus suggests that the major cause of error in these studies is the confounding effect of population stratification, with an over-representation of allele/haplotype frequencies in cases or controls due to underlying population differences. The most likely source of sub-stratification is through lack of homogeneity of ethnicity in the two study groups. This could occur by genetic admixture and migration. Ethnic differences in the marker and disease loci allele frequencies could give rise to positive associations that were not the result of LD between the disease and marker loci if there was

an increased frequency of both disease and marker alleles in one of the ethnic groups. Steps can be made to avoid some of these false postive results by checking for skews from non-random mating by calculating Hardy-Weinberg Equilibrium (section 3.8.3) and with the collection of control samples that are truly matched to cases for relevant factors such as ethnicity and geographical origin. Cases should also be as genetically homogenous as possible to prevent different associations in different ethnic groups masking their effect. However, stratification as a confounding factor has never been entirely proven and an alternative explanation is that high false positive rates observed result from a low prior probability that the few gene polymorphisms examined are causally related to the disease [Risch 2000].

Furthermore, the close proximity of a polymorphic marker locus to a disease locus does not necessitate that it is associated with the pathogenic mutations. The power to detect an association between a non-causative polymorphic marker and the disease phenotype is entirely dependent on the degree of linkage disequilibrium [Cox & Bell 1989]. The level of linkage disequilibrium being dictated by both the distance between marker and disease locus and the recent evolutional origin of the mutation, with both factors being dependent on number of recombination events. A disease association could remain undetectable if the candidate gene has multiple disease mutations associating with different alleles at a given locus, culminating in the same disease phenotype or if the study design lacks statistical power to detect the association through insufficient sample numbers. Hence a single population study is not definitive proof or exclusion and requires supporting evidence in the form of duplication or confirmation through other study designs.

Family association Studies:

Family based association tests were developed to circumvent some of the problems of population studies by using matched cases and 'pseudocontrols' within families. Tests such as the Transmission Disequilibrium Test (TDT) were developed by Spielman & Ewens [1996] from the haplotype relative risk method of Rubinstein et al [1981] and utilizes both linkage and linkage disequilibrium.

Disadvantages: To achieve a positive association, the disease allele must be associated (in LD) within the population with the test locus marker allele, but also simultaneously there must be linkage between the disease and marker for that association to manifest itself in a biased transmission to affected offspring. When subjects are related, as with multiple affected siblings, it is still a test of linkage, but as these subjects are not independent of each

other, it is difficult to know if there is evidence of LD in addition to linkage, and the test of association is invalid. The original TDT could only deal with two marker alleles but this has now been modified to cope with multiallelic markers in the extended TDT test (ETDT) [Sham & Curtis 1995]

Advantages: It is a model-free method and tests for both linkage and LD simultaneously [Spielman & Ewens 1996], and has an advantage over population case-control studies is that the test is equivalent to a matched case-control design, with case genotype as the affected offspring genotype and the 'control' genotype ie. the parental alleles not transmitted. Therefore it is not prone to the false positives due to population stratification. When the test is positive it implicates a very small chromosomal region and was introduced to reduce large candidate regions for a disease determined by linkage analysis.

Case-control and TDT study designs each have specific advantages and disadvantages, so ideally they should be utilised complimentarily if both family and population resources are available.

3.8.3 Hardy-Weinberg Equilibrium

Large completely mixed (panmictic) populations consist of a number overlapping generations or mating types, but because the genotypes of new members of the population are a function of the allele frequencies of the reproductive age class, the proportion of various genotypes at a given locus should remain unchanged from one generation to the next unless the population is subjected to selection forces such as non random mating, mortality, or migration, or mutation rate is not constant. Genotype frequencies should become homogenized across all age classes and sexes and this state is Hardy-Weinberg Equilibrium (HWE). Under certain circumstances the frequencies of the genotypes at the locus under study can be a function of the allele frequencies alone, and underlying selection forces could stratify the population. Therefore it has become standard practice to cross-check genotype frequencies in any given population with Hardy-Weinberg expectations as a departure from HWE could influence the validity of an association study. For example if the observed population allele frequencies significantly differ from the expected as result of increased heterozygosity or homozygosity, if not due to genotyping error, would indicate a degree of 'outbreeding' or 'inbreeding' respectively within the study population [REF].

Significant departure from HWE was assessed for all loci investigated using an MS-Excel sheet prepared by D.Curtis. This incorporated the HWE equation comparing observed and

expected genotype frequencies and calculated a Pearson chi-squared statistic with one degree of freedom [Pak Sham –Statistics in Human Genetics: Arnold 1998].

3.8.4 Analysis of Population Association Studies

In population association studies the frequencies of alleles/genotypes subjects were compared between the genotyped cases and controls using contingency tables and chi-squared tests (calculation of asymptotic p values based on a Pearson-Chi squared distribution). P-values were corrected using the Bonferroni test for multiple testing where appropriate. When alleles were rare or sample sizes too small (less than n=5 per cell) homozygous subjects were combined with heterozygous subjects, and presence of the mutant allele analysed. In addition, to guard against an overly conservative test, Monte Carlo procedures were employed. The SPSS (*v* 9.0) statistical package was used for all analyses.

3.8.5 Transmission Disequilibrium Tests

TDT: This test uses parents that are heterozygous or informative at the marker locus and compares the frequencies of the marker (M) alleles that are transmitted to the affected offspring with the frequencies of marker alleles that are not transmitted, using the test statistic $(n_{12}-n_{21})^2 /(n_{12}+ n_{21})$ with reference to table 3.8.1. An asymptotic chi-squared is generated with one degree of freedom when the disease and marker loci are unlinked. If an allele is transmitted on more than 50% of occasions to affected offspring, then there is evidence for both linkage and linkage disequilibrium between marker and disease loci.

Transmitted Marker (M) allele	Non-Transmitted Marker allele	
	M_1	M_2
M_1	n_{11}	n_{12}
M_2	n_{21}	n_{22}

Table 3.8.1: Basis of TDT Statistic:

TDT Haplotypes: To examine the association of haplotypes generated from multiple polymorphisms with disease phenotypes in families, the program ***TRANSMIT*** *(www.hgmp.mrc.ac.uk)* was employed [Clayton 1999]. This program tests for association

between marker and disease by examining transmission of markers to affected offspring as for TDT. The program can deal with multi-locus haplotypes even when phase is unknown, and unknown parental genotypes and produces asymptotic Chi-squared tests for excess transmission of individual haplotypes (1df) and a global statistic for association with H-1 df, where H=number of haplotypes. In the analysis untaken in this thesis, the recent additional bootstrapping option was also used (1000 bootstraps) to generate more accurate p-values than those generated from the chi-square approximations as suggested by Clayton [1999].

QTDT: In complex diseases such as TDM and Obesity, quantitative phenotypes can provide more information than can be provided by just dichotomous traits. Therefore programs have been designed to test for LD applicable in the analysis of quantitative traits in families. The Quantitative TDT program available at ***(www.well.ox.ac.uk/asthma/QTDT)*** suite contains the QTDT5 method of Allison, and orthogonal model of Abecasis based on the Fulker model for sib-pairs [Abecasis et al 2000; Fulker et al 1999]. These variance component approaches allow simultaneous modelling of means and variances, so all the information in a set of related individuals can be used to construct a test of association. The orthogonal model has the advantage over similar methods of optionally using data from both parental genotype and all available offspring, which if available can substantially increase power to detect associations. This test, as with other TDT tests is also free of the confounding effects of population-substructure, and robust to other biases including background familiarity regardless of the composition of the nuclear families. It can also be applied to markers with multiple alleles without modification, and was also capable of analyzing multiple haplotypes similarly with appropriate correction for multiple testing (personal communication Goncarlo Abecasis). Sex was included as a covariant in analyses where trait variances could be influenced by gender. In addition, the "total association" option in *QTDT* was used in some analyses to implement a non-TDT association test that incorporates multiple members of the same pedigree, whilst allowing for shared polygenic and environmental variances.

3.8.6 Power Calculations

Power Calculations for TDT analyses was performed using an MS Excel sheet kindly prepared by D.Curtis. Calculations were based on those published by Risch and Merikangas [1996] using a model that assumes a multiplicative relationship between the alleles and

accounts for incomplete penetrance. A genotype relative risk (GRR - γ), the increased chance that an individual with a particular genotype has the disease must be defined if known, or tested for different levels of risk with the degree of incomplete penetrance determined by the magnitude of γ. The frequency of the disease allele and number of test families must be provided, and the likely proportion of heterozygous parents are calculated. Power of 80% at a given significance value, is the probability of rejecting the null hypothesis when it is false.

3.8.7 Linkage Disequilibrium Tests

A marker can either be a causative mutation with a direct pathogenic role in the disease or in LD with mutations that are causative. Information on LD patterns between loci across a chromosomal region can provide useful information in the interpretation of relationships of markers/mutations with disease loci particularly in respect of power to detect that relationship. The extent of linkage equilibrium across a region will dictate the degree of sensitivity of a marker to detect a relationship with disease loci. LD and power being dependent on when the mutation/marker evolutionarily arose in the population, with the rate towards equilibrium not only affected by number of recombinations but also by the mutation rate of the marker itself. Information on the phase of the loci alleles allows some interpretation of marker loci and disease relationships. This can be especially important when a number of closely linked loci associate with the same disease phenotype, or different traits predisposing to the same phenotype, potentially LD data could reveal whether the markers are associated with the same or different disease causing mutations.

In the population collections the *EH* program (*ott@linkage.rockefeller.edu*) was used to assess LD between markers in haplotypes Xie & Ott 1993]. The program estimates alleles frequencies from randomly collected individuals for each marker. Haplotype frequencies are estimated with allelic association (H_1) and without (H_0) and an asymptotic chi-square value calculated.

3.8.8 Odds ratios and Relative Risks

Odds ratio (OR):

The odds ratio relates the odds of being a case to not being a case for those with the risk factor (a/b) to these same odds for those without the risk factor (c/d). When the odds in each group have equal risk, then odds ratio is equal to the unity.

OR= $\frac{a/b}{c/d} = \frac{a \times d}{b \times c}$ where a,b,c,d are as referred to in table 3.8.1

Relative Risk (RR):

$$RR = \frac{\text{incidence rate among exposed}}{\text{Incidence rate among non-exposed}} = \frac{a/(a+b)}{c/(c+d)}$$

where a,b,c,d are as referred to in table 3.8.2

This is a measure of association between exposure to a particular factor and the risk of a certain outcome. The relative risk, eg 5, means that an exposed person is 5 times as likely to have the disease than one who is not exposed. RR does not measure the probability that someone with the factor will develop the disease.

Population Attributable Risk (PAR):

The PAR estimates as a percentage the proportion of cases (eg T2DM) in the entire population that can be attributed to exposure to a particular factor, eg. variation at a gene locus or loci-haplotypes.

$$\frac{\text{Frequency rate among exposed} - \text{Frequency rate among non-exposed}}{\text{Frequency rate among exposed}}$$

$$PAR = \frac{[(a+c)/T] - [c/(c+d)]}{(a+c)/T}$$

where:

RiskFactor (gene variant allele)	Sample number Diseased	Sample number Non-Diseased	Totals	Risk
+	a	b	a+b	a/ (a+b)
-	c	d	c+d	c/ (c+d)
	a+c	b+d	a+b+c+d=T	(a+c)/ T

Table 3.8.2: Terms for derivation of OR, RR, and PAR calculations

3.8.9 Analysis of quantitative traits

The relationships between continuous variables when available and alleles were assessed using parametric statistics ie analysis of variance (ANOVA) in unrelated subjects using SPSS (v9.0). In each test, normal distribution was assessed by the Levene test for

homogeneity of variance. If this was significant, data values were logarithmically transformed and the non-parametric Kruskal Wallis test was used. When available co-factors was deemed to have an influence on the variance of a particular trait, such as age on BMI or WHR, then these were included as a covariant in the analysis (ANCOVA), alternatively in the case of known strong dimorphic effects such as gender on BMI and WHR, groups were separately analysed. RMR were analysed by ANOVA but were first adjusted for covariables by using a linear regression coefficient (a) according to Astrup [1999]. The linear regression coefficient (a) was derived from the regression line generated from the variable to be adjusted and known influencing factors ie RMR with FFM and FM, and leptin with BMI. Each value of RMR was then adjusted towards the mean value that intersects the regression line according to the equation as follows:

$RMR_{adjusted} = RMR_{actual} + [(FFM_{mean} - FFM_{actual}) \times a]$ (and also FM)

CHAPTER 4:

STUDY OF TWO UCP2 GENE VARIANTS IN CAUCASIAN OBESE AND SOUTH INDIAN TYPE 2 DIABETIC SUBJECTS

4.1 SUBJECTS

South Indian subjects were used both from the Chennai cross-sectional urban survey (n=453) and family collection (n=85 families). In addition a cohort of one hundred and fifty-six non-diabetic obese subjects and one hundred and five slim normoglycaemic volunteer controls. All previously described in chapter 3.

4.2 METHODS

4.2.1 PCR Analysis of UCP2 exon 8 45bp Insertion/ Deletion

PCR fragments of 412bp or 457bp were amplified using primers:

Forward Primer 5'-CAGTGAGGGAAGTGGGAGG-3'

Reverse Primer 5'-GGGGCAGGACGAAGATTC-3'

Standard 'Hotstart PCR' (3.3.5) in a 25µl reaction volume.

PCR profile: initial denaturing at 95°C for 3 min; 30 sec at 94 °C; 20 sec at 55°C; 20 sec at 72°C; for 40 cycles with a final extension of 72°C for 10 min.

PCR products were resolved on a 2% agarose gel. Homozygotes for the variant were verified by sequencing (3.6)

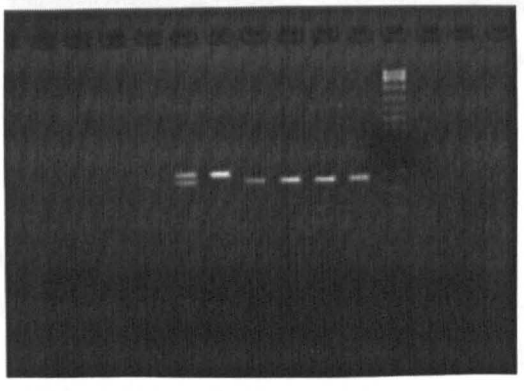

Figure 4.2.1: Image of agarose gel electrophoretic separation of UCP2 exon 8 variant. From left; Ins/Del Heterozygote, Ins/Ins, (4x) Del/Del, 100bp DNA Marker.

4.2.2 Analysis of the A55V variant

A PCR fragment of 127bp was amplified using primers:

Forward Primer 5'-GGGCCAGTGCGCGCTGCAG-3'

Reverse Primer 5'-CATTTGGCGCTGCAGGCCGG-3'

Standard 'Hotstart PCR' (3.3.5) in a 25µl reaction volume.

PCR profile: initial denaturing at 95°C for 3 min; 30 sec at 94 °C; 30 sec at 57°C; 30 sec at 72°C; for 35 cycles with a final extension of 72°C for 10 min.

PCR-gRFLP: A Bbv I restriction site engineered in the forward PCR primer: 5'GGGCCAGTGCGCGCTGCAG**C**CAGCG

Blue= Primer, G=replaced A in sequence to generate BbvI site. **C** –**T** polymorphism.

Bbv I Recognition Site 5'…GCAGC$(N)_{8....}$ 3'

3'…CGTCG$(N)_{12....}$5'

PCR Products (7µl) were digested in a 25µl volume, including 2.5µl of 10x NEB Buffer 2; 1mM spermidine and incubated 5 hours at 37 °C.

Restriction fragments were separated by on a 3% agarose gel G**C**C =Alanine cuts to give 100bp and 27 bp fragments, G**T**C= Valine 127 bp uncut.

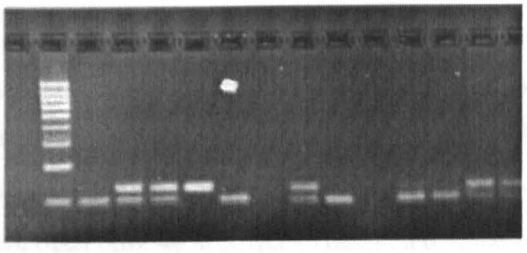

Figure 4.2.2: Gel Image of A55V polymorphism- BbvI restriction digest. Five lanes in order from left were; 100bp DNA marker; Ala/Ala; 2x Ala/Val; Val/Val.

4.3 RESULTS

4.3.1 Characterisation of the UCP2 exon 8 variant

Sequencing of five British Caucasians and five South Indians identified a 45bp insertion/deletion in exon 8 of the UCP2 gene, 158bp downstream of the the 3' TGA stop codon, and located in the non-coding 3'UTR. The insertion (Ins) comprised of a duplication of the preceding (5') 45 bp (figure 4.3.1)

5'CCCACCTCTTCCTTCCGCTCCTTTACCTACCACCTTCCCTCTTTCCCCACCTCTT
CCTTCCGCTCCTTTACCTACCACCTTCCCTCTTTCTACATTCTCA-3'

Figure 4.3.1: Insertion/Deletion sequence in UCP2 exon 8 3'UTR. Green = 45bp Insertion/Deletion; Sequence was essentially the same as subsequently published in French Caucasians [Otabe et al 1998] but with discrepancies at T=C and T =A compared to GDB sequences (Accession U76367) and in Hispanics [Walder et al 1998].

Three UCP2 genotypes (Ins/Ins, Ins/Del and Del/Del) were defined unambiguously according to length variation of exon 8 PCR product with insertion (457bp) or deletion (412bp). The frequency of alleles in the South Indian survey were; 0.81/ 0.19 for Del /Ins alleles accordingly. Similarly in the British Caucasian controls the deletion had a frequency of 0.82. The heterozygotes had a frequency of 28% and 30% South Indian and British Caucasian cohorts respectively, with homozygosity for the Ins allele at a frequency of 5% and 3.6% respectively. The observed frequencies did not significantly depart from those expected by Hardy Weinberg equilibrium in either the South Indian survey (p=0.07) or in British Caucasian study group (p=0.8).

4.3.2 Association studies of UCP2 45bpIns/Del with T2DM in South Indians

Family association studies using ETDT, revealed no association between T2DM and the 45bp Ins/Del variant, with 25 transmissions vs 30 non-transmissions observed for the Del allele from heterozygous parents to T2DM probands (Pearson χ^2 = 0.45, 1df, p= 0.5). Calculations of power for TDT carried out under the assumption that the disease related allele is the Ins allele with a frequency of 0.19 and with 85 families. There would have been 85% power to detect a p value $\geq$ 0.003 if the genotype risk ratio (GRR-γ) was equal to 2 or if the γ = 1.5 there would have been only 46% power to detect a p value $\geq$ 0.06.

In the South Indian urban survey the allele and genotype frequencies were not significantly different between either the T2DM or IGT/IFG subjects, and the normoglycaemic subjects, Pearson χ^2 = 2.41, 6 d.f, p= 0.88 (table 4.3.1). There was also no difference between family T2DM probands and survey non-diabetics (p=0.9).

There was also no significant difference in genotype frequencies between male and female normoglycaemic subjects p=0.76 (table 4.3.2). Comparison of genotype frequencies of both urban survey T2DM subjects and T2DM probands divided by gender either as individual

groups or as a single combined group, with all the normoglycaemic subjects or by gender, found no significant differences between the groups (p values in range 0.2 to 0.9).

		UCP2 exon 8 Genotype		
Subjects	n	Del/Del	Ins/Del	Ins/Ins
S Indian Normoglycaemics	318	67.3% (214)	27.4% (87)	5.3% (17)
S.Indian T2DM	80	68.8% (55)	27.5% (22)	3.8% (3)
S.Indian IGT/IFG	55	63.6% (35)	32.7% (18)	3.6% (2)
S.Indian T2DM probands	84	69.0% (58)	25.0% (21)	6.0% (5)

Table 4.3.1: Genotype Frequencies for UCP2 exon 8 variant in South Indians. Sample numbers for each genotype are in parentheses

		UCP2 exon 8 Genotype		
Subjects	n	Del/Del	Ins/Del	Ins/Ins
Male Normoglycaemics	162	65.4% (106)	29.0% (47)	5.6% (9)
Female Normoglycaemics	156	69.2% (108)	25.6% (40)	5.1% (8)

Table 4.3.2: Genotype Frequencies for UCP2 exon 8 variant in Normoglycaemic South Indians by gender.

4.3.3 Analysis of variant with quantitative traits in South Indians

In the survey South Indians, an association was found between the exon 8 variant and BMI in females only (figure 4.3.2, Table 4.3.3; $p = 0.018$), but not in males ($p = 0.33$). Females homozygous for the Ins allele had higher BMIs than either heterozygous or homozygous Del subjects. Re-analysis of data using a co-variate of age, still found the main effect was between BMI and genotype UCP2 ($p=0.015$; overall model $p=0.036$). Similar results were found when parents ($n=143$) from the South Indian families were analysed (table 4.3.3) ($p < 0.0001$) and also when fathers ($p=0.003$) and mothers ($p=0.002$) were analysed separately.

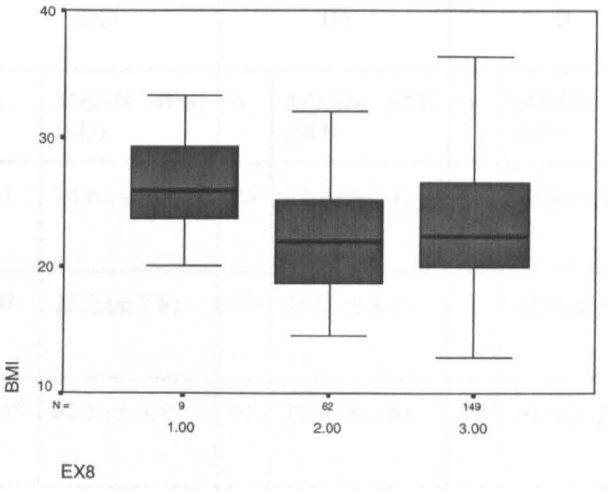

Figure 4.3.2: Boxplot - BMI with Exon 8 genotype in survey South Indian females. Genotype (x-axis) 1.0=ins/ins, 2.0 =ins/del, 3.0=del/del. BMI (y-axis) kg/m^2

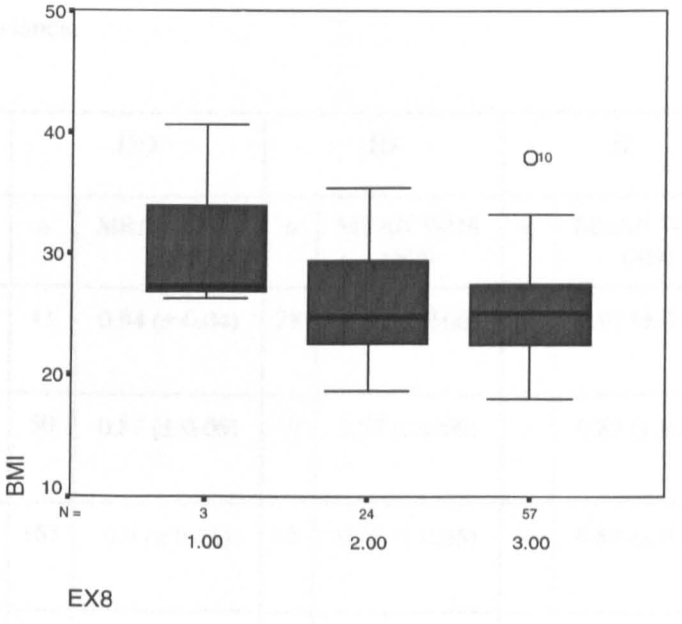

Figure 4.3.3; Boxplot -BMI with Exon 8 genotype in mothers from South Indian families. Key as for 4.3.2

	DD		ID		II		Pvalue
	n	MEAN BMI (SD)	n	MEAN BMI (SD)	n	MEAN BMI (SD)	
Family fathers	41	24.6 (± 3.0)	28	22.9 (± 3.4)	2	30.8 (± 2.5)	0.003
Family mothers	50	25.2 (± 3.8)	19	25.7 (± 4.1)	3	33.9 (± 7.1)	0.002
Survey Males	155	22.1 (± 3.7)	65	22.3 (± 3.6)	13	20.6 (± 3.8)	0.33
Survey Females	149	23.2 (± 4.6)	62	22.1 (± 4.3)	9	26.4 (± 4.5)	0.018

Table 4.3.3: Results of ANOVA analysis of BMI -exon 8 genotypes in South Indians. BMI was normally distributed in all four groups and were not significant for Levene Test of Homogeneity of Variance.

	DD		ID		II		Pvalue
	n	MEAN WHR (SD)	n	MEAN WHR (SD)	n	MEAN WHR (SD)	
Family fathers	41	0.94 (± 0.04)	28	0.94 (± 0.06)	2	0.97 (± 0.02)	0.57
Family mothers	50	0.87 (± 0.06)	19	0.87 (± 0.06)	3	0.82 (± 0.02)	0.31
Survey Males	153	0.9 (± 0.06)	65	0.92 (± 0.05)	13	0.88 (± 0.09)	0.018
Survey Females	147	0.84(± 0.06)	62	0.85(± 0.07)	9	0.82(± 0.04)	0.32

Table 4.3.4:_Results of ANOVA analysis of WHR and exon 8 genotypes in South Indians. Survey males were not normally distributed, values were log transformed and non-parametric Kruskal-Wallis Test used.

In respect of WHR, only the survey males had a significant association with the homozygous Ins subject group having a lower mean WHR (table 4.3.4). This association was not replicated in fathers or found in female subjects.

4.3.4 Analysis of variant with quantitative traits in South Indian families

The transmission of the insertion/deletion was also investigated with traits relating to T2DM and obesity in the South Indian families, using Quantitative TDT. Application of the Orthogonal model both with and without sex as a covariant found no significant associations with transmission of either Ins or Del alleles to offspring with, BMI, Waist, Hip, WHR, age of diagnosis, height, fasting plasma glucose levels, and 120 minute OGTT. The Total association method was also applied within the QTDT analysis program, with the null model variances based on a non-shared environment and polygenic disease, and again all traits tested above were non-significant.

4.3.5 Analysis of 45bp Ins/Del and A55V variants with obesity

In obese British Caucasians, both the 45bp Ins/del Insertion allele (0.27 obese vs 0.19 lean controls), and the A55V valine allele (0.27 obese vs 0.20 lean) were increased in frequency compared to lean subjects (tables 4.3.5 and 4.3.6).

	TOTAL	UCP2 45bp Ins/Del Genotype		
	n	Del/Del	Ins/Del	Ins/Ins
Lean subjects BMI< 27	103	68.0% (70)	28.2% (29)	3.9% (4)
Obese subjects BMI>30	152	53.3% (81)	41.4% (63)	5.3% (8)

Table 4.3.5: Genotype frequencies of exon 8 variant in British obese/lean subjects

Statistical analysis by 2x3 contingency tables found association of A55V variant significant, (Pearson $\chi^2 = 6.65$; 2 df, $p = 0.036$) and exon 8 variant borderline non-significant (Pearson $\chi^2 = 5.48$; 2 df, $p = 0.064$). However, the A55V association was not significant following correction for multiple testing.

	TOTAL	UCP2 A55V Genotype		
	n	Ala/Ala	Ala/Val	Val/Val
Lean subjects BMI< 27	104	55.8% (58)	38.5% (40)	5.8% (6)
Obese subjects BMI>30	167	40.1% (67)	49.7% (83)	10.2% (17)

Table 4.3.6:Genotype frequencies of A55Vvariant in British obese/lean subjects

Since it was impossible to generate haplotypes with the two variants, as allele phase was unknown. Instead an interaction was sought between both genotypes and phenotype. Female subjects only were analysed for the presence of both the Ins allele and Val allele (table 4.3.7). The presence of both the Ins and Val alleles was significantly increased in female obese subjects compared to female lean controls, Pearson $\chi^2 = 3.92$; 1 df, $p = 0.03$), with approximately two fold increase in risk to disease (OR =1.88; 95%CI 1.002-3.54.). There is strong linkage disequilibrium between the Ins and Val alleles (table 4.3.14).

	TOTAL	UCP2 45bp Ins/Del +A55V combinations	
	N	45bp Ins + A55V Val combination	45bp Ins + A55V Val combination not possible
Lean subjects BMI< 27	71	26.8% (19)	73.2% (52)
Obese subjects BMI>30	130	40.8% (53)	59.2% (77)

Table 4.3.7: UCP2 variant combination frequencies in British female obese/lean subjects.

4.3.6 Analysis of 45bp Ins/Del and A55V variants and traits of obesity

In the British Caucasoid obese subjects, both the exon 8 variant and the A55V were analysed with underlying traits associated with obesity. No association with BMI or WHR with either variant was found in female subjects (tables 4.3.8 and 4.3.9)

	UCP2exon 8 Ins/Del Genotypes			P values
	DD MEAN [SD] (n)	ID MEAN [SD] (n)	II MEAN [SD] (n)	
BMI	41.0 [± 6.4] (84)	39.3 [± 6.4] (66)	39.5 [± 5.2] (8)	0.24
WHR	0.84 [± 0.08] (77)	0.86 [± 0.07] (64)	0.88 [± 0.07] (7)	0.32

Table 4.3.8: ANOVA analysis of anthropometric traits in female British Caucasian Obese subjects with exon8 variant. Sample numbers are in parentheses. Levene test of homogeneity of variances for, BMI p= 0.87, WHR p=0.86.

	UCP2 A55V Genotypes			P values
	Ala/Ala MEAN [SD] (n)	Ala/Val MEAN [SD] (n)	Val/Val MEAN [SD] (n)	
BMI	39.8 [± 6.6] (61)	40.5 [± 6.9] (72)	40.3 [± 6.1] (11)	0.8
WHR	0.83[± 0.07] (56)	0.83 [± 0.06] (69)	0.87 [± 0.03] (10)	0.18

Table 4.3.9: ANOVA analysis of anthropometric traits in female British Obese subjects with A55V variant. Numbers in parentheses Levene test of homogeneity of variances for, BMI p= 0.4, WHR p=0.7

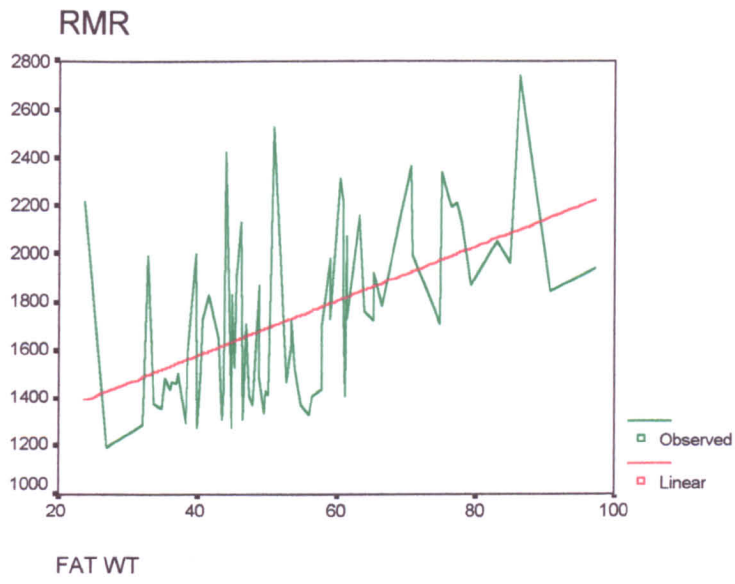

Figure 4.3.4: Linear regression of RMR correlated with FM

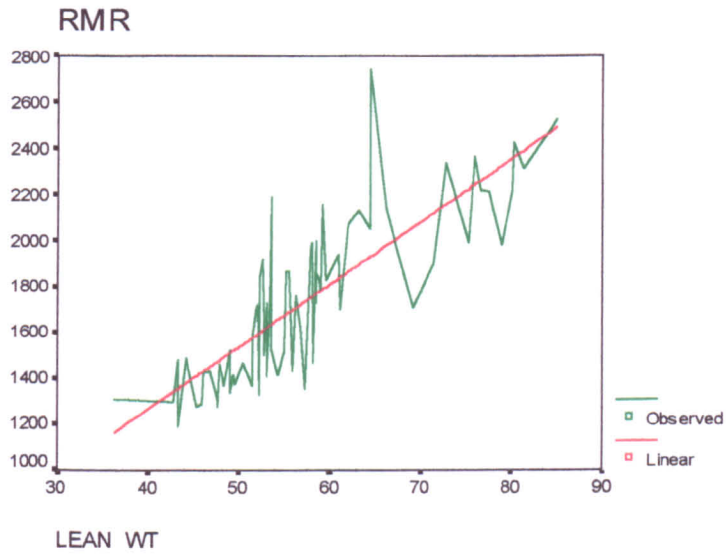

Figure 4.3.5: Linear regression RMR correlated with FFM

Seventy-four obese subjects had both RMR and bioelectrical impediance data allowing adjustment of RMR for both fat free mass (FFM) and fat mass (FM). Correction of RMR values was performed by simultaneous generation of linear regression coefficients, correlating both FFM and FM with RMR (both were significantly correlated Pearson

Correlation $p < 0.0001$ for both FFM & FM individual regressions figures 4.3.4 & 4.3.5) and application of correction equation (3.8.4). The regression coefficient values (a & b) generated for FFM were 24.227 and FM 6.645, and then used in conjunction with appropriate means, FFM 57.0 kg and FM 53.58 kg to correct actual RMR. ANOVA of adjusted RMR values (Levene test of homogeneity p=0.4) were not significantly associated with either the exon 8 Ins/Del or A55V variants (table 4.3.10 & 4.3.11).

	UCP2exon 8 Ins/Del Genotypes			
	DD MEAN [SD] n=37	ID MEAN [SD] n=31	II MEAN [SD] n=6	P value
RMR adjusted	1728 [± 174]	1735 [± 190]	1714 [± 106]	0.96

Table 4.3.10: Analysis of RMR adjusted for FFM and FM with UCP2 exon8 variant in Caucasian Obese subjects.

	UCP2 A55V Genotypes			
	Ala/Ala MEAN [SD] n=26	Ala/Val MEAN [SD] n=40	Val/Val MEAN [SD] n=8	P value
RMR adjusted	1734 [± 175]	1732 [± 182]	1707 [± 158]	0.93

Table 4.3.11: Analysis of A55V variant in Caucasian Obese subjects with RMR adjusted for FFM and FM.

In ninety-six female obese subjects fasting plasma leptin levels were available. Leptin levels were found to be associated with both exon 8 and A55V genotype using ANOVA, p=0.007 and p=0.03 respectively. Leptin levels are correlated with BMI (Pearson Correlation p=0.018), therefore leptin values were corrected by using BMI as a covariant in an ANCOVA analysis (tables 4.3.12 and 4.3.13).

In the obese females the lowest corrected fasting leptin values were significantly associated with the heterozygotes for both the 45bp Ins/del and A55V variants (figures 4.3.6 & 4.3.7).

Although the mean fasting leptin concentration in the four homozygotes for the exon 8 variant was higher than the heterozygotes, the 95% confidence limits spanned the confidence limits for both the heterozygotes and the homozygote wild.

	UCP2exon 8 Ins/Del Genotypes			P value
	DD MEAN [SD] n=45	ID MEAN [SD] n=36	II MEAN [SD] n=4	
Leptin	43.2 [± 15.4]	32.9 [± 13.7]	45.6 [± 16.9]	0.005

Table 4.3.12: ANCOVA for UCP2 45bp Ins/Del variant in female Caucasian Obese subjects for Leptin corrected for BMI. (Levene test of homogeneity p= 0.7)

	UCP2 A55V Genotypes			P value
	Ala/Ala MEAN [SD] n=34	Ala/Val MEAN [SD] n=45	Val/Val MEAN [SD] n=4	
Leptin	44.1 [± 13.7]	35.1 [± 16.6]	38.4 [± 8.1]	0.01

Table 4.3.13: ANCOVA of UCP2 A55V variant in female Caucasian Obese subjects for Leptin with BMI as covariant. (Levene test of homogeneity p=0.8)

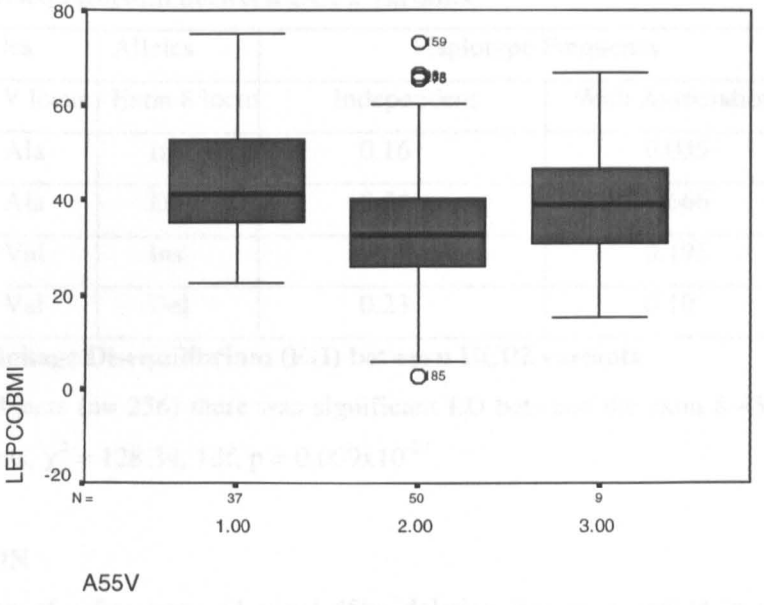

Figure 4.3.6: Boxplot -A55V variant and plasma Leptin levels corrected for BMI.
Genotype (x-axis) 1.0= Ala/Ala, 2.0 =Val/Ala, 3.0= Val/Val. leptin µg/l (y-axis)

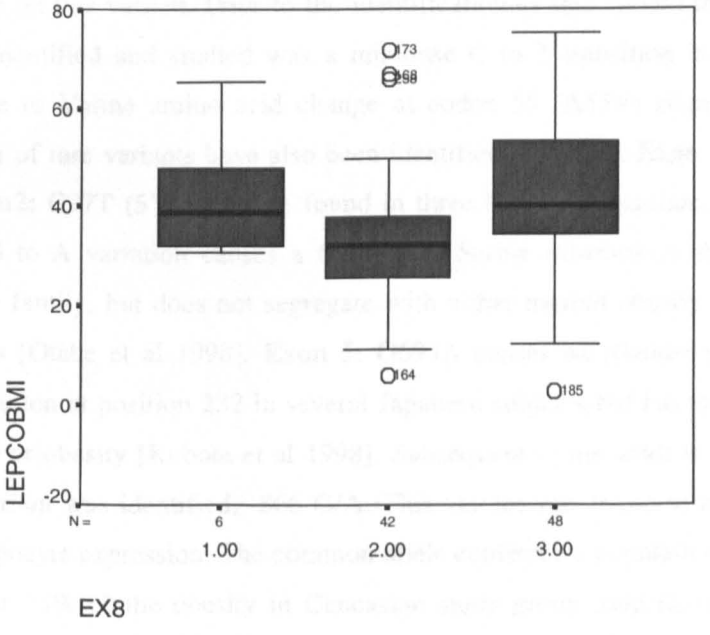

Figure 4.3.7: Boxplot –45bpIns/Del and plasma Leptin levels corrected for BMI
Genotype (x-axis) 1.0=Ins/Ins, 2.0 =Ins/Del, 3.0=Del/Del. leptin µg/l (y-axis)

4.3.7 Linkage Disequilibrium between UCP2 variants

Alleles	Alleles	Haplotype Frequency	
A55V locus	Exon 8 locus	Independent	With Association
Ala	Ins	0.16	0.035
Ala	Del	0.54	0.666
Val	Ins	0.068	0.195
Val	Del	0.23	0.10

Table 4.3.14: Linkage Disequilibrium (EH) between UCP2 variants

In Caucasian subjects (n= 256) there was significant LD between the exon 8 45bp Ins/del and A55V variants, $\chi^2 = 128.34$, 1df, $p = 0.009 \times 10^{-27}$.

4.4 DISCUSSION

The identification of a frequent and novel 45bp deletion /insertion variant in exon 8 (3' UTR) of the UCP2 gene following initial sequencing data and in response to *a priori* information (D.Ricquier and C. H. Warden *personal communication*) led subsequently to association studies on this variant. Prior to the identification of this variant the only other common variant identified and studied was a missense C to T transition in exon 4 that caused an Alanine to Valine amino acid change at codon 55 (A55V) (figure 4.4.1). In addition, a number of rare variants have also been identified including: **Exon 1:** C19T and C27G and in **Exon2:** C97T (5'UTR) were found in three French Caucasians [Otabe et al 1998]. **Exon 4**: G to A variation causes a Glycine to Serine substitution at amino acid position 85 in one family, but does not segregate with either morbid obesity or T2DM in French Caucasians [Otabe et al 1998]. **Exon 5:** G694A causes an Alanine to Threonine amino acid substitution at position 232 in several Japanese subjects but has no association with either T2DM or obesity [Kubota et al 1998]. Subsequent to the work in this thesis a novel promoter variant was identified, -866 G/A. This variant was found to be associated with enhanced adipocyte expression. The common allele conferred a population attributable risk accounting for 15% of the obesity in Caucasian study group used [Esterbauer et al 2001].

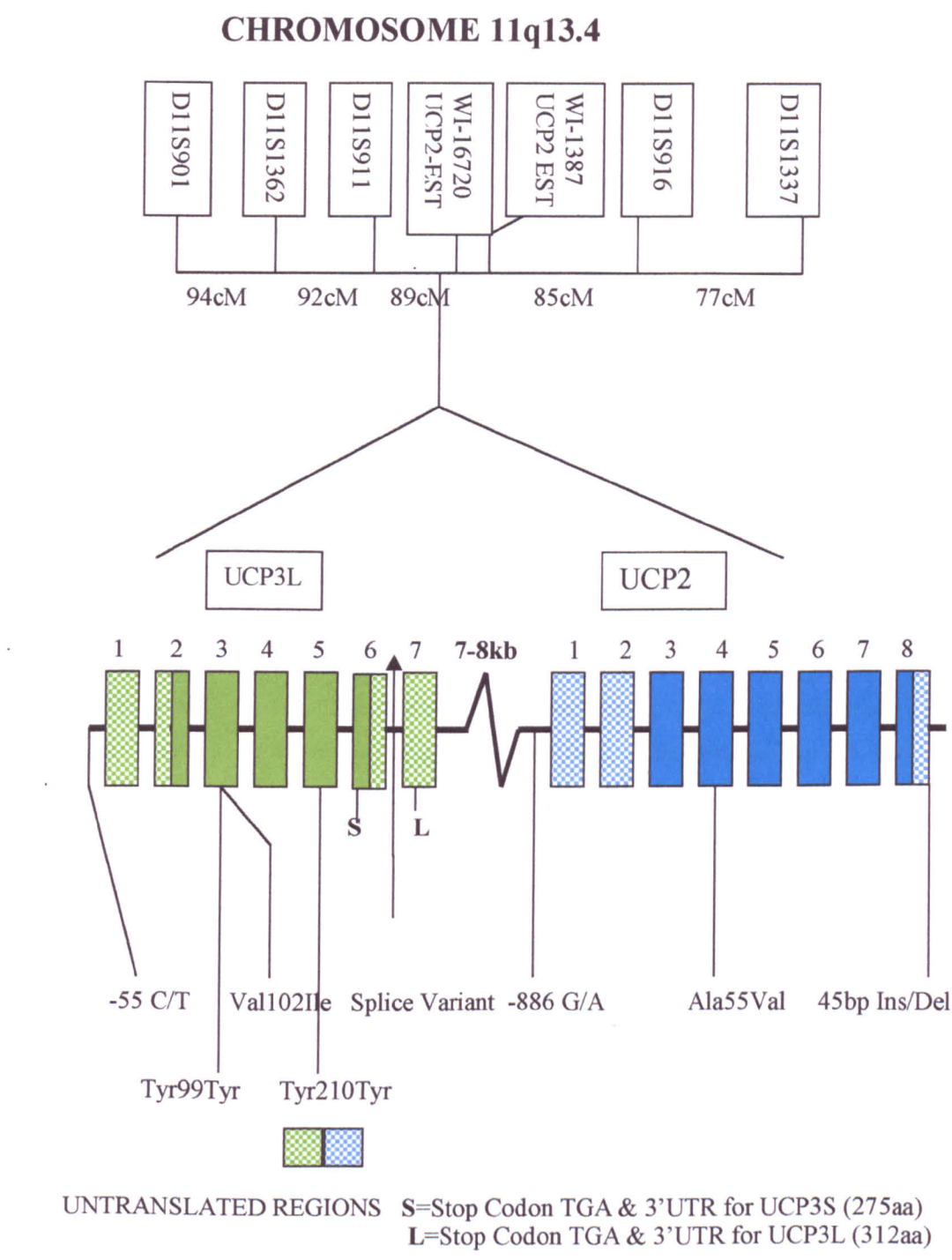

Figure 4.4.1: Exon structure and common variants of UCP3/UCP2 genes.

4.4.1 T2DM

Investigations in this thesis of the UCP2 45bp Ins/Del variant in both the South Indian urban survey and family collections found no evidence for association with either T2DM or IGT. Associations were also negative when analysed by gender. Power calculations for the number of families used suggested that there was 85% power to detect an association if this variant afforded a doubling of risk to disease to offspring, although if the variant afforded a lower relative risk then there would have been insufficient power to detect an association. However, the lack of association in the population based study supports the findings in the families. These observations are in accord with other reported studies, with no linkage with the UCP2/UCP3 cluster with T2DM in North European populations [Elbein et al 1997; Ghosh et al 2000; Watanabe et al 2000] or Pima Indians [Hanson et al 1998] and no association in obese French Caucasians, Japanese or Pima Indians [Otabe et al 1998; Shiinoki et al 1999; Walder et al 1998]. The A55V variant although not studied in diabetics in this thesis was found to be in strong LD with the 45bp Ins/Del variant in the British Caucasians. The A55V was also reported not associated with T2DM in several studies including obese French, Danish and Japanese subjects [Otabe et al 1998; Urhammer et al 1997b; Kubota et al 1998; Shiinoki et al 1999].

4.4.2 Obesity

A number of linkage studies using either overt obesity phenotype and/or related quantitative traits (mainly BMI) have found no evidence of linkage to the chromosome 11q13 [Norman et al 1998; Elbein et al 1997; Chung et al 1999; Comuzzie et al 1995; Hager et al 1998; Lee et al 1999]. However, a majority of these studies probably lacked the power to completely exclude the region. Nevertheless, one linkage study in French Canadians did find suggestive linkage with percentage fat mass ($p=0.02$) and also strong linkage with RMR ($p=0.00002$) [Bouchard et al 1997]. Furthermore, the first published linkage disequilibrium studies on variants of UCP2 (and UCP3) in Pima Indians found that heterozygotes of the 45bpIns/Del had both lower BMI and higher SMR [Walder et al 1998].

Case-control studies in British Caucasians in this thesis of the common exon 8 Ins/Del and A55V variants found the less common Ins allele (exon 8) and T-allele (A55V-Val) respectively both had increased frequencies in the obese subjects compared to lean controls. However, only the A55V variant reached formal levels of significance before correction for multiple testing. The observed simultaneous increased allele frequencies of both the exon 8

Ins/Del insertion allele and the Val allele is most likely a reflection of the LD that was found to exist between these alleles (table 4.4.14). This LD relationship has also been confirmed in other later studies [Yanovski et al 2000].

Comparison of British Caucasian subjects who would be capable of forming possible haplotypes with the positively associated insertion and Val (T) alleles (although actual chromosomal phase was unknown) with those subjects which could not, conferred a two fold increase in risk to obesity. In two contemporary studies in French and Danish Caucasians no association was also found between the 45bp Ins/Del variant and overt obesity [Otabe et al 1998; Dalgaard et al 1999]. Although in obese Caucasian subjects across a number of studies the frequency of the exon 8 Ins allele appeared to be reasonably consistent at 0.30, 0.25, 0.31and 0.27 in Danish, French, German and British subjects in this thesis respectively [Otabe et al 1998; Dalgaard et al 1999; Evans et al 2000]. In contrast, whereas the allele frequency in the control lean subjects were similar to obese patients in the Danish and French at. 0.29 and 0.23 respectively, in the British lean controls the frequency was substantially lower at 0.18. These observations might suggest that the small sample number in the British lean control group could have generated an unrepresentative allele frequency and hence a type 1 error. However, a similar allele frequency of 0.19 for the exon 8 insertion allele was also obtained from nearly a thousand South Indian samples although presumably this group are ethnically less similar to the British than the French or Danish Caucasians.

Disparity between the studies could also be due to the differences in gender ratio within each study population. The British subjects studied in this thesis were predominantly female (86%), whereas in the Danish study they consisted entirely of male subjects, and the French group were predominantly male (70%). Interestingly a study in German obese subjects that were also predominantly female (81%), the exon 8 insertion allele was significantly increased in morbid obese subjects (p=0.002) [Evans et al 2000]. Furthermore, the exon 8 variant frequencies in the German study in obese and lean controls (0.31vs 0.22) were more consistent with the findings in in this thesis than in the Danish or French studies.

In addition, a lack of replication in some contemporary studies may be the result of genotyping misclassification. In the French study no subjects were homozygous for the exon 8 insertion allele [Otabe et al 1998]. However, in both British and South Indian populations studied in this thesis between 3% and 5% were homozygous for this allele. Similarly the Ins/Ins genotype was detected at frequencies of 5% to 21% in many other studies including

Pima Indians, Danish, African/White-/Asian Americans, Japanese, and in three German populations [Walder et al 1998; Dalgaard et al 1999; Yanovski et al 2000; Shiinoki et al 1999; Evans et al 2000].

Investigations of traits associated with obesity and predisposing to T2DM in the South Indian ethnic group found a significant association with the 45bp Ins/Del variant Ins allele with increased BMI in the survey female subjects, but not in males. The homozygous Ins/Ins subject group had the highest mean BMI compared to either Ins/Del or Del/Del genotype groups, although heterozygotes had the lowest mean BMI overall. This association was further confirmed by analysis of the parents in the South Indian families, where both genders were highly significantly associated. In respect of WHR, a lower mean WHR in the survey males homozygous for the Ins allele was also observed. However, the association with WHR was not replicated in the families and neither of these findings were replicated in South Indian families using QTDT analysis, with the transmission of the Ins or Del alleles to offspring uncorrelated with any variance of anthropometric measurements used.

In studies in both Germans and Pima Indians the Ins allele of the 45bp Ins/Del variant was also similarly significantly associated with increased BMI, [Walder et al 1998; Evans et al 2000]. Moreover, in both studies this relationship was more significant when analysis was restricted to subjects greater than 41 (±8.0) and 45 years old respectively. When the trait data in the South Indian survey was re-analysed using a co-variate of age the main effect was still between BMI and UCP2 45bp Ins/Del variant ($p=0.015$).

Heterozygotes for the 45bp Ins/Del also had the lowest BMI in both Pima Indians [Walder et al 1998] and North European Caucasians [Cassell et al 2000] consistent with the observations in female South Indians. No association with BMI and the 45bp Ins/del variant was also observed in the all male Danish study, consistent with the findings in the South Indian survey [Dalgaard et al 1999]. In contrast a study in normal weight children comprising of a mixture of three ethnic groups, Asian, African American, and White found that heterozygotes for the 45bp variant significantly had the highest BMI, although del/del had the lowest BMI and Ins/Ins were the same as heterozygotes [Yanovski et al 2000]. This was also confirmed in that the percentage of overweight children was greatest in the subject group that possessed the Ins allele. Interestingly, in this study significant LD ($p=0.001$) was found between the exon 8 45bp variant and the exon 4 A55V with the same Ins-Val allele relationship as observed in this thesis. However, although children homozygous Val/Val (T/T) for the A55V variant had the highest percentage of overweight individuals consistent

with Val in LD with the Insertion allele. Ala/Val heterozygotes incongruously had a similar overweight frequency to the subjects with the Ala/Ala genotype. In a study of Danish Caucasian subjects a weak association was detected with the A55V variant in with juvenile onset obesity (p=0.04), and in a general population study (p=0.07) [Urhammer et al 1997b]. Interestingly, heterozygotes for the A55V variant had a lower BMI than A/A homozygotes, consistent with the LD relationship between the A55V and the exon 45bp Ins/Del.

The findings with BMI in the South Indians was not confirmed in the obese female British Caucasians cohort with either the 45bp Ins/Del or A55V variants, although there was evidence of association with obesity with these variants. However, the extremity of the overt obese phenotype in the Caucasian obese patients might possibly obscure the detection of subtle associations of traits with genotype.

The studies in this thesis have shown that variation at the UCP2 exon 8 45bp Ins/Del locus (or a variant in strong LD) could account for phenotypic differences in body mass. These findings have been replicated in a number of other studies in different ethnic populations [Walder et al 1998; Evans et al 2000; Cassell et al 2000; Yanovski et al 2000; Urhammer et al 1997b]. However, from the findings in this thesis and other studies it has become strikingly apparent that whatever the functional effect of the exon 8 variant (or a variant in strong LD) the effect of genotype on phenotype was more pronounced in female subjects.

Divergence of body mass and fat distribution between genders has been demonstrated both in Asian Indians and other ethnic groups, with differences reported being evident both well before puberty and in newborns [Snehalatha et al 1997; Taylor et al 1997; Guihard-Costa et al 1997]. Therefore it could be conceivable that there would be gender differences in the genes influencing adipose accumulation.

Correlations between UCP2 mRNA levels and BMI have been demonstrated in obese humans in both adipose tissue and skeletal muscle [Millet et al 1997&1998; Bao et al 1998; Nordfors et al 1998; Oberkofler et al 1998]. Similar differences in the expression of UCP1 in intraperitoneal tissue between obese and lean have been previously observed [Oberkofler et al 1997] and it was suggested that a common sequence variation in the UCP1 gene appears to account for variability of cellular UCP1 mRNA abundance [Esterbauer et al 1998].

A recent study found that subjects homozygous for the more frequent (0.63) G allele of a common G/A polymorphism at -866 in the promoter region of UCP2 associated with increased BMI in German Caucasians [Esterbauer et al 2001]. This novel variant and the

45bp Ins/Del variant appeared also to be in linkage disequilibrium and it was found that certain haplotypes of the two variants accounted for a significant increase in risk to obesity, with a population attributable risk of 15%. Functional studies found the -866 promoter variant associated with enhanced adipose tissue expression of UCP2 both *in vivo* and in reporter studies in adipocyte cell lines. It was further proposed that the promoter variant might influence weight gain by modulating the relative abundance ratio of UCP2 mRNA species containing either the insertion or deletion allele [Esterbauer et al 2001]. Interestingly, there was also further evidence of a gender difference in the allele frequencies of the -866 promoter variant.

4.4.3 RMR

In the cohort of British Caucasians studied there was no evidence of any association with either UCP2 variant with RMR. However, there is evidence for linkage of markers in the vicinity to UCP2 with RMR and the 45bp Ins/Del variant was found associated with sleeping metabolic rate (SMR) (adjusted for FFM and FM) and 24 hour EE in Pima Indians [Bouchard et al 1997; Walder et al 1998]. Pima subjects heterozygous for both the 45bp Ins/Del and A55V variant independently had higher SMR than either of the homozygotes. Walder et al (1998) suggested that this observed increase in SMR and 24hr EE could account for the significant association of the heterozygotes for the 45bp Ins/Del, with lower BMI in subjects over 45 years old, as was similarly found in the female South Indians studied in this chapter.

However, close examination of the published Pima Indian data revealed a disparity with both the South Indian findings and other contemporary studies [Evans et al 2000; Urhammer et al 1997b; Esterbauer et al 2001]. In the Pima Indian study, subjects that were homozygous for exon 8 Ins/Ins and A55V Val/Val independently have higher SMR and 24hr EE compared to Del/Del and Ala/Ala respectively. By inference from the same logical conclusion made for the heterozygotes, the Ins/Ins and Val/Val would have been expected to have a lower BMI than the homozygous Del/Del and Ala/Ala. This was not only contrary to the findings in the South Indians and other studies, but also within the Pima Indian study itself, with the highest mean BMI associated with the Ins/Ins genotype (mean BMI ±SD Ins\Ins 38.4 ±13.4, Del\Del 34.6 ±14.7, Ins/Del 33.7 ±13.6; p=0.04).

In contrast to the Pima Indian study and more consistent with observed BMI associations, a study in Danish Caucasians found both sleeping EE and 24 hr EE lower in the A55V

Val/Val carriers than either the Ala/Val and Ala/Ala subjects [Astrup et al 1999]. When measurements of 24hr spontaneous physical activity (SPA) were also taken into account, the Val/Val subjects had a normal 24hour EE for body size and composition, but with a 3% lower resting EE compared to other two genotypes. These findings were interpreted by the authors that carriers of the Val/Val genotype exhibited an enhanced metabolic efficiency and lower fat oxidation compared to other genotypes. These findings were further supported by the observation that in Danish subjects who were homozygous Valine at A55V had increased energy efficiency during exercise [Buemann et al 2001].

However, the lack of association with RMR in this thesis study was also complemented by two other negative association studies analyzing both common UCP2 variants and a linkage study [Klannemark et al 1998; Tu et al 1999a; Chung et al 1999]. Nonetheless, the measurement of RMR is not entirely equivalent to the measurements of SMR and 24 hour EE, as although RMR is an important determinant of 24hour EE, it only accounts for approximately 50-80% of daily energy expenditure [Ravussin et al 1986].

4.4.4 Leptin

Experimental findings for the obese British Caucasian females indicated that heterozygotes for both the 45bp Ins/Del and the A55V variant were significantly associated with a lower serum leptin concentration corrected for BMI, than either homozygous genotypes in the presence of extreme obesity. However, subjects homozygous for A55V Ala/Ala had the highest mean leptin levels, with a similar trend in homozygous 45bp Del/Del. These results suggest an altered interaction between BMI, leptin and two common UCP2 variants.

Subjects homozygous for the Ins/Ins (or Val/Val) were also associated with higher BMI in South Indian females, Germans [Evans et al 2000], Pima Indians [Walder et al 1998] and obesity in the British Caucasians (4.4.2). This may infer that the increased leptin levels observed in the Ala/Ala (Del/Del) subjects may be some way protective against weight gain. However, observations that subjects heterozygous for either the 45bp Ins/Del or A55V tended to have a lower BMI in South Indians, Northern Europeans, Pima Indians and Danish Caucasians, also had the lowest leptin levels in British Caucasian subjects was inconsistent with this hypothesis [Cassell et al 2000; Walder et al 1998; Urhammer et al 1997b].

In humans plasma leptin concentrations have been correlated with fat mass although there is considerable individual variation in levels with comparable degrees of obesity, suggesting that some individuals produce relatively lower leptin plasma levels [Ramachandran et al

1997c]. Variation in leptin levels could dictate eventual body mass outcome, with data in non-diabetic Pima Indians showing that lower leptin levels both precede an increased tendency to gain weight and are predictive of weight gain, independent of insulin secretion, insulin sensitivity and metabolic rate [Ravussin et al 1988 & 1997]. Gender also influences plasma leptin concentrations with levels observed to be higher in both obese and non-obese females compared to males matched in body composition [Saad et al 1997; Hellstrom et al 2000; Wauters & Van Gaal 1999]. The gender difference appears not to be entirely due to just an increased proportion of adipose tissue but also to higher production rates of leptin per unit mass of adipose tissue in females and influences related to reproductive processes. Ethnic differences on leptin levels have also been apparent with Japanese having increased leptin levels in subjects with a propensity to gain most weight with levels progressively increasing with age [Chessler et al 1998]. The age related increase presumably due to increasing leptin resistance with age, similar to that observed in rats [Li et al 1997].

In rodents a relationship definitely exists between leptin and uncoupling proteins. Leptin modulates UCP2 expression both positively in adipocytes (WAT) and pancreatic islets independently of dietary intake [Scarpace et al 1998; Zhou et al 1997] and negatively in WAT and hepatocytes in the *ob/ob* and *db/db* obese animal models [Gimeno et al 1997; Chavin et al 1999; Melia et al 1999; Larrouy et al 1997]. Observations that chronically infused intra-cerebroventricular (ICV) administration of leptin into rodents effects peripheral UCP2 transcript levels, suggests that leptin may regulate UCP2 by a form of central action [Cusin et al 1998]. However, ICV infusion of leptin also results in both the suppression [Combatsiaris & Charron 1999] and enhancement of UCP2 mRNA levels in WAT in rodents [Qian et al 1998; Cusin et al 1998]. A biphasic leptin effect has also been shown with younger rats suppressing UCP2 in WAT, whilst older rats have enhanced UCP2 expression [Qian et al 1998]. These conflicting findings might reflect the possible degrees of tissue resistance to leptin, as in older rats the effects on UCP expression were less dramatic with higher plasma leptin concentrations [Li et al 1997].

Leptin also appears to modulate UCP3 expression in rodent skeletal muscle, with most studies showing UCP3 expression consistently up-regulated by leptin, in some cases dramatically [Liu et al 1998; Boss et al 1998c]. UCP3 expression in muscle and BAT is highly sensitive to the effects of leptin in neonate mice [Villarroya et al 2001]. However, there are very few studies in humans investigating the role of leptin in pathways involving UCP2 and UCP3. One study shows a complete lack of any correlation between leptin levels

and UCP2 expression in intra-peritineal WAT of morbidly obese subjects [Oberkofler et al 1997]. However, in contrast increased leptin levels, and independently adiposity, were inversely correlated with UCP2 mRNA levels in human sub-cutaneous WAT biopsies [Pinkney et al 2000]. Moreover, interactions between leptin, PPARs and UCP expression have been established [Aubert et al 1997; Qian et al 1998], possibly implicating PPARs in mediating these effects via PPAR response *cis*-elements (PPRE) identified in the 5' regulatory regions of both UCP2 and UCP3 [Tu et al 1999b & 2000; Acin et al 1999; Pecqueur et al 1999].

It appears with some degree of certainty that leptin might influence uncoupling protein mRNA expression however there is currently no evidence to suggest that the reverse is also true. Although a quantitative trait locus influencing leptin levels has been identified in a mouse model of diet induced obesity and diabetes locating in the same chromosomal region as UCP2 and UCP3 [Surwit et al 1998].

Leptin receptors have been found on a variety of tissues including the central nervous system, pancreatic islet cells, haemopoietic cells and adipocytes. It has been suggested that uncoupling proteins could directly influence the function of cells or tissues by at least two separate mechanisms [Boss et al 2000]. Firstly, fat induces the expression of UCP2/UCP3 and this might be linked to a role in preventing the toxic effects of excessive fat metabolism. Increased fat oxidation causes an increased provision of electrons for the electron transfer transport chain in excess of ATP demands and results in the overproduction of apoptotic reactive oxygen species (ROS) [Chavin et al 1999]. Induction of UCP2 could limit this toxicity by reducing proton motive force and decreasing the back-pressure on the proton pumps in the electron transfer chain.

Alternatively, UCP2 could limit the effects of lipotoxicity by altering cellular fuel utilization [Wang et al 1999]. Excess calorie intake results in the overloading of adipose tissue and subsequently other tissues such as liver, pancreas and muscle and is characteristic in obese diabetics [McGarry & Dobbins 1999; Danforth 2000]. Increased lipid accumulation in various rodent tissues including pancreatic β-cells, and liver has been shown to impair their function [Unger et al 1999]. However, the over-expression of UCP2 in isolated lipid loaded pancreatic islets of the *fa/fa* rat clearly improved glucose stimulated insulin secretion [Wang et al 1999]. Impairment of overall uncoupling protein function, either by reduced expression, reduced mRNA turnover, or protein activity could compromise the function of other endocrine tissues such as adipose tissue, and hence leptin secretion.

Impairment of cellular function by ROS could also render cells less sensitive to the action of leptin. UCP2 is abundantly expressed in mouse brain tissue, including those tissues involved in leptin mediated neuropeptide Y expression [Richard et al 1998&1999]. UCP2 could by controlling ROS production influence the process of neural degeneration, post leptin receptor signalling transduction pathways, therefore modulating the neuro-endocrine appetite pathway [Wang et al 1997]. Reactive H_2O_2 have also been shown to induce UCP2 expression *in vitro* in pancreatic β-cells and protect against the detrimental effects of oxidative stress in this organ and possibly delay onset of T2DM [Li et al 2001].

However, all these mechanisms still remain unclear as up-regulation of UCP2 expression in rodents has also been demonstrated to impair pancreatic β-cell function, acting as a potent inhibitor of insulin secretion, possibly though the disruption of multiple pathways by decreasing cellular ATP production and availability [Chan et al 2001; Zhang et al 2001]. A relationship with variants of UCP2 with insulin was not observed in the studies in this thesis in South Indians (fasting and 120 min) and British Caucasians. In a number of other studies involving hyperinsulinaemic clamps in humans no modulation of UCP2/UCP3 mRNA levels by insulin was also observed [Urhammer et al 1997b; Millet et al 1997].

Leptin has been shown in rodents to activate energy expenditure, reducing food intake and increasing metabolic rate, possibly through changes in thermogenesis causing loss of body weight and fat mass [Halaas et al 1995; Pelleymounter et al 1995; Hwa et al 1996 & 1997]. Whether uncoupling proteins can enhance or disrupt this pathway and if truly there is an interaction between UCP2 genotype and leptin levels in humans still remains to be elucidated. However, a genetic determinant that could modulate leptin levels although possibly insufficient to lead to overt obesity by itself, but may be a contributor to weight gain or even weight loss.

4.4.5 UCP2 Variants-Functional Effects

The A55V amino acid substitution in exon 4 has been predicted to occur in the matrix orientated extra-membraneous loop between the first and second transmembrane domains based on a hydrophiliciy model [Vidal-Puig et al 1997; Klaus et al 1991]. The alanine residue is conserved at this position in the mouse UCP2 protein, but is replaced by tyrosine and leucine in the respective positions in human UCP1 and UCP3 proteins respectively. However, this is a conservative amino acid change and no significant functional aberrations

were observed when activity of the protein was assessed in a yeast expression vector [Fleury et al 1997].

The half-lives of most mRNA are influenced by the structure of the 3'UTR, a region that can work as an instability determinant independent of the remainder of the mRNA molecule [Ross 1995]. Insertion of sequences in the 3'UTR can change the positional relationships of important stability determining *cis*-elements. Therefore it could be envisaged that a 45bp insertion or deletion in the 3'UTR could potentially have an effect on mRNA stability. Furthermore, there could also be disruption of the intimate cell specific interactions of environmental factors affecting mRNA stability and longevity via *trans*-acting factors and hormones [Ross 1995].

Variation in mRNA stability as result of genotypic differences could affect protein levels and could either enhance or compromise the action of that protein in physiological pathways. However, studies by Walder et al [1998] did not find a significant difference in UCP2 mRNA levels in skeletal muscle of Pima Indians according to the 45bp Ins/Del genotype. Although this does not preclude a possible effect of the 45bp Ins/Del variant on post-translational modification, or translation of UCP2 mRNA, which could result in altered levels, and/or activity of mature protein, that might be tissue specific.

Alternatively he 45bp Ins/Del could be in LD with a causative mutation in the UCP3 gene, only 8249bp upstream of UCP2 [Pecqueur et al 1999] or with mutations in the important regulatory elements of UCP2 that extend 1400bp towards UCP3 [Esterbauer et al 2001; Pecqueur et al 1999; Tu et al 1999b].

In summary, the findings presented in this chapter show a consistent association between BMI and the less common insertion allele of a UCP2 gene exon 8 variant in South Indian female subjects. This variant is in LD with the exon 4 A55V variant and the presence of a combination of the less frequent Insertion allele and (T)-Valine allele may also confer an increased risk to obesity in British Caucasians. However, contemporary replication studies both support and refute these findings. The associations observed with the UCP2 variants may be the result of linkage disequilibrium with causative mutations either in the promoter of UCP2 or in UCP3. Studies in chapter 5 were designed to identify and analyse variants within the core promoter and coding regions of the UCP3 gene.

CHAPTER 5:

MUTATION SCREENING OF THE UCP3 GENE: IDENTIFICATION OF A NOVEL PROMOTER POLYMORPHISM.

5.1 SUBJECTS

A core group of eighteen subjects were sequenced comprising of six subjects identified as homozygous for the 45bp insertion at UCP2 exon 8, six homozygous for the deletion, and six heterozygous subjects. The group ethnically consisted of 50% South Indian (T2DM and non-T2DM) and 50% obese British Caucasian for each UCP2 genotype. However, during the course of sequencing extra randomly selected Caucasian and South Indian subjects were sequenced for certain exons to verify ambiguities. This resulted in some instances of over forty subjects being sequenced for certain regions.

Subjects used for subsequent screening of any identified variants of were the same as for chapter 4 and as described in chapter 3.

5.2 METHODS

5.2.1 Direct Sequencing of UCP3 Coding Region

The seven exons and intron/exon splice junctions of the UCP3 gene (figure 4.4.1) were sequenced in all eighteen subjects. PCR primers were designed using *PRIMER 3* program (www.hgmp.mrc.ac.uk) using the following Genome Database deposited sequences; Accession numbers, AF012196 for exon 1; AF012197 for exon 2; AF012198 for exon 3; AF012199 for exon 4; AF012200 for exon 5; AF012201 for exon 6; and AF012202 for exon 7. Direct sequencing was by standard methods using an ABI 373 DNA Sequencer and TaqFS Dye terminator cycle sequencing kits (Perkin-Elmer, Applied Biosystems, Warrington, UK) or Thermo Sequenase dye terminator cycle sequencing kit-(Amersham Life Science Inc, Ohio Cat No US79765). Primers were as for PCR.

PCR (25µl) reaction conditions were standardised for all exons with adjustments made to MgCl concentration as per as table 5.2.1.

Standard PCR profile with a pre-denature at 94°C for 5 minutes followed by 30 cycles of: 30seconds at 95°C; 30seconds annealing at temperatures as per table 5.2.1; and 60 seconds extension at 72°C; followed with a final 10 minutes at 72°C.

Exons	ANNEAL TEMP °C MgCl mmol/l	PCR PROD SIZE bp	PCR FORWARD PRIMERS 5'-3'	PCR REVERSE PRIMERS 5'-3'
Exon 1	56 (1.5)	153	AGGAGGGGCCATCCAATC	AAGGGATGAGGGAGGAGAAA
Exon 2a	60 (1.5)	247	AGCCAGGCCAGACATCAC	CAGTGGAAAGGTAACGAGG
Exon 2b	60 (1.5)	203	AAAGGGACTGGGCAGAGC	AGCCCCTCCTTCCATGTG
Exon 3	55 (2.0)	345	TGTGCACGCAGCCCCTTC	CTAGACTTCCCTGGTCTCTTGAC
Exon 4	55 (2.0)	381	GCAGCCCCGCAGAGAAC	ACGCCATGCTGGGAGTCC
Exon 5	55 (1.7)	245	CCATTTCTCCCATTTCCC	GCCCACTCCACGGAGTTC
Exon 6	55 (1.5)	242	TTTGCTTGTCCTTCCTCTGC	TTCTCTGGGAGGGAGTGCT
Exon 7	55 (1.0)	250	GCACTCCACCTGATGTGTTG	ATGTGTGGGTCTGTGTCCAT

Table 5.2.1: Primer sequences used for screening of the UCP3 gene exons

5.2.2 Identification and Sequencing of core promoter region:

Sequence data, but no functional information was available for the 5' UTR at the inception of these studies. Therefore the putative 5' regulatory region for UCP3 was predicted using several computer programs [UK HGMP Resource Centre]. Analysis of the UCP3 gene sequence (GDB Accession AF032871), using the *Prom Predict* program identified two main putative regulatory regions, numbered from the first codon, at -21 to-300 and -2200 to –2400. A second computer program, *Neural Net Promoter Prediction (NNPP),* identified a similar region encompassing part of exon 1 and 400 bases upstream, including both the putative TATAA and CCAC boxes. PCR Primers were designed using the *PRIMER 3* program (HGMP), to amplify a region to include the start of the coding region of exon 1 and 500 bp upstream.

Forward Primer 5'-AGAGACCTGGGAGAGCTGTG-3'

Reverse primer 5'-TGAAAGCCTCCAATGAAAGG-3'

Both primers had a Tm of 60°C. A standard 25µl PCR reaction was used (3.3.2)). The same PCR profile was used essentially as for the UCP3 exons in the preceding section except a 55°C annealing temperature was used. The 495 bp PCR product was sequenced in the same 18 subjects used for the coding region mutation screen.

5.2.3 PCR-RFLP Analysis

Exon 6 splice donor site variant:

PCR profile: HotStart PCR (3.3.5) with Standard PCR protocol (3.3.2) with an annealing temperature of (55°C) and 35 cycles. Primers used were:

Forward Primer 5'-GGGCACTGTGAGAGATATGGA

Reverse Primer 5'-CAGCTGACCCACGGTAG

Fok I Restriction Digest:

Recognition Sequence 5'...(13/9) CATCC...3'

Numbers in parentheses indicate point of cleavage as non-palindromic enzyme.

Restriction mix/sample 25µl:

Sterile, deionised water	10.5µl
Fok I RE 10xBuffer(Appendix I)	2.5µl
Spermidine (10mM)	1.0µl
FokI1enzyme (10units/µl)	1.0µl
PCR Product	10.0µl

Fok I digests were incubated overnight in at 37°C waterbath and products separated on a 3% Agarose gel (3.5.6). Product sizes were 396bp for uncut allele and, 316bp and 80bp cut for mutant allele.

UCP3 Val102 Ile, G304A variant:

PCR profile: HotStart PCR (3.3.5) with Standard PCR protocol (3.3.2) with an annealing temperature of (58°C) and 38 cycles.

Forward Primer; 5'-CCAGCAGGGTTCCTGTGC

Reverse Primer; 5'-CCTGGTCTGCCTCTGAGTCT

Tth III1 Restriction Digest:

Recognition sequence 5'...GACN/NNGTC...3'

Restriction mix/sample 25µl:

Sterile, deionised water	9.0µl
TthIII1 RE 10xBuffer (appendix I)	2.5µl
Spermidine (µg/µl)	1.0µl
TthIII1enzyme (4units/µl)	2.5µl
PCR Product	10.0µl

PCR products were digested overnight at 65°C and products separated on a 3% MetaPhor agarose gel, (3.5.6). Uncut product size was 376 bp. An internal non-polymorphic TthIII1

site produced two fragments in the A/A homozygote, of 295bp and 81bp. The G/G homozygote gave three fragments of 249, 81, and 46bp. The heterozygote should give four bands of 295, 249, 46 and 81bp (81bp being a double band). Positive African DNA controls kindly provided by Dr PJ Saker (Imperial College, London).

UCP3 promoter–55c/t polymorphism:

The –55 c➔t polymorphism was genotyped using a restriction–generating RFLP assay. PCR profile: HotStart PCR (3.3.5) with Standard PCR protocol (3.3.2) with an annealing temperature of 57 °C and 35 cycles.

Forward Primer; 5'- GGATAAGGTTTCAGGTCAGGC-3'

Reverse Primer; 5'- AAGGGATGAGGGAGGAGAAA-3'

A Hae III restriction site was engineered into the 194bp PCR product.

PCR primers Underlined, bold- forward primer with generated restriction site;

Uppercase –HaeIII restriction recognition sites;

Red base-C converted to G to create HaeIII site

Green base- C<> T transition SNP, T allele disrupts site.

ggataaggtttcaggtcaGGCCgtgtgtataagaccagtgccaagccagaagcagcagagacaacagtgaatgacaaggag gGGCCatccaatccctgctgccacctcctgggatggagccctagggagcccctgtgctgcccctgccgtggcaggactcacag gtaagaccccttctcctccctcatccctt

HaeIII Restriction Digestion:

Recognition sequence 5'...GG/CC...3'

Restriction mix/sample 25µl:

Sterile, deionised water	11.0µl
Hae III RE 10xBuffer (Appendix I)	2.5µl
Spermidine (10mM)	1.0µl
Hae III enzyme (10units/µl)	0.75µl
PCR Product	10.0µl

The 194bp PCR product was digested overnight at 37°C. A second naturally occurring non-polymorphic HaeIII site (uppercase-black) conveniently provided an internal control for digestion. The less frequent T variant allele only cuts at the non-polymorphic site giving bands of 84 and 110bp: The common C variant gives 20, 64 & 110 bp fragments. The heterozygote generating 110, 84,64 and 20 bp fragments. Restriction fragments were separated on 3.5 % Metaphor agarose gel, under Rapid gel run conditions for 1 hour (section 3.5.6).

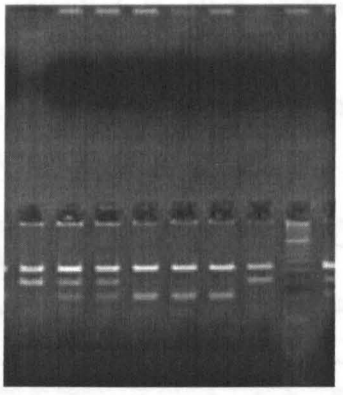

Figure 5.2.1: UCP3-55 promoter variant. Agarose gel separation of HaeIII digestion products. From left to right, homozygous T variant (110bp+84bp); (2x) Heterozygotes (110+84+64+20bp not visible); (3x) homozygous C wild type (110+64+20bp not visible); homozygous T variant; and DNA size marker.

5.3 RESULTS

5.3.1 Variants identified by Sequencing

Sequencing of the UCP3 in 18 subjects, revealed no new mutations in coding regions. Two previously described silent variants, T→C in exon 3 (codon 99 tyrosine to tyrosine) and C→T in exon 5 (codon 210, tyrosine to tyrosine) were identified in the sample group.

For the Tyr99Tyr, 16 of the 18 subjects had the T allele; the one remaining subject had the variant C allele and was a homozygote for the UCP2 45bp Del allele (one no data). In respect of the Tyr210Tyr the most common allele in the 18 subjects (South Indian and British Caucasoid) was the T allele present in 5 of the 6 UCP2 45bp Ins/Ins homozygotes (one no data), 4 of 6 heterozygotes (one no data) and 2 of 5 Del homozygotes (one no data).

Although numbers sequenced were small there was little evidence to support that the exon 3 Tyr99Tyr polymorphism was in linkage disequilibrium (LD) with the UCP2 45bp Ins/Del variant, and therefore it was studied no further. However, there was a suggestion that the C allele of Tyr210Tyr may be in LD with the 45bp Del allele, as the C allele appears to be absent in Ins/Ins homozygous subjects, although this also could not be tested formally due to small numbers. The Tyr210Tyr variant was also not pursued as no contemporary published had found any association with this variant with any disease phenotypes or traits.

In the initial sequencing only the wild type common G allele was observed in both the exon 3 Val102Ile variant and the exon 6 splice donor mutation in the eighteen obese

Caucasian/South Indian sample. This analysis was further extended by PCR-RFLP (5.3.2). In addition, analysis of data for all subjects sequenced found only wild type alleles for all other rarer UCP3 variants identified in other studies including, Val9Met (3 Obese Caucasians, 9 Asians), C288T (8 Obese Caucasians, 8 Asians), G732C (30 Obese Caucasians, 17 Asians), Arg282Cys (25 Obese Caucasians, 9 Asians), and C/T intron 4 mutation (7 obese Caucasians , 4 Asians).

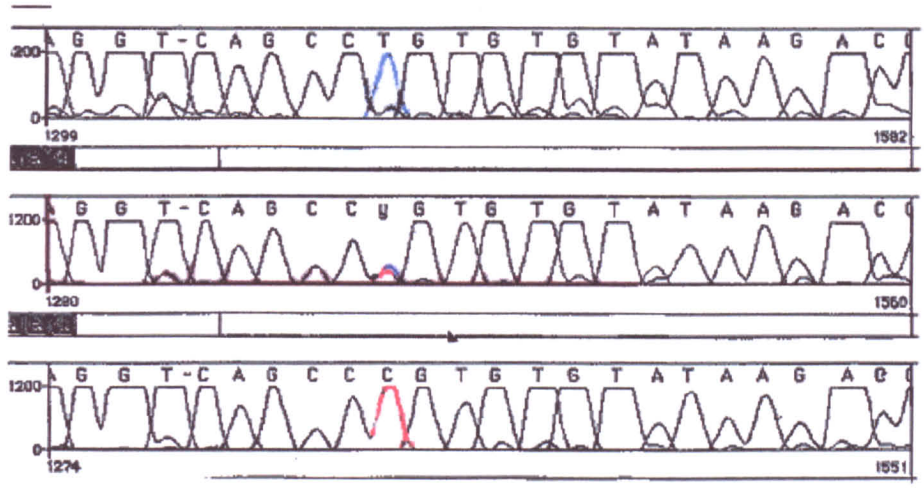

Figure 5.3.1: Sequencing Electropherogram showing UCP3 promoter C-T transition: from top: homozygous T, heterozygote, Homozygous C, 6bp upstream of putative TATA box and 4bp downstream of a putative PPAR/RXR site.

```
2641 AAGAAATGACATTATTATTAAGAGACCTGGGAGAGCTGTGCCCAGCCTATCGTGGGAGGC
2701 CTTGACCTTTGGACTCAAAAGTGGCAGCAGGTCCACCCCCCCATACACCCTTGTCACCAA
2761 GGAAGCGTCCACAGCTTAAAGGAGCTATATTAAAGCACCCCAAGTCAAGAGGACTGAACC
2821 AGATCTGGAACTCACTCACCTCCCCTCTCACCTCACTGCCCTCACCAGCCAGCCTCTTGT
2881 CAAGTGATCAGGCTGTCAACCAACTTCTCTAGGATAAGGTTTCAGGTCAGCCCGTGTGTA
2941 TAAGACCAGTGCCAAGCCAGAAGCAGCAGAGACAACAGTGAATGACAAGGAGGGGCCATC
3001 CAATCCCTGCTGCCACCTCCTGGGATGGAGCCCTAGGGAGCCCCTGTGCTGCCCCTGCCG
3061 TGGCAGGACTCACAGGTAAGACCCCTTTCTCCTCCCTCATCCCTTCCCCTCTCCCTCTCC
3121 CTTCTCCTTGTTCTCCCTTTCATTGGAGGCTTTCAGAGAGCAGCCCCGAGCAGTCAGGGC
```

Figure 5.3.2 UCP3 Promoter sequence showing position of novel mutation. From GDB Accession AF032871: Homo sapiens UCP3 gene, exon1 and partial exon 2. PCR and sequencng Primers, putative **tata box at 2939-2945,**

2988=1 (Start of exon 1, first base transcibed) Novel **c to t** polymorphism at-55.

However, in the putative regulatory region (exon 1 and 400 base pairs upstream) a novel variant was identified consisting of a C to T transition located 6 bases upstream of the putative TATA box (figures 5.3.1 & 5.3.2) [Vidal-Puig 1997; Solanes 1997], at nucleotide position –55 within the putative core promoter of the UCP3 gene. LD with the UCP2 45bp Ins/del variant was difficult to ascertain at this stage due to small numbers, but preliminary data suggested the T allele could be weakly in LD with the Del allele (table 5.3.1). The location of this polymorphism and its possible association with UCP2 prompted further investigation.

5.3.2 RFLP analysis of UCP3 Variants

The mutant A alleles of the common African exon 3 variant Val102Ile and the important exon 6 splice donor mutation were not observed by sequencing in either South Indian or British Caucasian subjects, but as numbers were small both variants were further analysed by PCR-RFLP. RFLP analysis revealed no variant alleles in either the Val102Ile (total subjects including sequencing; 0/9 Obese Caucasians, 0/39 Asians) or the exon 6 splice donor site (total subjects; 0/30 Obese Caucasians, 0/38 Asians). This further confirmed that both variants are probably absent or at an extremely low frequency in South Indian and White North European populations.

5.3.3 –55 variant association with diabetes in South Indians

The frequency of the variant t-allele was 18% in the South Indian urban survey, 21% in the South Indian trios and 21% in unrelated British Caucasians. There was no statistically significant deviation from the expected genotype values for Hardy-Weinberg equilibrium in either the South Indian survey, χ^2 =0.58, p=0.44, or British Caucasians, χ^2 =2.94, p=0.09.

TDT analysis in the South Indian families revealed no evidence for excess transmission of either allele to the T2DM offspring. In 58 heterozygous parents, 31 transmitted the t-allele and 27 the c-allele (χ^2 =0.184, p=0.67). Power calculations were performed under the assumption that the disease related allele was the t allele with a frequency of 0.21 (n= 85 families). Using a muliplicative model, with a γ (GRR) equal to 2 (double the risk), there would an 87% power to detect a p value $\geq$ 0.002, however if γ = 1.5, there would be only

48% power to detect $p \geq 0.05$. If the disease related allele was the C allele at a population frequency 0.79, then if GRR $\gamma = 2$, then power to detect a $p \geq 0.01$, would be 74%.

Ethnic group	UCP245bp Ins/Del	-55C/T variant	Exon 3 T297C Tyr99Tyr	Exon3 G304A Val102Ile	Exon 5 C630T Tyr210Tyr
BOC	DD	C C	T T	G G	C C
BOC	DD	C T	C C	G G	T T
BOC	DD	C T	T T	G G	- -
SI	DD	C C	T T	G G	C C
SI	DD	C C	T T	G G	T T
SI	DD	C C	T T	G G	C C
BOC	ID	C C	T T	G G	- -
BOC	ID	C T	T T	G G	C T
BOC	ID	C T	T T	G G	T T
SI	ID	C C	T T	G G	T T
SI	ID	C C	T T	G G	T T
SI	ID	C T	T T	G G	T T
BOC	II	C C	T T	G G	T T
BOC	II	C C	- -	G G	T T
BOC	II	C T	T T	G G	T T
SI	II	C C	T T	G G	T T
SI	II	C C	T T	G G	T T
SI	II	C C	T T	G G	- -

Table 5.3.1 Comparison of UCP3 variants with UCP2 45bp Ins/Del variant

BOC=British Obese Caucasian; SI= South Indian

In the South Indian survey, the t allele had a frequency of 17.6% in normoglycaemic subjects, 15% in IGT, and 21% in T2DM subjects There was no significant association between –55 c➔t genotype and glucose tolerance status (table 5.3.2; survey NGT, IGT/IFG, T2DM, and T2DM probands) for both genotypes and allele frequencies, Pearson $\chi^2 = 1.7$, 4df p=0.80; and $\chi^2 = 1.44$ 2df, p=0.49 respectively. When subjects were separated by gender, no difference (p=0.8) was observed in the genotype frequencies between sexes in the survey normoglycaemic subjects (table5.3.3). There was also no significant difference in the genotype frequencies between male and female T2DM subjects (survey and probands n= 98; p=0.6; survey and probands n=67; p=0.7 respectively) compared to normoglycaemics.

	UCP3 –55 C/T Genotype			(n)
	CC	CT	TT	
Survey NGT	67.3% (212)	29.2% (92)	3.5% (11)	315
Survey IGT/IFG	72.7% (32)	25.0% (11)	2.3% (1)	44
Survey T2DM	62.8% (49)	32.1% (25)	5.1% (4)	78
T2DM probands	61.4% (51)	37.3% (31)	1.2% (1)	83

Table 5.3.2: UCP3 –55 variant frequencies in South Indian subjects. Sample numbers in parentheses. NGT= Normoglycaemic.

	UCP3 –55 C/T Genotype			(n)
	CC	CT	TT	
Survey male NGT	66.9% (109)	30.1% (49)	3.1% (5)	163
Survey female NGT	67.8% (103)	28.3% (43)	3.9% (6)	152

Table 5.3.3: UCP3 –55 variant frequencies between sexes in South Indian survey normoglycaemic subjects.

5.3.4 -55 variant analysis with quantitative traits in South Indians.

Analysis of quantitative traits in both South Indian families and survey subjects revealed that the less frequent UCP3 -55 t-allele was associated with increased WHR (table 5.3.4; Figures 5.3.3 & 5.3.4). This observed association was only significant in female subjects but was similar across three groups, mothers ($p = 0.036$), female probands ($p=0.032$), and survey females ($p=0.039$). This association was not seen in any complementary male groups (table 5.3.4). Families were further analysed using the "total association" method implemented by QTDT program irrespective of offspring gender, but including sex as a covariate. Shared environment effects were also taken into account in this analysis. Interestingly, the association between the UCP3–55 variant and WHR was confirmed ($p=0.029$). However, using QTDT (Allison's QTDT5 model) to assess transmission from heterozygous parents to offspring, no significant effects were seen for WHR.

		UCP3 –55 c➔t genotype		P values
	n	cc	ct/tt	
South Indian trios				
Fathers	85	0.95 (0.05)	0.95 (0.06)	0.81
Mothers	85	0.87 (0.07)	0.90 (0.08)	0.036
Male probands	54	0.93 (0.04)	0.95 (0.05)	0.10
Female probands	31	0.84 (0.07)	0.89 (0.06)	0.032
South Indian urban survey				
Males	235	0.91(0.06)	0.91(0.06)	0.63
Females	220	0.84(0.06)	0.86(0.07)	0.039

Table 5.3.4: ANOVA analysis of UCP3 –55 variant with WHR in South Indians. Homozygous t-allele subjects were very rare (1.4%), so were pooled with the heterozygous subjects. WHR data is given as mean (SD).

No association was found between the UCP3 –55 variant and BMI with either gender (table 5.3.5). However, surprisingly given the absence of any genotype-related differences in the BMI means (table 5.3.5), evidence for the skewed transmission of the –55 c➔t variant was seen for BMI (p=0.028 with sex as covariate) in families using QTDT, with higher BMI being associated with transmission of the t-allele.

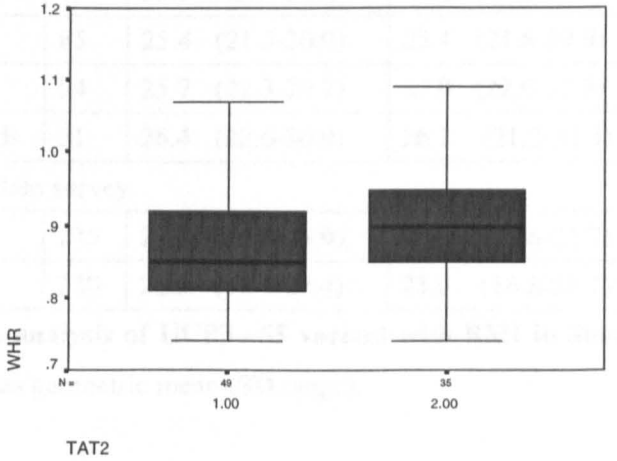

Figure 5.3.3: Boxplot of association of –55 variant with WHR in South Indian mothers. 1.00=C homozygous, 2.00=presence of T allele (C/T & T/T)

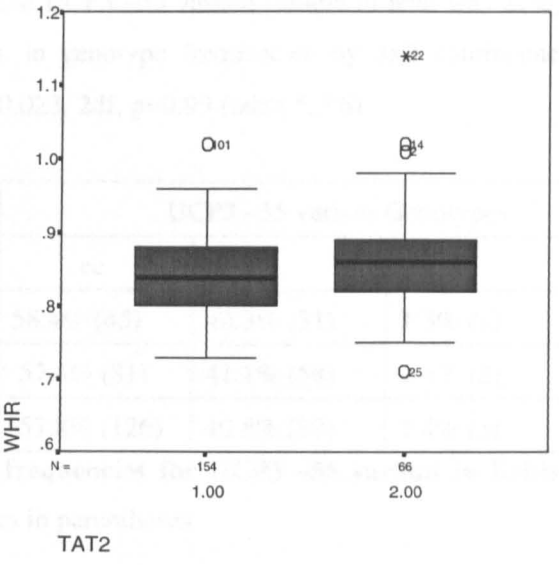

Figure 5.3.4: Boxplot of association of –55 variant with WHR in South Indian survey females. 1.00=C homozygous, 2.00=presence of T allele (C/T & T/T)

		UCP3 –55c➔t genotype		P values
	n	cc	ct/tt	
South Indian trios				
Fathers	85	23.9 (20.6-27.8)	24.2 (21.2-27.6)	0.74
Mothers	85	25.4 (21.5-30.0)	25.4 (21.6-29.9)	1.0
Male probands	54	25.7 (22.3-29.7)	27.0 (22.6-32.3)	0.28
Female probands	31	26.4 (22.6-30.9)	26.3 (21.2-32.5)	0.94
South Indian urban survey				
Males	235	21.6 (18.0-25.9)	21.9 (18.6-25.7)	0.57
Females	220	22.5 (18.4-27.4)	23.0 (18.8-28.1)	0.40

Table 5.3.5: ANOVA analysis of UCP3 –55 variant with BMI in South Indians. BMI data is given in kg/m^2 as geometric mean (SD range).

5.3.5 Case/control study of -55 variant in obese Caucasians

The allele frequency of the UCP3 -55 variant t-allele in lean was 21%, and in obese 22%. Analysis for differences in genotype frequencies by 3x2 contingency tables, were not significant, Pearson χ^2=0.023, 2df, p=0.99 (table 5.3.6)

	UCP3 -55 variant Genotypes			TOTALS (n)
	cc	ct	tt	
Lean Subjects	58.4% (45)	40.3% (31)	1.3% (1)	77
Obese Subjects	57.4% (81)	41.1% (58)	1.4% (2)	141
TOTALS (n)	57.8% (126)	40.8% (89)	1.4% (3)	218

Table 5.3.6: Genotype frequencies for UCP3 -55 variant in British Obese and lean subjects. Sample numbers in parentheses

5.3.6 Study of variant with intermediate traits in obese subjects

Quantitative traits related to obesity were assessed in unrelated female obese subjects only. RMR was adjusted for both FFM and FM, and fasting leptin was corrected for BMI in subjects with available data. No significant association was observed with any of the traits analysed with the UCP3 -55 variant (table 5.3.7)

	UCP3 -55 c-t Genotypes			P values
	cc MEAN [SD] (n)	ct MEAN [SD] (n)	tt MEAN [SD] (n)	
BMI	39.4 [±6.3] (78)	40.5 [±6.9] (52)	41.9 [±0.0] (1)	0.63
WHR	0.83[±0.06] (72)	0.84 [±0.06] (49)	0.78 [±0.00] (1)	0.51
leptin (adj)	39.0 [±16.7] (52)	38.1[±14] (41)	40.3 [±0.0] (1)	0.95
RMR (adj)	1703.7[±156] (40)	1765 [±195] (33)	(0)	0.14

Table 5.3.7: Analysis of quantitative traits with UCP3 -55 variant, in female British Obese. Levene test pvalues BMI=0.8, WHR=0.3, RMR= 0.4, Leptin=0.3.

5.3.7 Linkage Disequilibrium with UCP2 variants

Linkage disequilibrium (LD) between alleles of UCP2 exon8 Ins/Del and the UCP3-55 variant was explored using the *EH* program by comparing the frequencies of the haplotypes with that expected from allelic frequencies, under a null hypothesis assuming no LD.

Alleles	Alleles	Haplotype Frequency	
UCP2 45bp I/D	UCP3-55 c-t	Independent	With Association
I	C	0.18	0.22
I	T	0.48	0.01
D	C	0.61	0.57
D	T	0.16	0.19

Table 5.3.8: Linkage Disequilibrium UCP3 –55 and UCP2 Ins/Del variants.

Alleles	Alleles	Haplotype Frequency	
UCP2 A55V	UCP3 –55 c-t	Independent	With Association
A	C	0.55	0.57
A	T	0.14	0.12
V	C	0.23	0.21
V	T	0.06	0.08

Table 5.3.9: Linkage Disequilibrium UCP3 –55 and UCP2 A55V variants.

In the South Indian survey (n=443), the less common t-allele at UCP3 –55 was in significant LD with the more frequent UCP2 45bp Del allele, $\chi^2 = 14.7$, p=0.00012 (table 5.3.7). In the British Caucasian cohort (n= 243) this was also confirmed, $\chi^2 = 11.79$, 1D.F p = 0.0006. However, there was no significant LD between the UCP2 A55V variant and UCP3-55 in the British Caucasian cohort (n= 239), $\chi^2 = 2.78$, 1D.F p = 0.095 (table 5.3.8), despite the fact the two UCP2 variants were shown to be in strong LD (4.3.7).

5.3.8 UCP2-UCP3 haplotype analysis in South Indian families

Haplotypes of UCP2 45bp Ins/Del and UCP3 –55 variants were analysed for excess transmission to T2DM offspring using the *TRANSMIT* program. No significant association/linkage was observed with a Global p-value of 0.56 (table 5.3.9)

Haplotypes UCP2:UCP3	Observed Transmissions	Expected Transmissions	Chisq 1df	P value 1000bs
Ins / c	26.77	27.2	0.019	0.87
Del / c	104.8	99.9	1.22	0.23
Ins / t	2.2	3.2	0.79	0.37
Del / t	26.1	29.6	1.0	0.25

Table 5.3.10: UCP2 45bp Ins/Del-UCP3-55 haplotype analysis in South Indian families.bs= number of bootstrapping calculations performed.

5.4 DISCUSSION

The associations found with two common variants of UCP2 in chapter 4 with intermediate quantitative traits leading to obesity and T2DM in South Indians and British Caucasians respectively, suggested that variants of UCP2 may influence certain physiological pathways to predispose an individual to weight gain. Alternatively the findings with UCP2 variants might represent linkage disequilibrium with causative mutations in other genes nearby. The UCP3 gene was found to be in close proximity to UCP2 and therefore could be considered a strong potential candidate gene [Pecqueur et al 1999]. Consequently, the coding and core promoter regions of the UCP3 gene were sequenced for novel variants in this chapter.

The subsequent identification of several common UCP3 gene coding variants and a number of rare or ethnically specific variants in contemporary studies also allowed the incorporation of these into the thesis study where justified.

Known UCP3 variants are listed below. Amino acids and nucleotides are numbered 1 from the first residue Methionine, using the sequence submitted by Boss et al (GDB; Accession number U84763).

Missense Mutations:

Exon2: Val9Met; G25A; Rare, only identified in one morbidly obese French Caucasian [Otabe et al 1999], one lean, two obese Caucasians [Chung et al 1999b].

Exon 3: Gly84Ser; Rare, only identified in two Danish healthy controls [Urhammer et al 1998]

Exon 3: Val102Ile (G304A); Common in Africans [Argyropoulos et al 1998b]

Exon 4: Arg143X; Rare, premature stop codon in one African family. [Argyropoulos et al 1998b]

Exon 7: Arg282Cys (C844T); In one lean Asian, three lean and three obese Caucasians, one lean hispanic [Chung et al 1999b].

Exon 7: Arg308Trp; Rare, one morbidly obese French Caucasian [Otabe et al 1999]

Silent Mutations:

Exon 3: C288T; Rare, one obese Hispanic subject [Chung et al 1999b]

Exon 3: Tyr99Tyr (TAT-TAC) T297C: Common in several ethnic groups [Chung et al 1999; Otabe et al 1999; Urhammer et al 1998].

Exon 3: Ala83Ala; Rare, one control subject [Otabe et al 1999].

Exon 5: Tyr200Tyr (TAC-TAT) C630T. Common variant in Europeans [Otabe et al 1999; Urhammer et al 1998].

Exon 6: G732C; Rare, one obese Caucasian [Chung et al 1999b]

Intronic variants:

Intron 4: C-T 36bp upstream of 5' splice site of exon 5 in French Caucasians [Otabe et al 1999].

Intron 3: Four rare single nucleotide polymorphisms identified in Danish subjects [Urhammer et al 1998]

Other Variants:

Splice donor site exon 6: (GGT-GAT) 1 bp downstream of exon 6 3' splice site, generating a transcript and truncated protein equivalent to the UCP3S transcript found in populations of African ethnicity [Argyropoulos et al 1998b; Chung et al 1999b].

5.4.1 UCP3 variants and LD with UCP2 45bpIns /Del

Sequencing of the coding regions of UCP3 in both obese British Caucasians and South Indians did not identify any new variants, but as the mutation screen was limited to small numbers, mutations at very low frequencies (<5%) could have been missed.

Studies comparing the biochemical activity of the truncated UCP3S protein generated by the exon 6 splice donor site [Chung et al 1999b; Argyropoulos et al 1998b] found that UCP3S had a greatly reduced uncoupling activity [Hagen et al 1999] and might be responsible for a reduced efficiency in fat oxidation in obese subjects [Argyropoulos et al 1998b]. The Arg282Cys mutation, also converts a basic residue to a reactive cysteine residue, and appears to slightly reduce uncoupling capacity, whereas neutral variations at Val102Ile and Val9Met have no effect on uncoupling activity [Hagen et al 1999]. The mutant alleles of the variants Val9Met, Arg282Cys, Val102Ile, and the exon 6 splice variant, were not detected in

either the South Indian or British Caucasian subjects studied. The absence or very low frequency of the Val102Ile and the exon 6 splice variants in South Indians and British Caucasians, was also further confirmed in North Europeans [Cassell et al 2000] and other ethnic groups [Chung et al 1999b; Argyopoulous et al 1998b]. These findings suggest that these two variants are exclusive to populations of African extraction.

In respect of the known common silent variants, only one individual was identified with the C allele of the Tyr99Tyr variant, an Obese British Caucasian subject who was homozygous C/C and also homozygous Del/Del for the UCP2 45bp variant. All others typed (n=16) were T homozygous, irrespective of the UCP2 genotype. French Caucasians homozygous C/C for the Tyr99Tyr variant were previously found weakly associated with BMI within obese subjects [Otabe et al 1999], but in the South Indians and British Caucasians it is the UCP2 45bp Ins allele that associated with increased BMI and obesity, not the deletion allele. However, the association in the French obese cohort was not statistically significant following correction for multiple testing and was also not replicated in Danish subjects with juvenile onset obesity [Otabe et al 1999; Urhammer et al 1998]. Superficially there appears to be little evidence of LD between UCP2 45bp Ins/Del and UCP3 Tyr99Tyr variants, although numbers were too small to evaluate formally.

In respect of the second common silent coding polymorphism in exon 5, numbers were also small, but there was some evidence to suggest LD with the UCP2 variant. The C allele of the Tyr210Tyr was only present in individuals carrying the UCP2 45bp Del allele. It was also more frequent in the Del/Del homozygotes and completely absent in subjects homozygous for Ins/Ins in both British Caucasians and South Indians (table 5.3.1). No associations with disease phenotypes or disease predisposing traits have been reported with the UCP3 Tyr210Tyr variant to date.

5.4.2 Novel UCP3 –55 variant -T2DM and obesity

Sequencing of 500bp of the putative core promoter region of UCP3 identified a novel C to T transition at position -55 relative to the first codon. According to the original designation of transcription initiation [Vidal-Puig et al 1997; Solanes et al 1997], this –55 c/t polymorphism lies in the proximal promoter immediately adjacent and 6 bp upstream to the putative TATA box [Schrauwen et al 1999b; Otabe et al 2000].

The initial sequencing data and subsequent genotyping of a large number of South Indian and British Caucasoid subjects found the common UCP2 45bp Del allele was in LD with the less frequent t-allele of the UCP3 –55 C/T variant. However, the UCP3-55 variant in British Caucasians was unexpectedly not in LD with the UCP2 A55V variant, which is in strong LD with the UCP2 45bp Ins/Del variant and is chromosomally positioned between 5'of UCP3 and the UCP2 3'UTR. This data might suggest that the UCP2 A55V and the UCP3-55 variants arose phylogenetically at different times in the evolutional history of the British population.

The relationship between the variation at the UCP3 –55 variant and susceptibility to diabetes, obesity and related anthropometric traits was further investigated in both the South Indian study groups and the British Caucasoid Obese/lean cohort used for the UCP2 dataset. The t-allele had an approximate frequency of 0.18- 0.21 in both South Indians and British Caucasians, however t/t homozygotes were relatively rare in both ethnic groups. However, neither UCP3 allele was associated with either T2DM or IGT in the South Indians. Furthermore the promoter variant was not associated with obesity in the British Caucasians.

One contemporary study in French Caucasians has found a weak association with T2DM and the UCP3 –55 variant with that the t-allele being less frequent in diabetics compared to controls in two separate datasets. In contrast, in a second study in a French population no association with T2DM was found [Otabe et al 2000; Meirhaeghe et al 2000]. In the positive French study frequencies of 0.13 *vs* 0.22 and 0.18 *vs* 0.25 were obtained in diabetics and controls respectively [Meirhaeghe et al 2000]. This frequency of the t-allele was also similar (0.21) in the British Caucasian cohort non-diabetic lean subjects studied and also in two other studies using non-diabetic British and Danish Caucasians, at 0.21, and 0.23-0.33 respectively [Halsall et al 2001; Dalgaard et al 2001]. However, these findings are in contrast to the South Indian data. The frequency of the t-allele was 0.18 in the survey non-diabetic subjects, similar to that of the French diabetics, but with a non-significant increased frequency of 0.21 in the South Indian T2DM patients. Similar frequencies were also observed in diabetic parents from the South Indian families utilized and also in North European Caucasian families, at 0.21and 0.24 respectively [Cassell et al 2000]. This suggests that there may be ethnic differences in allele frequencies of this variant, although all groups referred to above are of Caucasian extraction, the t-allele does appear to be at a lower frequency in the South Indian populations.

In another replication study using White North European T2DM families (n=150) there was also no excess transmission of either allele to T2DM offspring (55 transmitted the t-allele, and 71 the c-allele p=0.15) confirming the findings in South Indian families in this thesis [Cassell et al 2000]. These findings suggest that the UCP3-55 variant is unlikely to be causative or in LD with a causative mutation for T2DM.

Analysis of intermediate traits in the South Indians identified a significant association with WHR, but not BMI in female South Indians (mothers, female probands and females in the urban survey). The presence of the less frequent t-allele of UCP3 -55 was associated with an elevated WHR compared to subjects in which it was absent. The Ins allele of the UCP2 45bp variant was found to be associated with increased BMI (but not with WHR) in female South Indian subjects in chapter 4. Since the UCP2 45bp less common Ins allele is in LD with the common C allele of UCP3-55, the pattern of linkage disequilibrium suggests that the trait associations seen with the UCP2 45bp Ins/Del and UCP3 –55 variants are independent of each other. However, another study in a predominantly female French cohort found that homozygosity for the t-allele associated with increased BMI within both obese and control cohorts (but without any difference in allele frequencies between the two groups) [Otabe et al 2000]. This association apparently conferred a 1.75 fold increased risk to higher BMI within the obese (OR1.75, p=0.03). The t-allele was also in LD phase with the C allele at exon 3 Tyr99Tyr variant and it was suggested that this might explain the previously reported weak association between this variant and BMI [Otabe et al 1999]. In the South Indians and British Caucasians studied, although numbers were small, there was no evidence of LD between the UCP3 Tyr99Tyr and the –55 variant. However, in European families a weak association was observed in male probands, with the UCP 45bp Del allele and increased BMI (p=0.02) [Cassell et al 2000] and given that the Del allele is in phase with the UCP3-55 t-allele this would support the observations in the French Caucasians [Otabe et al 2000] but not by the South Indian data.

In contrast in another replication study using 'free-living' female and male British Caucasians, the Ely collection, the t-allele was associated with a reduced BMI [Halsall et al 2001]. Based on the LD observations in this thesis for both the British Caucasian and South Indian populations, these findings would be in agreement with the UCP2 exon 8 data. The UCP-55 variant t-allele segregates with the UCP2 45bp Del allele and is congruent with the association of the UCP2 45bp Del allele with a lower BMI in female South Indians. In

another contemporary study of Danish Caucasians with the UCP3 –55 variant comprising of several study groups, there was no association with either BMI or % body fat [Dalgaard 2001]. However, the largest group in this study was entirely male.

In replication studies involving the –55 variant, none have investigated the effect of genotype on fat distribution (WHR) [Otabe et al 2000; Halsall et al 2001; Dalgaard et al 2001]. It also cannot be excluded that the either one or both of the positive results with UCP2 or UCP3 in the South Indians are spurious and there may be the result of confounding effects of population substructure that can give rise to Type 1 errors.

QTDT analysis, that should theoretically circumvent this confounding problem, did not provide additional support for the WHR association. Albeit, the lack of replication in the families might be due to the very few informative meioses (31), as a result of low parental variant heterozygosity. The fact that the WHR association was confirmed in the South Indian families using the Total Association analysis option, suggests this interpretation was correct. Moreover, a concurrent parallel study on the –55 promoter variant in a large number of European Caucasian T2DM families also found a significant association with the t-allele with increased WHR in mothers (cc: 0.86(0.07) *vs* ct/tt 0.88(0.08), p=0.037) and by restricted analysis of trios with female probands (p=0.0025) [PJ Saker -Cassell et al 2000]. Furthermore, a similar pattern of linkage disequilibrium between UCP2-UCP3 variants was observed in the Europeans. This consistent relationship with the t-allele and raised WHR in female subjects, across two ethnically distinct populations and three independent datasets argues in favour of the association with WHR being genuine.

Finally, analysis of the UCP3 –55 variant in the British Caucasian cohort found that it did not account for the positive correlations observed with the UCP2 exon 8 Ins/Del variant with leptin. No correlation was also observed with RMR, and together with UCP2 gene variants did not provide any evidence that either UCP locus influences metabolic rate.

5.4.3 Functional roles of UCP3 variants

The original sequencing data of the promoter region of UCP3 [Vidal-Puig et al 1997; Solanes et al 1997] positions the –55 variant at 6bp upstream of the putative TATA box. However, further detailed analysis of the 5' flanking region has suggested that the first exon extends a further 581bp upstream than previously thought, placing the site of potential transcription initiation further upstream [Acin et al 1999]. Potentially the UCP3 –55 variant

still might influence UCP3 function and/or regulation, by possible effects on *cis*-acting elements within the 5'UTR. In non-diabetic Pima Indian subjects homozygous for the –55 c-allele, subjects had reduced skeletal muscle UCP3 mRNA expression although there was no difference in genotype frequencies between obese and non-obese subjects [Schrauwen et al 1999b]. Nevertheless, this study design did not localise the effect to the UCP3-55 polymorphism specifically. In a study in French Caucasians it was suggested that this reported t-allele related increased expression may be protective against insulin resistance as its allele frequency was significantly reduced in T2DM patients [Meirhaeghe et al 2000]. The authors postulated that a diminished induction of UCP3 expression due to hyperinsulinaemia suppression of NEFA levels could in part influence progression to T2DM. However, in contrast in the same study French subjects who were homozygous for the t-allele also had a more atherogenic lipid profile, characterised by significantly higher total plasma cholesterol, LDL-cholesterol and apolipoprotein B concentrations [Meirhaeghe et al 2000].

Whereas studies in humans show that the –55 t-allele may be associated with increased expression of UCP3 which may detemine either an increased or decreased BMI, lower risk to T2DM or increased tendency to central obesity (increased WHR) as found in the study in this chapter [Schrauwen et al 1999b; Meirhaeghe et al 2000; Otabe et al 2000; Halsall et al 2001]. In transgenic mice the over-expression of UCP3 in skeletal muscle causes the mice to become hyperphagic but they weigh less than their littermates. The striking reduction in adipose tissue mass observed was also combined with lower fasting plasma glucose and insulin levels and increased glucose clearance rates. This has provided evidence that in rodents an increased expression of UCP3 in skeletal muscle potentially can influence metabolic rate, glucose homeostasis and influence overall body mass [Clapham et al 2000].

Although studies have demonstrated that UCP3 displays uncoupling activity [Liu et al 1998; Gong et al 1997; Boss et al 1998] and skeletal muscle UCP3 expression has been negatively correlated with BMI, and positively correlated with metabolic rate in Pima Indians [Schrauwen et al 1999a]. The physiological importance of UCP3 in human energy balance remains controversial [Chung et al 1999]. Studies of UCP3 expression have also proved hard to interpret, with publications variously reporting that UCP3 mRNA levels are increased [Bao et al 1998], decreased [Schrauwen et al 1999b] or unchanged [Millet et al 1997 &1998; Boss et al 1998a; Nodfors et al 1998] in obese individuals compared to

controls. Thus, whilst some have interpreted elevated mRNA levels as indicative of compensatory UCP3 up-regulation in the face of obesity [Bao et al 1998], others have suggested that reduced UCP3 expression contributes to the pathogenesis of obesity [Schrauwen et al 1999b]. Studies in healthy male humans suggest that expression of skeletal muscle UCP3 (and UCP2 in both adipose and muscle tissues) is poorly correlated with variability in resting energy expenditure [Boivin et al 2000]. UCP3 expression in humans also consistently increases during acute fasting when thermogenesis is decreasing and when there is a decrease in resting and non-resting energy expenditure [Millet et al 1997; Boss et al 1998a; Weigle et al 1998; Leibel et al 1995].

Gender differences in associations with phenotypic traits were also observed with the UCP3 promoter variant as with UCP2 in chapter 4 and similar arguments could also be applied. In addition, human muscle sympathetic nerve activity also appears to be greater in males due to increased abdominal-visceral fat deposition, suggesting both gender and fat depot differences could determine muscle thermogenic activity [Jones et al 1996]. Sympathetic activity is an important regulator of expression of UCP3 (and UCP2) in skeletal muscle [Boivin et al 2000], particularly β-adrenergic stimulation [Oliver et al 2000], and the degree of SNS activity in muscle is strongly correlated with %body fat in humans [Jones et al 1996]. Gender differences have also been observed in β-adrenergic stimulation of BAT thermogenesis in response to high fat diet in rats, with males having a greater thermogenic capacity and less weight gain. The more pronounced effect of UCP3/UCP2 genotype in female humans in determining increased fat mass and fat distribution compared to males observed in this thesis may reflect this gender determined diminished muscle sympathetic innervation and decreased thermogenesis. It is possible that under conditions of suppressed muscle uncoupling activity in females the ability to detect the effect of UCP2/UCP3 genetic aberrations could be greater as they would be more phenotypically penetrant. Moreover, the combined effect of a UCP2/UCP3 genetic defect and a gender/fat deposition determined reduction in UCP3-UCP2 expression /thermogenic activity could exacerbate an individual's propensity to weight gain.

Furthermore, some of the tissue specific differences in UCP2/UCP3 expression particularly evident in different muscle types, and with the influence of adipose deposition patterns and gender effects [Schrauwen et al 2001; Jones et al 1996] this may account for the inconsistencies observed in many mRNA expression studies.

It has been hypothesised that the primary physiological role of UCP3 may be more related to fatty acid metabolism rather than non-shivering thermogenesis [Samec et al 1998a]. This based on observations that skeletal muscle UCP3 expression (and UCP2) is up-regulated by *in vivo* administration of fatty acids in both humans and rodents [Khalfallah et al 2000; Weigle et al 1998; Brun et al 1999b] and also high fat diet has a more pronounced effect in type IIA fibres [Schrauwen et al 2001]. In addition, no major impairment of regulatory thermogenesis was found after targeted disruption of the UCP3 gene in mice, despite altered mitochondrial proton leak [Gong et al 2000; Vidal-Puig et al 2000]. However, these mice did appear to have an impaired starvation-induced shift in muscle fatty acid partitioning between oxidation and storage [Muoio et al 1997]. Studies on UCP3 mRNA expression in human skeletal muscle biopsies also found no relationship with either RMR or insulin induced thermogenesis although expression positively correlated with carbohydrate oxidation and respiratory quotient (RQ), and negatively correlated with lipid oxidation [Willi et al 1998]. Interestingly this was only observed in female subjects. Furthermore, over-expression of UCP3 in primary human muscle cell lines promotes nutrient oxidation and enhances fatty-acid-dependent inhibition of glucose oxidation [Garcia-Martinez et al 2001], further supporting a role of UCP3 in fuel partitioning. The UCP3 exon 6 splice variant that generates a protein equivalent to the UCP3S isoform has also been associated with an increased RQ and reduction in fat oxidation [Argyropoulos et al 1998b].

The UCP3S isoform is still capable of inserting itself into the inner mitochondrial membrane and is probably biochemically active but may have either reduced or enhanced activity [Hagen et al 1999; Hinz et al 1999b; Renold et al 2000; Bouillard et al 1994]. Differential expression of the two splice isoforms could potentially modulate skeletal muscle uncoupling activity. The ratio of the UCP3S isoform to the UCP3L isoform in muscle appears to be significantly increased in diabetics compared to normoglycaemic controls [Vidal et al 1999].

However, further studies on the relevance of the splice variants and common gene variants on mRNA or protein abundance and function are necessary. In this respect preliminary analysis of the UCP3-55 promoter variant's effect on transcription factor binding was untaken in Chapter 6.

CHAPTER 6:
FUNCTIONAL ANALYSIS OF UCP3 PROMOTER POLYMORPHISM

6.1 SUBJECTS

Allelic specific dsDNA EMSA probes were either generated by PCR amplification of the UCP3 –55 variant in randomly selected subjects homozygous for the variant or dsDNA probes were constructed by annealing synthesised oligonucleotides.

6.2 METHODS

A Homo sapiens UCP3 gene, exon1 sequence GDB Accession AF032871 [Solanes; Bowen] flanking the UCP3 promoter variant at –55 was scanned for possible putative transcription factor *cis*-acting elements using the *Transcription Element Sequence Search* program (*TESS*) using *Transfac database* v3.3, and *MatInspector* (v3.2) computer programmes (www.hgmp.mrc.uk).

6.2.1 EMSA UCP3 –55 probe generation

A 92bp double-stranded EMSA DNA probe was generated by PCR (figure 6.2.1 (a)). This probe encompassed both the -55 variant and flanking putative *cis*-elements. Fifteen 25µl replicate PCR reactions were carried out to generate sufficient probe using Hotstart PCR (3.3.5) reactions and Standard PCR programme (3.3.2) with a 50°C annealing temperature as described in Chapter 3.7.1. Reactions were combined, lyophilised to reduce volume, loaded on 1% agarose gels and bands extracted (3.6.4).

Subsequently short 20bp ds UCP3 promoter probes were generated (3.7.1) containing both the putative PPAR and TATA *cis* binding sites with a probe representing either the T or C allele at -55 (figure 6.2.1 (b)). Specific and non-specific competitor ds DNA fragments were also generated from oligonucleotides (figure 6.2.1 (c-f)).

6.2.2 UCP3-55 EMSA assay

Electrophoretic mobility shift assays (EMSA) were carried out as described in Chapter 3.7.4. Poly IC was found to be optimal for the labelled kit control probe with an OCT2A binding site. However inclusion of Poly AT showed a marked reduction in non-specific binding with the UCP3-55 PCR generated probe compared to Poly IC, hence this was used instead. In this experiment the UCP-55 probe had the wild-type C allele.

(a) UCP3 promoter EMSA PCR generated ds probe

TCCCCTCTCACCTCACTGCCCTCACCAGCCAGCCTCTTGTCAAGTGATCA

GGCTGTCAACCAACTTCTCTAGGATAAGGTTTCAGGTCAGCCCGTGTGTA

TAAGACCAGTGCCAAGCCAGAAGCAGCAGAGACAACAGTGAATGACAA

GGAGGG

KEY: Probe underlined; PCR Primers and -55 C-T variant. Putative *cis*-acting elements: PPRE; TATA box; MEF3;

(b) Sequence of two short UCP3 promoter probes generated from oligonucleotides

5'-TAAGGTTTCAGGTCAGCCC/TGTGTGTATAAGAC
ATTCCAAAGTCCAGTCGGG/ACACACATATTCTG

(c) Specific competitor fragment for PPAR (DR1) binding site

5'-TCTAGGATAAGGTTTCAGGTCAG
AGATCCTATTCCAAAGTCCAGTC

(d) Specific competitor fragment for TFIID binding site (TATA binding protein)

5'-GCAGAGCATATAAGGTGAGGTAGGA
CGTCTCGTATATTCCACTCCATCCT

(e) Non-specific competitor fragment for consensus OCT2A binding site (bold type)

5'-GTACGGAGTATCCAGCTCCGTAGC**ATGCAAAT**CCTCTGG
CCTCATAGGTCGAGGCATCG**TACGTTTA**GGAGACCAGCT

(f) Non-specific competitor fragment for consensus AP2 binding site

5'-GATCGAACTGACCGCCCGCGGCCCGT
CTAGCTTGACTGGCGGGCGCCGGGCA

Figure 6.2.1: EMSA probes generated

ddH20 to 10µl final reaction volume	1	2	3	4	5	6	7
Binding Buffer (Roche)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
PolyAT	--	--	--	0.5	0.5	0.5	0.5
Poly IC	0.5	0.5	0.5	--	--	--	--
Poly L-lysine	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Labelled -55 frag-(c) 5ng/µl	--	--	--	2.0	2.0	2.0	2.0
Unlabelled-55 frag (c) 20ng/µl	--	--	--	--	--	3.0	--
Hela Nuclear Extract	--	1.0	1.0	--	2.0	2.0	2.0
Labelled OCT2A probe	2.0	2.0	2.0	--	--	--	--
Unlablleled OCT2A	--	--	1.0	--	--	--	1.0

Table 6.2.1: EMSA UCP3 Experiment 1 reaction conditions

ddH20 to 10µl final reaction volume	1	2	3	4	5	6	7
Binding Buffer	2.0	2.0	2.0	2.0	2.0	2.0	2.0
PolyAT	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Poly L-lysine	0.5	0.5	0.5	0.5	0.5	0.5	0.5
labelled Short 20bp probe (t) 4ng/µl	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Unlabelled- short probe (t) 33ng/µl	--	--	2.0	--	--	--	--
Unlabelled- short probe (c) 33ng/µl	--	--	--	2.0	--	--	--
Hela Nuclear Extract	--	1.0	1.0	1.0	1.0	1.0	1.0
Unlab PPARfragment 50pmole/ µl	--	--	--	--	0.2	--	--
Unlab TFIIDfragment 1.75pmole/ µl	--	--	--	--	--	2.0	--
Unlablleled AP2 1.75pmole/ µl							2.0

Table 6.2.2: EMSA UCP3 Experiment 2 reaction conditions

ddH20 to 10µl final reaction volume	1	2	3	4	5	6	7	8
5x Binding Buffer	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
PolyAT	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Poly L-lysine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
labelled short probe (t) 4ng/µl	2.0	2.0	2.0	2.0	--	--	--	--
labelled- short probe (c) 4ng/µl	--	--	--	--	2.0	2.0	2.0	2.0
Unlabelled- short probe (t) 33ng/µl	--	--	2.5	--	--	--	2.5	--
Unlabelled- short probe (c) 33ng/µl	--	--	--	2.5	--	--	--	2.5
Hela Nuclear Extract	--	1.0	1.0	1.0	--	1.0	1.0	1.0

Table 6.2.3: EMSA UCP3 Experiment 3 reaction conditions

6.3 RESULTS

The *TESS* program identified the nearest putative transcription factor binding site 4 bp upstream to the variant at -59 AGGTCA-64, peroxisome proliferator-activated receptor element (PPRE). This *cis*-acting element binds to members of the peroxisome proliferator-activated receptor (PPAR) family of *trans*-acting factors. This was overlapped by several other putative binding sites including (1) TGT3 (TGTTTGT) motif a weak affinity site for hepatic nuclear factor 3 (HNF3); (2) Urokinase transcription factor binding element (URTF) also referred to as an AP1/CRE like binding site; (3) an orphan receptor of the nuclear receptor family chicken ovalbumin upstream promoter (COUP); (4) a Retinoid-X receptor (RXR-alpha) element, RXR a member of the hormone receptor superfamily that is activated by retinoic acid. The *MatInspector* program (v3.2) identified a Retinoic acid receptor (RAR) related orphan receptor alpha 1 (-69 GGTC), and at–70 to –74 TAAG a putative muscle enhancing factor –3 MEF3 site. The putative TATA box was also identified by both programs 6bp downstream to the –55 variant as an Avian C-type TATA box.

Experiment 1:

PROBE	OCT2			UCP3 (C) ALLELE				
HeLa NF extract	-	+	+	-	+	+		+
Competitor	-	-	+	-	-	C		Oct2

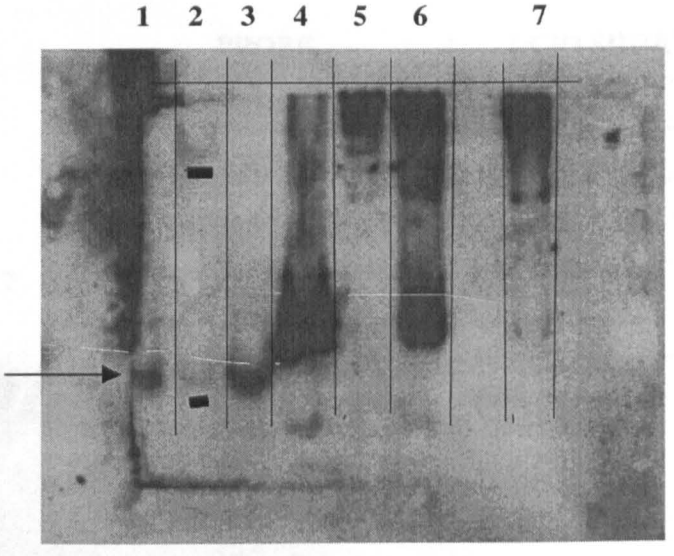

Figure 6.3.1: EMSA (Exp1) using 92 bp UCP3 promoter fragment (C).

Control Lanes 1-3: Labelleled OCT2A probe mobility was retarded with nuclear factor extract present (Lane 2-Bands underlined) compared to lane 1 without (arrowed). Bound complexes were successfully disassociated by competition with a specific competitor in Lane 3 (125x fold excess unlabelled OCT2A DNA probe). Control experiment confirms assay conditions are capable of supporting stable interaction between specific proteins and DNA (figure 6.3.1).

UCP3 promoter fragment lanes 4-7:

Labelled 92bp UCP3 –55 wild type (c) probe had unhindered mobility in Lane 1 and was retarded in lane 2 with HeLa cell nuclear extract presumably because of formation of DNA/Protein complexes. The appearance of multiple bands (at least 4) suggests the binding of more than one *trans*-acting factor.

Addition of excess specific competitor (25 fold excess of unlabelled UCP3 (c) probe) in lane 6 showed successful competition for interaction with proteins. However, some DNA/protein complexes remained intact possibly as a result of insufficient excess of unlabelled specific

competitor. Competition with an excess of unlabelled non-specific DNA competitor (125 fold excess OCT2A probe) in lane 7 did not disassociate DNA/protein complexes. These results suggest that nuclear factors within HeLa nuclear extract specifically bind sites within this UCP3 promoter fragment. This experiment was repeated and similar results were obtained.

Experiment 2:

PROBE	**UCP3 SHORT (T) ALLELE**							
HeLa NF extract	-	+	+	+		+	+	+
Competitor	-	-	C	T		PPAR	TBP	AP2
	1	2	3	4		5	6	7

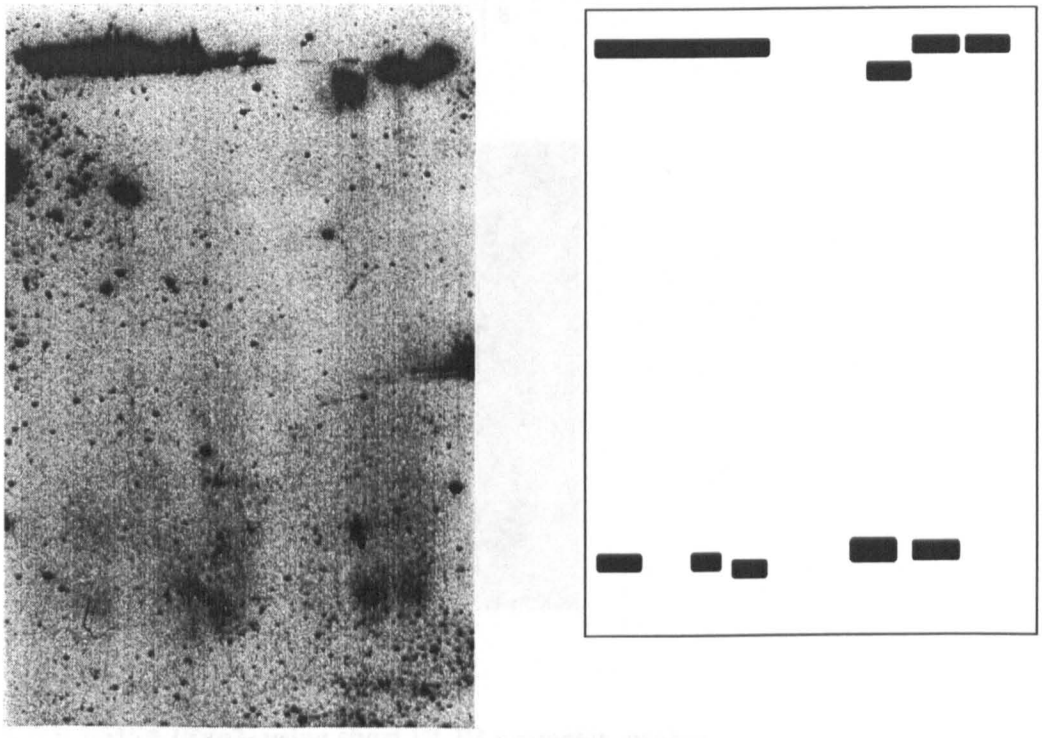

Figure 6.3.2: EMSA (Exp2) using short UCP3 promoter probes.
For easy interpretation a diagrammatic representation was produced to right.

Competition with excess of either of the specific short probes (c or t allele) in lanes 3 and 4 specifically prevented retardation of the labeled 't' probe by bound protein complexes compared to the retardation observed in lane 2 with no competitors. Competition appeared to be specific in that the AP2 consensus oligo did not compete for bound protein complexes in lane 7.

DNA fragments containing motifs specific for either PPAR or TATA binding protein (TFIID) sites also successfully competed for nuclear proteins that bound the UCP3 't' probe (lanes 5 and 6 respectively). Notably the upper retarded bands in these lanes 5 had different mobilities suggesting competition was for different nuclear proteins.

Experiment 3:

UCP3 T allele					**UCP3 C allele**				**PROBE**
-	+	+		+	-	+	+	+	**Hela Nuclear Extract**
-	-	T		C	-	-	T	C	**Competitor**
1	2	3		4	5	6	7	8	

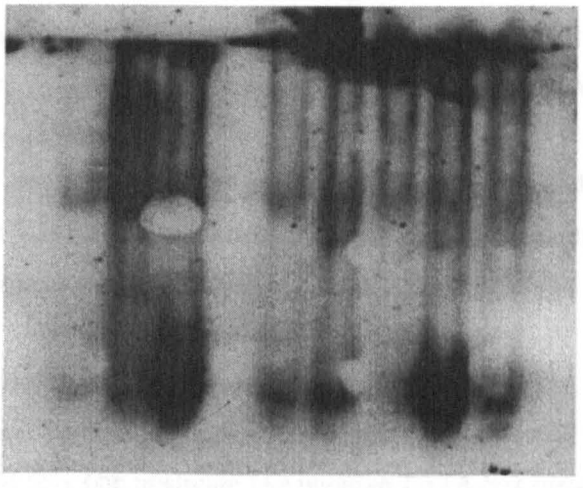

Figure 6.3.3: EMSA (Exp3) using short UCP3 promoter probes.

Lanes 1 to 4, and lanes 5 to 8 were duplicate sets using the 't' allele and 'c' allele short UCP3 probes respectively. The intensity of the non-retarded DNA bands were greater when

either the 't'allele or the 'c' allele probes were in competition with excess unlabelled 't' short probe (lanes 3 and 7 respectively) compared to competition with unlabelled 'c' probe at identical concentrations (lanes 4 and 8). This was also confirmed by pixel densitrometry (table 6.3.1) with increase in intensity of over 25% of the non-complexed DNA probe when competing with unlabelled 't' allele relative to the 'c' allele competitor. This suggested that the UCP3 promoter fragment with the 't' allele had a higher binding affinity for certain nuclear factors in HeLa cell nuclear extract compared to the wild-type 'c' fragment.

Non-retarded UCP3 probe band	Labelled probe	Excess unlabelled Competitor	Integrated Density Value
Lane 3	T	T	21,358
Lane 4	T	C	16,779
Lane 7	C	T	22,277
Lane 8	C	C	16,630

Table 6.3.1: EMSA band densitrometry values. Intensity values were derived using a digital camera and software to quantify pixels in a constant band sample area from autoradiographs (Alpha Innotec Inc).

In summary, nuclear proteins from HeLa cells that would include an array of *trans*-acting factors bind to elements within the UCP3 gene promoter in close proximity to the –55 sequence variation. Evidence was produced to suggest that two factors (possibly PPAR and TBP) might be targeting this region. Furthermore, the complexing of at least one, but possibly more nuclear factors to this UCP3 promoter region appeared to be compromised by the sequence variation at –55.

6.4 DISCUSSION

The novel previously unpublished UCP3 –55 promoter variant discovered in Chapter 5 was located only 6bp upstream to a putative TATA box identified and positioned at –45 to –49 in several studies [Solanes et al 1997; Vidal-Puig et al 1997; Tu et al 2000]. However, two further studies on UCP3 transcription initiation suggested that there could be second or alternative initiation sites with exon 1 extending upstream by a further 581bp or an alternative TATA box at –187 [Acin et al 1999; Tu et al 2000]. Although there appears to be some disparity in the exact structure of the 5' UTR, the scan of DNA sequence including and flanking the UCP3 –55 promoter variant by software that can identify sequence motif's

to putative *trans*-acting factor *cis* elements, identified a number of potential candidate sites. Putative TATA box, PPRE, RAR, RXR and MEF3 sites were all identified in close proximity to the variant. The presence of all these *cis*-element motif's within a few base pairs of the –55 variant suggests that variation of this polymorphism could modify the binding of nuclear *trans*-acting factors to their respective DNA sites and potentially exert an effect on transcription of the UCP3 gene.

The work carried out in this chapter was a preliminary investigation to establish whether *trans*-acting factors could potentially bind to a UCP3 promoter region in close proximity to the–55 variant. The preliminary EMSA experiments carried out strongly suggested that at least one but possibly more *trans*-acting factors contained within the HeLa nuclear extract bind in a highly specific manner to this promoter region. The subsequent experiments using a short promoter probe that was more restricted to DNA sequence harbouring the putative PPRE/RXR, MEF3, TATA and variant confirmed that there were nuclear factors that have affinities for both the PPRE/RXR and TATA box *cis*-elements. Nuclear factors such as these are ubquituous and found in many cell types. However, the utilization of a HeLa cell nuclear extract instead of a skeletal muscle nuclear extract in which UCP3 specifically expresses would mean some other interactions could be missed, such as with the muscle specific MEF3. However, the location of the variant between two different *trans*-acting binding sites could potentially make it very important in the transcriptional activity of the UCP3 gene.

The *trans*-acting factor search programs identified a putative PPRE site within 4bp of the -55 variant, and a RXR site also in very close proximity. RXR frequently heterodimerises with PPAR to form a DNA bound protein comlex [Mangelsdorf & Evans 1995]. Putative PPAR sites have been reported in the promoter region of UCP2 [Tu et al 2000; Pecqueur et al 1999]. A putative PPARγ site amongst others has been suggested to locate to between –67 to –56 adjacent to an upstream putative thyroid response element (TRE) at –71 to –65 [Tu et al 2000]. Members of the PPAR family are expressed at high levels in human skeletal muscle with PPARα and PPARβ the predominant sub-types [Auboeuf et al 1997; Costet 1998]. UCP2 and UCP3 expression in rodents muscle and adipose cell lines has been separately regulated by PPARα, PPARß and PPARγ agonists such as thiazlidinediones/troglitazone, bezafibrate, WY-14.643, and known fatty acid ligands of PPAR's such as Linoleic acid [Camirand et al 1998; Aubert et al 1997; Matsuda et al 1998; Gong et al 1997; Brun et al 1999a; Pedraza et al 2000; Cabrero et al 1999; Teruel et al 2000]. In human skeletal muscle PPAR's are potential candidates mediating the effects of

fatty acids on uncoupling proteins. However, the regulation of UCP2 and UCP3 could be controlled by intracellular metabolism and oxidation of lipids, without recruitment of specific nuclear receptors or these nuclear receptors could influence transcription without the involvement of fatty acids. Furthermore, thyroid hormone is recognised as a thermogenic hormone thought to exert its effect through a generalised stimulation of the metabolic activity of many tissues. Thyroid hormones consistently up-regulate UCP expression [Lanni et al 1999; de Lange et al 2001].

During the course of my investigation, an extensive functional study of the UCP3 gene promoter in muscle cell lines was reported [Solanes et al 2000]. Deletion and point mutation analysis of the human UCP3 promoter revealed an important direct-repeat element separated by one base pair (DR1) between positions –71 to –59. The DRI element locates to a previously described putative TRE-PPRE site [Tu et al 2000]. Using EMSA this study demonstrated that the DR1 site bound nuclear proteins from muscle cells and that the DR1 site is most likely comprised of both RAR and RXR *cis*-elements [Solannes et al 2000]. Furthermore, in deletion mutant reporter gene studies retinoic acid (RA) was found to be a transcriptional activator of the UCP3 gene transcription, but only when the DR1 element was present in the promoter fragment. Interestingly, this is the same site that the transcription factor element identification program suggested that putative PPRE, RAR and RXR receptors *cis*-elements were located. However, DR1 elements are highly promiscuous and could be mediating responsiveness either as a retinoic acid response element (RARE) with a heterodimer of RAR/RXR, or alternatively as a heterodimer with PPAR/RXR, or with HNF-4 or COUP-TF or even as a RXR homodimer. In the studies of Solanes [2000] the observed increased response from all-*trans* RA over that of 9-*cis* RA, suggests that DR1 site is most likely to be a RARE consisting of a RAR/RXR heterodimer. The DR1 element is only 4bp upstream to the –55 variant and was included in the sequence of the EMSA probe used in my study. RAR an RXR nuclear factors would not be restricted to muscle tissue alone and would almost certainly be present in the HeLa cell nuclear extract. It is therefore highly probable that one or several of the nuclear factors binding to the UCP3 probes could be doing so via the DR1 site.

As mentioned above the limitations in experimental design in this thesis would make it incapable of demonstrating binding to some other important putative *cis*-elements that were recognized by the transcription element search computer software. In this respect a putative muscle enhancer factor (MEF3) *cis*-element was predicted in close proximity to the –55

variant and immediately adjacent and upstream to the DR1 element at –71 to –74. Although MEF3 is an important nuclear factor in muscle cell specific transcription of some genes it is unlikely to be present in the Hela cells. In the Solanes [2000] study UCP3 expression was largely dependent on the action of the MyoD factor via an E-box site, an important factor that appeared to a prerequisite for muscle specific expression of this gene. The putative MEF3 motif was also located in deletion fragments that appeared to be important in regulating UCP3 transcriptional activity [Solanes et al 2000; Tu et al 2000]. Although the distance of the –55 variant from this site makes it unlikely to affect binding affinities. A replication of EMSA experiments in this chapter using skeletal muscle nuclear extract could reveal other yet undetected DNA/protein complexes.

Preliminary studies in this thesis also showed that a UCP3 promoter fragment with the –55 t-allele appeared overall to have a greater affinity (> 25%) for nuclear factors compared with a promoter fragment with the c-allele. Although, these experiments could not identify which *trans*-acting factor complexes proteins were affected by the –55 variation. The differences in binding capacity observed could potentially either increase or decrease transcription rates depending on whether the *trans*-factors acted in an enhancer or suppressor mode. In the Solanes et al [2000] study, as far as can be ascertained, the human UCP3 promoter clone used possessed the t-allele of the –55 variant but the effect on binding to the DR1 element by the –55 variant was not investigated. However, in the Solannes deletion mutant transcription studies the most important transcription regulatory elements were found to be located upstream 3kb 5' of base –61 [Solanes et al 2000; Tu et al 2000]. Moreover, elements that might be present in DNA sequence downstream of –61 (-61 to +47) appeared only to have minor effects on transcription rates above basal levels suggesting that the –55 variants may have relatively little effect on regulation of transcription [Solanes et al 2000]. In contrast, studies in humans have shown that in non-diabetic Pima Indian subjects homozygous for the –55 c-allele there is a reduced skeletal muscle expression of UCP3 mRNA [Schrauwen et al 1999b]. Further EMSA studies are necessary to identify which of the *trans*-acting factors has its binding affinity icompromised by the –55 variation, and *in vitro* transcription assays are required to determine whetherit actually influences the regulation of UCP3 gene expression.

CHAPTER 7:

CALPAIN 10 GENE; TYPE 2 DIABETES SUSCEPTIBILITY GENE IN A SOUTH INDIAN POPULATION

7.1 SUBJECTS.

Linkage Studies: Two hundred and twenty-four pedigrees (471 affected siblings with 278 possible sib pairs) of North European Caucasian extraction were studied from the BDA Warren 2 ASP repository as described in chapter 3. These families were part of the initial collection of the Warren 2 and were derived from three of the six collecting centres.

Association Studies: Subjects used were from both the South Indian urban survey (n=468) and family collection (n=97 families). These have all been described in Chapter 3.

7.2 METHODS.

7.2.1 Microsatellite markers for Linkage Study

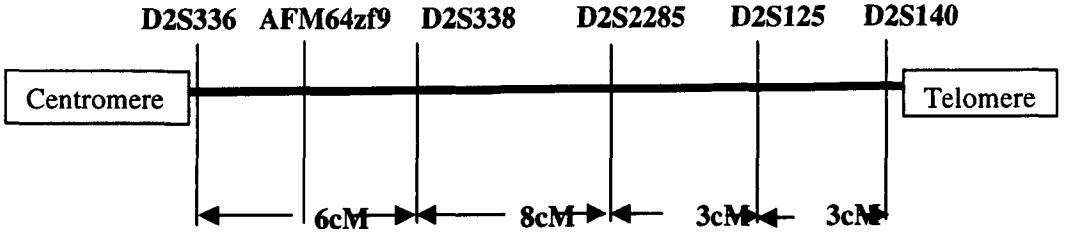

Figure7.2.1: Map of six linkage markers used on for studies on Chromosome 2q

Fourteen microsatellite markers (table 7.2.1), including D2S125, were originally selected with reference to the Mexican American (MA) genome scan [Hanis et al 1996]. In the MA study markers D2S338 MLS=0.84, D2S140 MLS=1.30, and D2S395=1.78 all had nominal evidence for linkage [Hanis et al 1996]. Markers with high PIC values within the 20cM region spanning the telomeric region were selected from Genebank/Genethon databases. Six microsatellites (Figure 7.2.1) were finally selected (table 7.2.1*) by map position, robustness of PCR, ability to score alleles unambiguously and appropriateness for multiplexing. Microsatellite markers were labelled, Touchdown PCR amplified and genotyped as per Methods section in Chapter 3. Two CEPH controls were used on every gel to control for inter-gel variation.

Marker ID	Product Size	Fluorescent label-ABI	Forward Primer Tm	Reverse Primer Tm
D2S125*	95 bp	TET	62	64
D2S336*	134bp	FAM	64	52
AFMB333XG9*	150bp	HEX	56	52
D2S395	155bp	TET	54	56
D2S140*	167bp	FAM	58	64
D2S2338*	169bp	HEX	52	54
D2S407	194bp	FAM	58	68
AFMA064ZF9	227bp	TET	60	56
D2S2253	229bp	HEX	66	58
D2S2202	234bp	FAM	58	58
D2S427	245bp	FAM	58	58
D2S345	256bp	HEX	48	56
D2S2285*	262bp	HEX	52	52
D2S338	282bp	FAM	60	58

Table 7.2.1: Microsatellite markers for NIDDM1 linkage analysis.

7.2.2 Typing the UCSNP43G/A polymorphism

Subjects were genotyped using the MS-PCR method (3.5.4).

Primer sequence:

Common forward primer 5'-CATCCATAGCTTCCACGCCTC

Allele 1 (G) 5'-GCTTAGCCTCACCTTCAATC

Allele 2 (A) 5'- ATCCTCACCAAGTCAAGCGTTAGCCTCACCTTCAAGT

Allele specificity indicated in red and underlined nucleotides show bases mismatched with the template to improve the allele specificity.

The common primer was used at a concentration of 1000 nmol/l and allelic specific forward primers were used at a ratio of 10:1 (short allele 1 primer 1000 nmol/l: long allele 2 primer 100 nmol/l).

Hot-Start PCR (3.3.5) was carried out in a 10µl reaction volume:

10X PCR Buffer	1.0µl 1.5mmol/l $MgCl_2$,
dNTPS (25mM)	0.1µl 200µmol/l each dNTP

Primer Forward (100pmole/µl)	0.1µl	1000 nmol/l
Short Reverse Primer (100pmole/µl)	0.1µl	1000 nmol/l
Long Reverse Primer (100pmole/µl)	0.01µl	100 nmol/l
Qiagen Hotstar Taq Polymerase (5u/µl)	0.1µl	
UV treated HPLC water	3.6µl	
Genomic DNA (5-10ng/µl)	5.0µl	

Standard PCR amplification profile used (3.3.2) with an annealing temperature of 57°C. Cycling conditions were 96 °C for 15 min; and 35 cycles of 94 °C for 30sec; 57 °C for 30sec; and 72 °C for 30sec; followed by 10 min at 72 °C. PCR fragments were separated using a 3% Metaphor agarose gel (3.5.6). PCR product sizes were 134bp (allele 1) and 152bp (allele 2) (figure 7.2 2)

7.2.3 Typing the UCSNP44T/C polymorphism:

This polymorphism was also typed using a MS-PCR method (3.5.4)

Primers:

Common reverse primer: 5'-CTCATCCTCACCAAGTCAAGGC,

Allele 1 (T) primer 5'-CAGGGCGCTCACGCTTGCTAT,

Allele 2 (C) primer 5'-GTGGGCAGAGGACTGGTGGGCGCTCACGCTTGCTTC.

The reaction PCR cycling conditions were the same as for UCSNP43 except annealing at 60°C. PCR fragments were size separated on a 4% Metaphor agarose gel (3.5.6). PCR product sizes were 60bp (allele 1) and 75bp (allele 2) (Figure 7.2.3).

7.2.4 Typing the UCSNP19 Ins/Del polymorphism

PCR Primer sequences:

Forward 5'- GTTTGGTTCTCTTCAGCGTGGAG

Reverse 5'- CATGAACCCTGGCAGGGTCTAAG

Hot-start PCR (3.3.5): PCR was carried out in a 20µl reaction volume with 40ng of genomic DNA. PCR profile (3.3.2): 94°C for 12 min, and 35 cycles of 94°C for 30sec; 60°C for 30sec; and 72°C for 30sec; followed by 10 min at 72°C The 32bp insertion/deletion was size separated on a 3% Metaphor agarose gel (Flowgen) (3.5.6). PCR product sizes were 155bp (allele 1) and 187bp (allele 2) (figure 7.2.4).

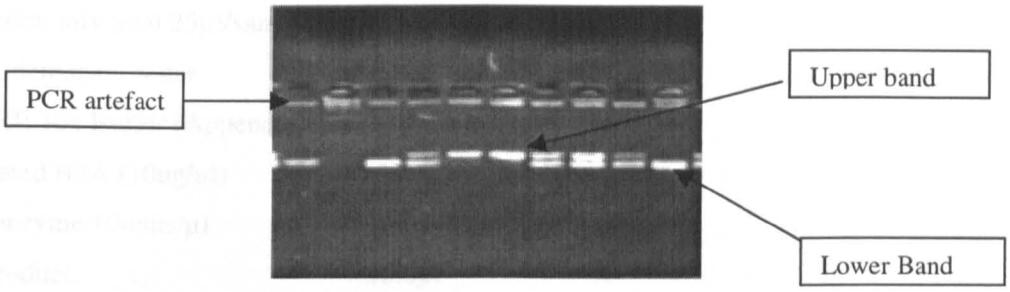

Figure 7.2.2: UCSNP43 MS-PCR genotyping gel. Homozygotes allele 1 upper band (134/134bp), heterozygotes two bands, and homozygotes allele 2 lower bands (154/154bp)

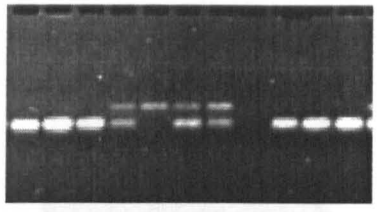

Figure 7.2.3: UCSNP44 MS-PCR genotyping gel. Homozygous allele 2 (75/75bp) lower bands, homozygous allele1 (60/60bp) upper band, and heterozygotes two bands.

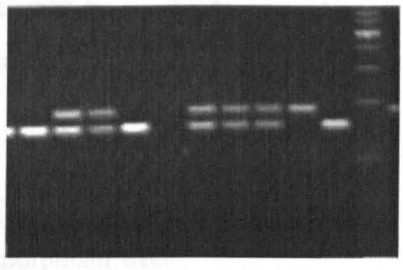

Figure 7.2.4: UCSNP19 Ins/Del Genotyping gel. Lower band=homozygous (155/155bp), upper band=homozygous insertion (187/187bp), and heterozygotes have both bands.

7.2.5 Typing the UCSNP63C/T polymorphism:

PCR Primer sequences:

Forward 5'-AAGGGGGGCCAGGGCCTGACGGGGGTGGCG

Reverse 5'-AGCACTCCCAGCTCCTGATC

PCR conditions were the same as for UCSNP19 except that the annealing temperature was 62°C, Standard PCR protocol (3.3.2) and HotStart PCR (3.3.5).

PCR-RFLP -Hha I Restriction digest:

Recognition Sequence 5' ...GCG/C...3'

Restriction mix total 25µl/sample comprised of:

Sterile, deionised water	11.5µl
Hha I RE 10x Buffer (Appendix I)	2.5µl
Acetylated BSA (10ug/µl)	0.25µl
Hha I enzyme 10units/µl	0.75µl
PCR product.	10.0µl

Restrictions digests were incubated overnight at 37°C and products separated on a 3% MetaPhor agarose gel (3.5.6). Digest product sizes were 162bp + 30bp for allele 1 (C) and 192bp for allele 2 (T) (figure 7.2.5).

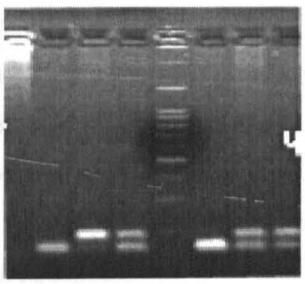

Figure 7.2.5: UCSNP63 PCR-RFLP genotyping gel. Upper band=homozygous allele1 (192/192bp), lower band=homozygous allele2 (162/162bp, 30bp bands not visible), and heterozygotes two bands.

7.2.6 Typing the UCSNP110 (T504A) polymorphism:

Primer sequences for this polymorphism were:

forward 5'-CGCCATCAGGGCAGTGGCCAAGAACAGC

reverse 5'-CAGAGTGATGCGGACGCAGCG.

PCR was carried out as for UCSNP19, PCR-RFLP HhaI digest as for UCSNP63. Products were separated on 3% Metaphor agarose (Flowgen), the common unrestricted allele is 196bp, variant is digested to 172bp + 24bp.

7.2.7 Additional Statistical Analysis

Statistical analysis for linkage, association, and quantitative traits was carried out as previously described in Chapter 3.8. In addition some analyses had to be modified or extended and these are described below.

Haplotype assignment in urban survey subjects:

Haplotype frequencies and phase in the South Indian families was deduced unambiguously from the genotype data utilising both parental and multiple sibling genotype data with the *TRANSMIT* program (Chapter 3). Haplotype frequency information from the families also permitted unambiguous deduction of haplotype frequencies and phase in the vast majority of South Indian survey subjects without family data. In a small number of the population based subjects more than one haplotype phase combination was possible. Within this group the percentage of individuals with particular haplotype combinations were estimated based on the haplotype frequencies derived from the families. The assignment of haplotypes to the survey subjects was carried out without the use of a population haplotype phase estimation computer programs because programs that were widely available were based on the EM algorithm such as the *EH* program [Xie & Ott 1993]. These programs have a bias towards estimating in favour of the common haplotypes with a tendency to ignore rare haplotypes (< 1% frequency). In addition these programs do not allow the inclusion of important *a priori* information, such as knowledge of haplotype frequencies from ethnic family data to improve efficiency in estimating haplotype frequencies.

Analysis for transmission of 'haplotype combinations: Available TDT methods were only capable of analysing the effect of transmission of a single allele or haplotype to affected offspring in families. To allow analysis of disease transmission in families as result of haplotype combinations a novel statistical approach was designed by Dr D.Curtis. Families were selected on the basis that the parents must carry at least one of the two haplotypes comprising the 'disease risk' haplotype combination and could potentially create the 'at risk' haplotype combination in offspring. In order to detect transmission distortion of a haplotype combination, the number of transmissions were counted for a haplotype combination to affected offspring as a proportion of the total number of parents who could potentially passed the combination to their offspring (i.e. those where one parent had at least one haplotype and the other parent the other haplotype).

7.3. RESULTS

7.3.1 Linkage Results

The study in this thesis found no evidence of linkage with any marker on chromosome 2q. An exclusion map (Figure 7.3.1) generated by plotting non- parametric linkage (NPL) scores

against map distance, shows that NPL scores were negative across the entire 20-25cM region (maximum NPL score of –0.79). If the T2DM siblings had shared more of their alleles than would be expected by Mendelian inheritance, the NPL score would have been positive with a threshold significance value of NPL= 2.0 or greater. There were some inconsistencies in the map order with relative distances of the six markers dependent on the source of map information (map order in Figure 7.2.1is based on the Whitehead Institute YAC map). Compared to the Genethon map, markers D2S140 and D2S125 were reversed and D2S140 placed approximately 16cM from D2S338. However this variability made no difference to the final results.

7.3.2 Allele/haplotype Frequencies in South Indians

Allele frequencies in the urban survey for the four bi-alleleic variants were (allele1/2), UCSNP44, 0.81/0.19, UCSNP43, 0.86/0.14, UCSNP19, 0.44/0.56, UCSNP63 0.97/0.03 and UCSNP110 0.81/0.19. Similarly in the parents from the families by gene counting the allele frequencies were UCSNP44 0.85/0.15; UCSNP43 0.89/0.11; UCSNP19 0.41/0.59; UCSNP63 0.97/0.03.

UCSNP110 was found to be in perfect LD with UCSNP44 and as it provides no additional information this locus was not analysed further.

Three loci UCSNP44 (p=0.96), UCSNP43 (p=0.84), and UCSNP63 (p=0.36) were in Hardy Weinberg equilibrium, whereas UCSNP19 was not (p=0.009). This was unlikely to be due to genotyping error as analysis of the 32bp Ins/Del UCSNP19 was easy to interpret (figure 7.2.4). The deviation from HWE was also not due to inbreeding, as this skew observed resulted from increased heterozygosity rather than homozygosity (UCSNP19 genotype frequency AA/Aa/aa, observed 77/260/132, and expected 91/231/146 values). In addition within the same population group there was no deviation from HWE either in the other three CAPN10 loci that are in very close proximity, or with any polymorphisms identified in both UCP2 and UCP3 in chapters 4 and 5. This tends to suggest that the deviation from HWE for UCSNP19 is most likely due to chance.

Haplotypes were constructed from the four loci in order UCSNP44, -43, -19, and –63. The assignment of haplotypes in those South Indian families with only one possible parental haplotype, found only 8 of the possible 16 haplotypes represented. Furthermore, only five of the 8 representative haplotypes had a frequency greater than 1% (table 7.3.1). The

identification of only five common haplotypes allowed the unambiguous assignment of haplotypes in a vast majority of survey subjects. In these subjects for all but four genotype configurations there was only one possible haplotype phase and in the four exceptions there was just two possible phases. Based on the family haplotype frequencies the proportion of individuals with one or other phase was estimated. In these groups, one possible phase always included one of the three rare haplotypes that in reality only applied to more or less a single individual. Compared with other ethnic groups (Table 7.3.1) the absence of 8 haplotypes was consistent with other populations of similar ethnic background, with haplotype frequencies in the South Indians not unlike those found in Northern Europeans.

CM	NPL	p	info
-5.0	-1.33	0.92526	0.65342
-4.0	-1.39	0.93389	0.66971
-3.0	-1.45	0.94177	0.68823
-2.0	-1.51	0.94943	0.70956
-1.0	-1.58	0.95629	0.73480
0.0	-1.64	0.96237	0.76818
0.2	-1.67	0.96478	0.76454
0.4	-1.70	0.96727	0.76277
0.6	-1.73	0.96932	0.76194
0.8	-1.76	0.97163	0.76195
1.0	-1.79	0.97345	0.76321
2.0	-1.72	0.96876	0.74572
3.0	-1.65	0.96296	0.73852
4.0	-1.58	0.95639	0.73898
5.0	-1.51	0.94902	0.74749
6.0	-1.44	0.94096	0.76911
7.6	-1.35	0.92752	0.73457
9.2	-1.25	0.91091	0.71682
10.8	-1.14	0.89125	0.70997
12.4	-1.03	0.86874	0.71359
14.0	-0.93	0.84160	0.73167
14.6	-0.92	0.84039	0.72505
15.2	-0.92	0.84007	0.72157
15.8	-0.91	0.83811	0.72014
16.4	-0.91	0.83687	0.72080
17.0	-0.90	0.83498	0.72495
17.6	-0.87	0.82606	0.70828
18.2	-0.83	0.81537	0.69510
18.8	-0.80	0.80569	0.68361
19.4	-0.76	0.79500	0.67339
20.0	-0.73	0.78425	0.66433
21.0	-0.70	0.77549	0.64861
22.0	-0.67	0.76528	0.63496
23.0	-0.64	0.75636	0.62283
24.0	-0.62	0.74737	0.61197
25.0	-0.59	0.73811	0.60221

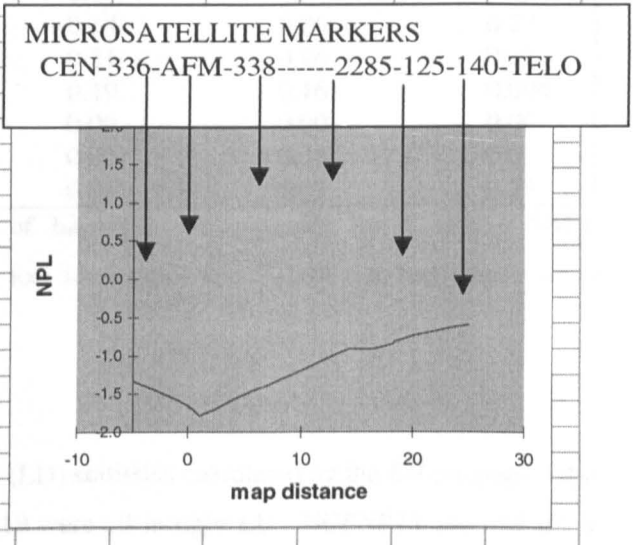

Figure 7.3.1: Exclusion map for telomeric region of Chr 2q. Marker order D2S336-AFM-D2S338-D2S2285-D2S125-D2S140

Two three locus haplotypes, 121 and 112 constituted the Mexican-American 'at risk' haplotype combination [Horikawa et al 2000]. Using haplotypes inclusive of UCSNP44 these haplotypes are more or less represented by the 1121 and 1112 haplotypes here (table 7.3.1). This conclusion was based on the lack of a 2112 split of 1112, and the 2121 haplotype that would split the 1121 only had a frequency of 0.3%-0.5%. The latter split affecting only two or three subjects in the total study.

Haplotypes	Estimated Haplotype Frequencies			
44/43/19/63	South India Family parents (n=172)	South India Survey (n=452)	† UK Europeans	‡Mexican Americans
1121	0.37	0.42	0.36	0.32
2121	0.00	0.005	0.00	NK
1221	0.18	0.14	0.26	0.27
1111	0.19	0.21	0.16	0.17
2111	0.19	0.19	0.16	0.006
2211	0.00	0.00	0.00	0.00
1122	0.007	0.00	0.00	0.01
1112	0.03	0.03	0.07	0.23

Table 7.3.1: Population comparison of haplotype frequencies for CAPN10 SNPs: Mexican American data based on three loci haplotypes and T2DM risk haplotypes are in bold.

7.3.3 Linkage disequilibrium of SNP's

Pairwise linkage disequilibrium statistics (LD) statistics calculated by the *EH* program (table 7.3.2) found that UCSNP's43, -44 and –19 were all in tight LD. UCSNP43 and –44 are in weak LD with UCSNP63, whereas UCSNP19 is in tight LD with UCSNP63; this is consistent with their respective map positions.

SNP's	UCSNP44	UCSNP43	UCSNP19	UCSNP63
UCSNP44	-	p<0.0001	p<0.0001	p=0.034
UCSNP43	p<0.0001	-	p<0.0001	p=0.015
UCSNP19	p<0.0001	p<0.0001	-	p<0.0001
UCSNP63	p=0.034	p=0.015	p<0.0001	-

Table 7.3.2: Linkage Disequilibrium of four calpain 10 loci in South Indians.

7.3.4 Association/linkage in families

Family based association studies by ETDT in South Indians did not reveal any excess transmission from heterozygous parents for any of the four individual SNP's to probands with T2DM (Table 7.3.3).

TDT-Type II	Transmitted	Non Transmitted	p value
UCSNP44	21	31	0.17
UCSNP43	25	26	0.89
UCSNP19	31	42	0.2
UCSNP63	11	8	0.49

Table 7.3.3: TDT Analysis in South Indian Families

Haplotype analysis (tables 7.3.4 and 7.3.5) in the families using the *TRANSMIT* program also found no evidence of excess transmission of any haplotype to T2DM or IGT/IFG offspring (global p values 1.0 and 0.3 respectively, generated by a 1000 bootstrap iterations). Individually (1df) the MA 'at risk' combination haplotypes, the 1121 (p=0.18), and 1112 (p= 0.56) were also negative.

Four locus Haplotypes 44/43/19/63	Observed Transmissions to T2DM offspring	Expected Transmissions to T2DM offspring	South Indian Families (n=95) p values ; 1df
1121	91	100	0.18
2121	1.0	1.5	0.26
1221	40	39	0.81
1111	45	41	0.32
2111	53	49	0.34
2211	< 1	<1	0.29
1122	<1	1.3	0.28
1222	<1	<1	1.0
1112	12	10	0.56
GLOBAL p value based on a 1000 bootstrap sample=1.0			

Table 7.3.4: Transmission of CAPN10 haplotypes to T2DM offspring

Four locus Haplotypes 44/43/19/63	Observed Transmissions to IGT/IFG offspring	Expected Transmissions to IGT/IFG offspring	South Indian Families (n=28) p values ; 1df
1121	27	25	0.57
2121	<1	<1	0.28
1221	13	11	0.23
1111	5	8	0.24
2111	10	10	0.73
2211	< 1	<1	0.26
1122	<1	<1	1.0
1222	<1	<1	1.0
1112	<1	1	0.26
GLOBAL p value based on a 1000 bootstrap sample=0.3			

Table 7.3.5: Transmission of CAPN10 haplotypes to IGT/IFG offspring

7.3.5 Population Association studies

Analysis of the five common haplotypes in survey subjects and family probands, found the frequency of the 1112 haplotype significantly increased in the survey IGT/IFG subjects (Global chi square p=0.001, 4df, 99% CI 0.0001-0.001) and the probands (Global chi square p= 0.004; 4df; 99% CI 0.002-0.005) compared to controls. There was also a slight non-significant increase in frequency of the 1112 in survey diabetics (Table 7.3.6). No other observed haplotype frequencies were significantly different from survey normoglycaemic subjects.

HAPLO >1% FREQ	URBAN-NGT	URBAN-IGT/IFG	URBAN-T2DM	T2DM PROBANDS
1121	42.8% (267)	42% (48)	41.6% (70)	35% (66)
2111	19% (120)	13% (15)	18.4% (31)	22.9% (43)
1221	13% (82)	15.8% (18)	12.5% (21)	17.5% (33)
1111	23% (145)	19.3% (22)	24.4% (41)	18% (34)
1112	1.6% (10)	9.6% (11)	3.0% (5)	6.3% (12)

Table 7.3.6: Haplotype frequencies for Case control studies. NGT=normal glucose tolerance.

To tease out whether the association observed with the 1112 haplotype was due to an individual SNP, these were analysed individually. In the contingency table analysis if

homozygosity for any locus allele was rare (< 5/cell), then subjects were analysed for the prescence of the uncommon allele, ie heterozygous and uncommon homozygous genotypes were combined. Analysis of the individual polymorphisms revealed no difference in frequencies between the genotype frequencies for normoglycaemic controls, IGT/IFG and T2DM for the UCSNP44, -43 and –19 variants (table 7.3.7).

	UCSNP44		UCSNP43		UCSNP19			n
	1/1	1/22/2	1/1	1/22/2	1/1	1/2	2/2	
NGT	64.4%	35.6%	75.5%	24.5%	17.2%	53.2%	29.6%	323
IGT/IFG	72.9%	27.1%	71.2%	28.8%	13.6%	59.1%	27.2%	60
T2DM	65.9%	34.1%	75.3%	24.7%	14.8%	61.7%	23.5%	85
P values	0.45		0.77		0.33			

Table7.3.7: Survey case-control genotype frequencies for UCSNP44, -43, -19

However the presence of the uncommon allele 2 (T) of UCSNP63 was significantly increased in both survey IGT/IFG subjects, with a frequency of 16.7% (p=0.001) and in T2DM probands 11.7% (p=0.005) compared to 4% in controls (Table 7.3.8). There was also again only a slight non-significant increase in frequency in survey T2DM subjects (5.9%). T2DM parents from the families also had a significant increase in the frequency of the UCSNP63 allele 2 (11.9%) compared to controls (p=0.002), although they cannot be considered strictly a separate group from the probands. This positive association with UCSNP63 allele 2 was not entirely surprising as this allele is almost invariably (99.5%) carried on the 1112 haplotype.

UCSNP63 Genotype	URBAN-NGT	URBAN-IGT/IFG	URBAN-T2DM	T2DM PROBAND
1/1	96% (310)	83.3% (50)	94.1% (80)	88.3% (83)
1/2 2/2	4% (13)	16.7% (10)	5.9% (5)	11.7% (11)
	P values	0.001	0.55	0.005

Table 7.3.8: UCSNP63 case-control genotype frequencies

7.3.6 Analysis of Haplotype Combinations

Global chi-square analysis of 14 haplotype combinations identified in South Indians, found the Mexican-American 'at risk' haplotype combination 1112/1121 to have a significantly increased frequency in the survey IGT/IFG subjects (9.0%) compared to normoglycaemic controls (0.9%), with a global p value [99% confidence limits] p=0.02 [0.019-0.023] (Table 7.3.9). Furthermore, there was also a significant increase in the frequency of the same haplotype combination in the T2DM probands (5%) global p= 0.015 [0.012-0.018] and a similar trend in survey T2DM subjects (6%) global p value= 0.27 [0.26-0.28]. Further analysis found the 1112/1121 combination associated with an increased risk in survey IFG/IGT subjects (Odds Ratio (OR) 10.74; Yates corrected chi-square (1df), p=0.001 and Relative Risk (RR)=4.4 [1.75-6.6]), T2DM subjects (OR 6.52; p=0.015; RR=3.07 [1.23-4.5]) and probands (OR 5.78; p=0.025; RR=2.79 [1.13-4.11]) (table 7.3.9). A second combination 1112/1221 in the survey was completely absent in both the normoglycaemic and T2DM subjects, but was present in both the IGT/IFG subjects (global p=0.018) and the probands (global p=0.003). This suggested that a heterozygous haplotype combination of the 1112 haplotype with either 1121 or 1221 might influence susceptibility to glucose tolerance. In diabetic parents (n=132) there was also a concomitant increase in risk attributed to both the 1112/1121 and 1112/1221 haplotype combinations (OR 4.05 [95% CI 0.83-21.7] and OR 6.58 [95%CI Infinite] respectively, although these subjects cannot strictly be treated as a separate dataset from the probands. In addition, in the parents the 1121 haplotype in combination with 1221 also contributed to significantly increased risk (OR 1.88, [95%CI 1.02-3.45] p=0.04), but in combination with 2111 appeared to decrease risk (OR 0.49, [95%CI 0.25-0.94] p=0.03).

TDT analysis of haplotypes (*TRANSMIT*) revealed no excess transmission of any haplotype (Table 7.3.4 and 7.3.5). However, this analysis could not address the effect of an increased transmission of the 1112/1121haplotype combination to affected offspring. Therefore a novel statistical approach was applied (7.2.7). Four sets of parents were identified and three T2DM probands did receive the 'at risk' haplotype combination and one did not compared with the one quarter expected proportion with no excess transmission (p=0.05). Although the number of families were small, the observed frequency of the simultaneous transmission of both haplotypes to the T2DM probands was greater than the expected one-quarter probability for transmissions under the null hypothesis that affection is not associated with

the haplotype combination. In addition, there was transmission of the 'at risk' haplotype combination from a fifth set of parents where one parent was homozygous for 1121 and the other parent had the 1112 haplotype.

To estimate what proportion of diabetics in the entire South Indian population that could be attributed to exposure to the 'at risk' haplotype combination, the population attributable risk (PAR) was calculated. The PAR's for survey T2DM subjects and T2DM probands were 6.3% and 7% respectively.

7.3.7 Quantitative Trait analysis

Analysis of the five common haplotypes in the families using the QTDT Orthogonal model [Abecasis et al 2000] and sex as a covariant, found that the transmission of the 1121 haplotype to offspring was associated with a decrease in BMI (p=0.019), a narrower hip size (p=0.007), and weakly with a decreased FBG (p=0.03) (Table 7.3.10). In contrast transmission of the 1221 was associated with larger hips p=0.008. Following correction for the number of haplotypes analysed, only the hip remained significantly associated. Tests for parent of origin effect, found the 1121 and 1221 haplotype associations with hip, were both paternal in origin (p=0.02, p=0.016, respectively). Transmission of the 1112 haplotype to offspring was associated with a decrease in WHR (p=0.03), further confirmed by the "total association" analysis option within the QTDT program (p= 0.003) incorporating adjustment for non-shared environment and polygenic disease (table 7.3.11). Analysis of the individual CAPN10 polymorphisms by QTDT, also with sex as a covariant, found the transmission of the allele 1 of UCSNP43 to T2DM offspring was associated with a decrease in BMI (p=0.04), and hip (p=0.0056). Analysis of the individual variants using the "Total Association" option, found that UCSNP63 was associated with variance in both waist circumference (p=0.02) and WHR (p=0.004) (table7.3.12). Following correction for the four loci analysed, only associations with hip and WHR remained significant confirming the haplotype data.

Analysis of the same intermediate quantitative traits in the urban survey samples found no relationships with either individual loci or the four loci haplotypes, including weight, height, BMI, waist circumference, hip, WHR, fasting blood glucose, and 2-hour glucose. Haplotype combinations were similarly assessed and associations were also all negative

HAPLO COMBS	NGT n=297	Survey IGT/IFG n=55		Survey T2DM n=84		T2DM PROBANDS n=92	
	FREQ (n)	FREQ (n)	OR (95%CI)	FREQ (n)	OR (95%CI)	FREQ (n)	OR (95%CI)
1111/1111	0.054 (16)	0.018 (1)	0.28 (0.01-2.0)	0.036 (3)	0.57 (0.13-2.1)	0.05 (5)	0.86 (0.27-2.55)
1111/1112	0.017 (5)	0.018 (1)	1.11 (0.05-10.1)	0.00 (0)	0.0 (0.84-6.6)	0.01 (1)	1.33 (0.17-7.9)
1111/1121	0.19 (56)	0.20 (11)	1.14 (0.53-2.4)	0.23 (19)	1.23 (0.65-2.28)	0.098 (9)	0.44 (0.2-0.98)
1111/1221	0.067 (20)	0.09 (5)	1.29 (0.4- 3.82)	0.11 (9)	1.58 (0.64-3.79)	0.05 (5)	0.74 (0.23-2.14)
1111/2111	0.067 (20)	0.036 (2)	0.54 (0.08-2.5)	0.08 (7)	1.32 (0.49-3.47)	0.09 (8)	1.36 (0.52-3.39)
1112/1121	0.009 (3)	0.09 (5)	10.7 (2.0-55.2[1])	0.06 (5)	6.52 (1.3-35.3[2])	0.05 (5)	5.78 (1.18-31.2[3])
1112/1221	0.00 (0)	0.036 (2)	inf (1.385-inf[4])	0.0 (0)	0.00 (0.00)	0.04 (4)	inf (2.22-inf[5])
1112/2111	0.007 (2)	0.018 (1)	2.8 (0.1-40.5)	0.0 (0)	0.00 (0.78-15.2)	0.01 (1)	1.66 (0.06-23.6)
1121/1121	0.18 (54)	0.125 (7)	0.66 (0.26-1.63)	0.17 (14)	0.93 (0.46-1.85)	0.12 (11)	0.62 (0.29-1.29)
1121/1221	0.11 (32)	0.14 (8)	1.4 (0.56-3.42)	0.05 (4)	0.48 (0.12-1.3)	0.13 (12)	1.2 (0.57-2.62)
1121/2111	0.20 (58)	0.16 (9)	0.8 (0.34-1.8)	0.17 (14)	0.82 (0.42-1.62)	0.18 (18)	0.97 (0.52-1.8)
1221/1221	0.017 (5)	0.018 (1)	0.93 (0.04-7.99)	0.012 (1)	0.61 (0.03-5.2)	0.03 (3)	1.68 (0.32-7.74)
1221/2111	0.05 (15)	0.018 (1)	0.36 (0.02-2.68)	0.07 (6)	1.52 (0.5-4.36)	0.06 (6)	1.35 (0.45-3.85)
2111/2111	0.037 (11)	0.018 (1)	0.49 (0.02-3.84)	0.02 (2)	0.66 (0.1-3.28)	0.05 (5)	1.54 (0.45-4.95)

Table 7.3.9: Frequencies and Odds Ratios for CAPN10 haplotype combinations in South Indian subjects

	HAPLOTYPES >1% POPULATION FREQUENCY				
TRAIT	**1121**	2111	1221	1111	**1112**
BMI	**0.019**	NS	NS	NS	NS
Waist	NS	NS	NS	NS	NS
Hip	**0.007**	NS	**0.008**	NS	NS
WHR	NS	NS	NS	NS	**0.03**
age diag	NS	NS	NS	NS	NT
Height	NS	NS	NS	NS	NS
FBG	**0.03**	NS	NS	NS	NS

Table 7.3.10: Quantitative Trait haplotype Analysis in South Indian families. NS = Not statistically significant; NT = Not tested due to low haplotype frequency/numbers

	CAPN 10 Four Loci haplotypes				
Traits	**1121**	2111	1221	1111	**1112**
BMI	0.079	NS	NS	NS	NS
Waist	NS	NS	NS	NS	NS
Hip	NS	NS	NS	NS	NS
WHR	NS	NS	NS	NS	**0.003**
age diag	NS	NS	NS	NS	NS

Table 7.3.11: Total Association analysis of five common CAPN 10 haplotypes

	CAPN 10 loci			
Traits	UCSNP44	UCSNP43	UCSNP19	UCSNP63
BMI	NS	NS	NS	NS
Waist	NS	NS	NS	**0.041**
Hip	NS	NS	NS	NS
WHR	NS	NS	NS	**0.002**
Age Diag	NS	NS	NS	NS

Table 7.3.12: Total Association analysis of four CAPN 10 loci individually

7.3.8 TDT POWER CALCULATIONS

Analysis of the power to detect an association in the TDT studies was examined for UCSNP63. Based on the genotype frequencies and relative risk conferred by UCSNP63 in the South Indian survey there would have been only 39% power to detect an effect significant at p=0.05 with 97 families used.

7.4 DISCUSSION

7.4.1 Linkage data

The linkage study in this thesis failed to find excess allele sharing in 278 T2DM affected sib pairs, although this sample number almost certainly lacked sufficient power to exclude the NIDDM1 region on Chromosome 2q. However, an extension of my study using an additional 556 affected sibling pairs from the Warren 2 repository (a total of 834 ASP) also produced a negative non-parametric linkage score (NPL) of -0.31 *(*Zlr score-*GENEHUNTER-PLUS)* confirming my result [Evans et al 2001]. Concurrent studies with the original MA genome scan also found no evidence of linkage with D2S125 in both non-Hispanic whites (147 ASP's) and Japanese (140 ASP's) with MLS scores of 0.03 and 0.00 respectively [Hanis et al 1996]. Three other linkage studies in MA populations, including one using samples from the same geographical region as the Hanis [1996] study, also failed to detect linkage to Chromosome 2q [Stern et al 1996a & b; Duggirala et al 1999]. However, a fourth genome scan for T2DM in MA families did find nominal evidence of linkage with D2S125 (LOD score 0.88) and a combined T2DM and Impaired Glucose homeostasis phenotype (IH; consisting of both IGT and IFG) in the primary phase of analysis with a marker density of 10cM intervals [Ehm et al 2000]. This finding was not entirely surprising as a high proportion of the nuclear families used in this study were the same as for Hanis study. Furthermore, analysis of additional markers at a density of 5cM intervals, although increasing the LOD score to 1.66 shifted the peak 27cM centromeric from position 259cM (D2S125-260.6cM) to position 232cM, near marker D2S362 [Ehm et al 2000]. The majority of linkage studies in other ethnic groups have not replicated the Chromosome 2q linkage result of Hanis et al, with negative studies in Botnian Finnish [Mahtani et al 1996], American-whites, -blacks, -Japanese [Ehm et al 2000], European Caucasians from Utah [Elbein et al 1999], Sardinians [Ciccarese et al 1997], Finnish [Ghosh et al 2000; Wanatabe et al 2000], and British Caucasians [Thomas et al 1997; Evans et al

2001]. In a relatively early study in Finnish families although the distal telomeric region of Chromosome 2q was excluded, positive single-locus MLS values with the proximal markers D2S126 (MLS 1.05; positioned at 214.4cM) and D2S338 (MLS 0.92; positioned at 240.8cM) were found [Ghosh et al 1998]. Interestingly, D2S126 had the second strongest evidence for linkage after D2S125 in the MA genome scan and nominal evidence of linkage was observed for D2S338 (MLS of 1.15 ($p<0.025$) in non-Hispanic white subjects [Hanis et al 1996]. Furthermore suggestive linkage was found in close proximity to D2S126 in another MA study [Ehm et al 2000]. Although these markers have been positioned 10.2cM-16cM proximal to D2S125 [Ghosh et al 1998; Hanis et al 1996] it was suggested that a weak susceptibility locus might be present in this region in non-Hispanic Caucasians [Ghosh et al 1998]. In respect of the markers more proximal to D2S125 studied in this thesis, NPL's for D2S338 and the most centromeric marker used D2S336, which is between 6cM-24cM distal to D2S126 (depending on the map used) were less than a NPL score of –1.5. Linkage studies in T2DM French Caucasian families failed to find linkage to D2S125, but did found some evidence of linkage with a marker D2S140, 3cM distal to D2S125 [Hani et al 1997]. Although the markers used in this thesis generated progressively less negative NPL's approaching the telomeric region of Chromosome 2q, the only marker D2S140, used thought to be distal to D2S125 had a negative NPL score of –0.7.

The lack of replication of contemporary genome scans, assuming that the positive evidence of linkage for Chromosome 2q is not merely due to the 5% false positive rate considered to be present in genome scans [Landers & Kruglyak 1995] could be due to several reasons. Firstly, there might be ethnic differences at this locus with respect to predisposition to T2DM as well substantial genetic heterogeneity underlying the phenotypic expression of T2DM in diverse ethnic populations. Secondly, there also may be differences in family ascertainment and diagnosis of T2DM between the studies in addition to stochastic factors [Suarez & Hampe 1994]. Finally, there are power considerations whereby the number of sibling pairs would only have the power to detect a locus that contributes a specific variability to T2DM, and therefore could not exclude genes within the NIDDM1 region from having a minor role in the disease process. Simulation studies on interval mapping suggest that the probability of falsely excluding region is very small for a range of λ_s values [Hauser et al 1996]. However, the majority of the linkage studies mentioned above including the present study in this thesis, most probably had insufficient power to exclude a gene with

a relative sibling risk of λ_s 1.37 that was found in Mexican Americans. Nevertheless, three recent studies have provided evidence for exclusion of the region in UK and Finnish Caucasians at sibling relative risk ratio levels of λ_s 1.25 and λ_s 1.37 respectively [Evans et al 2001; Ghosh et al 2000; Watanabe et al 2000].

Indeed, the subsequent degree of association seen with CAPN10 UCSNP43 variant with T2DM in MA [Horikawa et al 2000], would render all studies including that of Hanis et al inadequate in terms of power to detect the increased risk afforded to the increased frequency of G allele of UCSNP43 (MA controls 0.75 vs T2DM MA 0.80). Altshuler et al [2000] suggested that a population group of 100,000 affected sib pairs would be necessary to have been able to attribute this association with the 4.03 lodscore obtained originally by Hanis et al [1996]. Never the less subsequently a putative T2DM gene (CAPN10) was positionally cloned to the NIDDM1 region [Horikawa et al 2000]. Furthermore, in many of the ethnic populations that failed to find evidence of linkage to the NIDDM1 region, the utilization of linkage disequilibrium based studies have since demonstrated positive associations with T2DM and variants of the CAPN10 gene. Examples include studies in Botnian Finnish, Saxon Germans, Utah Caucasians and UK Caucasians [Horikawa et al 2000; Schwarz et al 2001; Ren et al 2000; Evans et al 2001]. Therefore supporting the view that most of the different linkage studies discussed above most probably lacked the statistical power to detect minor diabetogenic loci. However, in the few studies in which there was sufficient power for exclusion of linkage at levels of λ_s of 1.25, it is consistent with CAPN10 being a minor susceptibility gene in those populations.

7.4.2 CAPN10 variant haplotype frequencies

In South Indian families only 8 of the possible 16 four loci haplotypes were present, with only five haplotypes with a frequency of greater than 1%. In reference to the available population data, globally there only appears to be four common three locus haplotypes (UCSNP43, -19, -63) [Horikawa et al 2000; Schwarz et al 2001; Evans et al 2001]. Inclusion of a fourth SNP, UCSNP44, causes a near equal split of haplotype 111, and therefore five common haplotypes are observed in both South Indians and UK Caucasians [Evans et al 2001]. These findings suggest that there is still very strong disequilibrium across the CAPN10 gene in most ethnic populations. This was also confirmed in this thesis for the South Indians, implying overall that these mutations are relatively recent in human

evolution. However, there are differences in the population frequencies of certain haplotypes, which is often reflected in the ethnic ancestry of the individual populations. The South Indians, ethnically Caucasian, unsurprisingly have frequencies not unlike those present in European Caucasians [Horikawa et al 2000; Evans et al 2001]. One of the haplotypes the constitutes the MA 'at risk' haplotype combination 121, represented by the 1121 haplotype in the South Indians, is the most common haplotype in virtually all populations so far investigated with frequencies of 0.32 in MA, 0.42 in Germans, 0.56 in Chinese/Japanese Asians, 0.42 in USA Europeans, 0.36 in the Pima Indians [Horikawa et al 2000], 0.36 in UK Europeans [Evans et al 2001], and 0.37-0.41 in South Indians in my study. However in Botnian Finnish and the Zapoteca Indians the 111 (0.35), and 221 (0.52) respectively are the most frequent, but the 121 haplotype is still common at 0.29 and 0.17 respectively. In contrast, the second haplotype of the MA 'at risk" haplotype combination, the 112, is relatively common (0.23) in the MA population, and in Pima Indians, Zapoteca Indians, Chinese and Japanese with frequencies of 0.29, 0.24, 0.31 and 0.25 respectively. However, the 112 haplotype, represented solely by 1112 in the South Indians is at a very low frequency (0.05) similar to that found in Europeans (0.03-0.07) [Horikawa et al 2000; Evans et al 2001].

7.4.3 Case-Control Studies

The most important finding from the studies in this chapter, is that in the South Indian epidemiological based urban survey, the presence of the 1112/1121 heterozygous haplotype combination confers both an 10.7 fold increase in risk to abnormal glucose tolerance and a 6.3 fold and 5.8 fold increased risk to T2DM in unrelated subjects and probands respectively. Furthermore, the risk for T2DM was also increased in the T2DM parents from the families (4 fold) although failing to reach conventional levels of statistical significance. In the seminal publication analysing CAPN10 [Horikawa et al 2000] the modest association of UCSNP43 in MA could not alone account for the original linkage data [Hanis et al 1996], so haplotypes were tested with combinations of other CAPN10 SNP's identified. In MA the two most important haplotypes identified were, 121 and 112 which both have the disease associated G allele of UCSNP43 but also included two other variants, UCSNP19 and UCSNP63. UCSNP19 is in near perfect LD with a cluster of four intronic SNP's UCSNP56, -59, -30, and –65 and one coding mutation in exon 11, UCSNP48 a silent mutation at codon

A620. UCSNP63 was also found individually associated with an increased risk to T2DM in Botnian Finnish subjects. In MA the heterozygous combination of these two haplotypes conferred a three fold increased (Odds Ratio's 2.8 and 3.58, from two datasets) risk to T2DM. In the Horikawa et al [2000] study it was suggested that there was also a concomitant 2.5 fold and 5.0 fold increase in risk to T2DM with the 112/121 haplotype combination in both Finnish and Germans respectively, although the odds ratio's achieved were not statistically significant. Nevertheless in the same Saxon German ethnic population but combined with a Czech study group found a 3.48 fold increased risk to T2DM, hence confirming these findings [Schwarz et al 2001]. The 1112/1121 combination in this South Indian study is equivalent to this Mexican American 'at risk" 112/121 combination, as explained in the results section. Therefore these haplotype associations therefore both replicate and extend those previously published in the seminal publication of Horikawa et al [2000]. In the South Indian population a second haplotype combination, 1112/1221 also appeared to be associated with increased risk to both AGT and T2DM, although the numbers involved are very small and the haplotype combination is completely absent in the normoglycaemic subjects (n=312). The 1221 haplotype in combination with the 1121 also afforded a 2.0 fold increase in risk to T2DM in the parents of the T2DM probands. Homozygosity for these haoplotypes (1121, 1112, 1221) were not associated with an increased risk for disease, although only 2 subjects in the study were homozygous for the 1112 haplotype. These findings are in agreement with the MA data, emphasising the importance of heterozygous combinations of CAPN10 gene haplotypes in determining susceptibility to disease [Horikawa et al 2000]. However, contrary to the findings of Horikawa, I found no underlying association for T2DM with the G allele of UCSNP 43, or individually with UCSNP44 and UCSNP19.

In my study in a South Indian population the presence of the uncommon allele 2 (T) of UCSNP63 was significantly increased in both IGT/IFG subjects (p=0.001) and T2DM probands (p=0.005). This allele was also concomitantly increased in T2DM parents from the families (p=0.002). There are only two haplotypes detected in South Indians that carry the allele 2 (T) of UCSNP63; these are 1112 and 1122. The 1122 haplotype has only a frequency of 0.005 in the South Indians, with a similar low frequency in Mexican Americans (0.01) and UK Europeans (undetected) [Horikawa et al 2000; Evans et al 2001]. Therefore not surprisingly the 1112 haplotype, was also significantly increased in both

IGT/IFG subjects (p=0.0001, OR 6.5) and T2DM probands (p=0.001, OR 4.1) (Table 7.3.5). Allele 2 of UCSNP63 is in linkage disequilibrium with allele 1 of the other three SNP's USNP44, -43 and -19. The G allele of UCSNP43 was the strongest underlying variant within the 'at risk' haplotype combination that associated with an increased risk to T2DM in MA's, Germans and Botnian Finns [Horikawa et al 2000]. However in the South Indians, the LD relationship between UCSNP63 and UCSNP44/ -43 is much weaker, suggesting that the uncommon T allele (2) of UCSNP63 may be marking a separate disease susceptibility determinant to UCSNP44 and UCSNP43, and additionally contributes to disease susceptibility associated with the 'at risk' haplotype combination. Allele 2 of UCSNP63 was also strongly associated with increased risk to T2DM in Botnian Finnish subjects, but not in Germans or MA's, suggesting that there is some divergence in the importance of individual variants between ethnic populations possibly as a result of variation in LD marker relationships with causative mutations [Horikawa et al 2000]. This view is further supported in that, whereas in the MA population study the affected sib pairs who were conconcordant for the UCSNP43 G/G genotype accounted for all the 'evidence for linkage' to NIDDM1 region [Horikawa et al 2000]. In contrast, investigations of UCSNP43 and 'evidence for linkage' to the NIDDM1 region in UK Europeans using the Warren 2-NIDDM1 linkage data generated in the linkage studies in this Chapter, found no increase in allele sharing at NIDDM1 (p=0.92) in a subset of 79 families (maximum 122 sib pairs) in which the probands were UCSNP43 homozygous for the G allele [Evans et al 2001]. Furthermore, in the UK Europeans there was also no evidence of epistatic interactions, such as those found with Chromosome 15 in the MA studies [Cox et al 1999]. Case/control studies in the UK population using the affected sibpair probands (n=269) also found no difference in the UCSNP43 G/G allele frequency between T2DM subjects and controls [Evans et al 2001].

An extension of these studies to the Warren 2 UK European family trios also confirmed the lack of association with the UCSNP43 (or UCSNP63) variant, and also found no associations with the MA 'at risk' haplotypes. This study was estimated to have 90% power to detect the odds ratio of 2.86 seen with the 112/121 combination in MA at a one tailed p value of 0.05 [Evans et al 2001]. However, in the family trios a significant increased transmission of UCSNP44 allele 2 to T2DM offspring was observed. The haplotype combination 2111/2111(p=0.038) in contrast to both the MA and South Indian data was

found to have the most important effect, although its effect was entirely due to the UCSNP44 locus. The result was further replicated in the European families with the UCSNP110 variant (p=0.033), which is in 100% LD with UCSNP44, but not replicated in the case-control group [Evans et al 2001]. UCSNP44 and UCSNP110 were also found to be in perfect LD in South Indians in this thesis, although no association with disease was observed with UCSNP44/110 variants either in families or in the urban survey samples. It was suggested by the authors that one possible explanation for the lack of association with the 'at risk' haplotype combination in UK Europeans might be due to the low frequency of the 1112 in this population (0.07) compared to 0.23 in MA [Evans et al 2001]. However, in my studies a significant association was detected in the South Indians despite only a frequency of 0.05 for the 1112 haplotype. Similarly in the German/Czech study positive associations with T2DM were also achieved with population frequencies ranging from 0.02 to 0.08 for 1112 [Schwarz et al 2001].

Stimulation studies comparing the relative power of haplotype and SNP analysis [Bader 2001], suggest that under certain circumstances haplotype analysis is more powerful than analysis of individual SNPs, this being dependent on the number of 'causative' SNP's within the haplotypes and number of common haplotypes present (> 5% in frequency). Bader [2001] illustrated this by using data from one particular study [Liggett et al 2000], and demonstrated the effectiveness of haplotype-based analysis by finding positive association with phenotype amongst the four common haplotypes, in spite of there being no individual association with any of the 13 SNP loci that were constituent of these haplotypes. Under these circumstances the effect of individual loci on phenotype was undetectable unless the 'additive' contribution of individual variant alleles were analysed as a whole ie. haplotype effect. In this thesis study although there was an association with phenotype with UCSNP63 individually. However, interpretation of the results in the manner suggested by Bader [2001], haplotype-based analysis should have provided increased power to detect an association, and this appears to be the case with the 1112 haplotype.

From these observations it might be postulated that the overall susceptibility to T2DM and related phenotypes results from the compounded 'additive' effect of a number of 'independent' causative mutations present either in the CAPN10 gene or in another gene in close proximity, in which the relative importance of individual variants has been ethnically determined. Furthermore, there also appears to be synergistic effect of different haplotypes

on each other in detemining the overall risk to disease, and this is discussed later in section 7.4.6.

The (1)112/(1)121 haplotype combination in MA type2 diabetics was estimated to account for 14% of the population attributable risk to T2DM, but this was only 4% in two European population groups, namely Botnian Finns and Saxon Germans [Horikawa et al 2000]. Further studies in Saxon Germans and Czechs suggested the PAR might be as high as 13% using combined data [Schwarz et al 2001]. However in South Indians with AGT/T2DM, the population attributable risk appears to be intermediate for this haplotype combination calculated at between 6% and 7%.

7.4.4 Family association studies

Family association studies in the South Indians found no excess transmission of any SNP allele or haplotype to T2DM offspring using the *ETDT* and *TRANSMIT* programs respectively [Sham & Curtis 1995; Clayton et al 1999]. These results do not confirm the association study findings for UCSNP63 and haplotype 1112 in South Indians. At present there have only been two contemporary family-type association studies for CAPN10 variants performed and results have been inconsistent. In one study using UK European Caucasian parent-T2DM offspring trios, only the uncommon C allele of UCSNP44 was excessively transmitted to T2DM offspring [Evans et al 2001]. In the second family study, also in subjects of Northern European extraction (Mormons, Utah, USA), there was an excess transmission of the less common A allele of UCSNP43 from diabetic parents to diabetic offspring, but this was only significant when all parents were used and when only transmitted to male diabetic offspring (p=0.003) and not to female diabetics offspring [Quianfang et al 2000]. This latter result is in complete contradiction of the MA data for UCSNP43 [Horikawa et al 2000].

The failure to detect an association with T2DM using TDT based methods for either the 1112 or 1121 haplotypes individually or SNP63 allele 2 could be for several reasons. Firstly, a calculation of power for the TDT study based on the UCSNP63 allele frequencies from the South Indian population study group, found that there would be insufficient power (39%) to detect an association at this locus. The 1112 haplotype was found at a substantially lower frequency in the South Indians, as in European populations [Evans et al 2001] compared to the Mexican Americans [Horikawa et al 2000] and this could have also further impeded the

TDT analysis. Secondly, family association methods could also have a reduced power to detect a disease association, under other specific circumstances. An 'at risk' haplotype combination (1)112/ (1)121 is attributed to the greatest risk to T2DM in both MA and South Indians with homozygosity for the individual haplotypes not associated with an increased risk to T2DM in either ethnic group [Horikawa et al 2000]. Furthermore, in MA other haplotype combinations with either the 'at risk' 112 or 121 haplotypes included were found either to be neutral or protective to disease susceptibility. Consequently, unless the independent transmission of the two individual haplotypes can be assessed collectively, then observations of association in the offspring are less likely unless an individual haplotype association is strong enough. Interestingly, using a novel statistical approach to examine families where simultaneous transmission of both 'at risk' haplotypes was possible, there was a significant increased transmission to offspring with T2DM. Although the number used in the analysis was small it complements and confirms the positive case-control haplotype combination data.

7.4.5 Quantitative Trait analysis:

T2DM is likely to be polygenic disease in which susceptibility determinants are determined by associations between intermediate traits and diabetes susceptibility genes. Having found associations between CAPN10 and diabetes it was therefore important to determine whether any of the associations can be accounted for association with related intermediate traits. Quantitative trait analysis of haplotypes in the South Indian families applying the Orthogonal model [Abecasis et al 2000] within the *QTDT* program revealed significant associations with only three haplotypes. Coincidentally these three haplotypes were the same as those implicated in increased risks to both AGT and T2DM in both case-control and family studies in South Indians. The transmission of the 1121 haplotype was associated with a decrease in BMI, and narrower hip size. Independently the 1221 also associated with larger hip measurements. Analysis of the individual variants with traits provided evidence that UCSNP43 may contribute in part to this association, as UCSNP43 allele 1 was individually also associated with decreased BMI and hip measurement. The transmission of the 1112 to offspring was also associated with a reduction in the WHR, which in part was due to the presence of less common allele 2 of UCSNP63 that individually significantly associated with variance in WHR and waist circumference. From these findings one might

predict that South Indian subjects with the 1112/1121 haplotype would have a low BMI and WHR, with a smaller hip measurement. However, contrary to the findings in South Indians, lower sleeping metabolic rate was associated with the UCSNP43 G/G genotype in Pima Indians [Baier et al 2000] and therefore this allele might be expected to be associated with an increased body mass. This association was also supported by a Chinese study in which control subjects homozygous for allele 1 (G/G) of UCSNP43 had increased BMI and WHR [Ji et al 2001]. Although increased BMI, waist circumference and WHR are widely recognised as predisposing factors towards the development of T2DM, this is not the case for hip measurements. Nevertheless, it has been suggested that narrow hips reflect a decrease in leg muscle mass and this has been found to be a predisposing factor for T2DM independent of waist circumference and irrespective of stature, particularly in males [Seidell et al 1997]. It was suggested that the more apparent effect in males was possibly due to a reduced gluteal subcutaneous fat mass compared to females. However the findings in female subjects also suggested that the associated increase in risk attributable to WHR was not only a result of larger waists but independently also smaller hips than would be expected. Furthermore, in another study comparing body tomographic cardiovascular risk factors between Swedish and Asian Indian subjects the higher frequency of impaired glucose tolerance in the Asian Indian subjects, was not related to the preponderance of visceral fat but to a lower leg muscle to total muscle ratio [Chowdhury et al 1996]. It has been further advocated that the specific effects of girth measure are poorly captured with WHR [Seidell et al 2001]. Waist and hip measure different aspects of body composition and fat distribution, and in relation to cardiovascular disease risk factors have been shown to have both independent and often opposite effect [Seidell et al 2001]. Therefore, the observed trait associations with CAPN10 variants, although they do not necessarily conform to what might be expected, might represent a genuine genotype-phenotype relationship and should not be unjustifiably disregarded.

7.4.6 Functional effects of UCSNP 63 and haplotype combination

There is no direct evidence of causality for UCSNP63 as these studies have yet to be carried out. UCSNP63, UCSNP43 and UCSNP44 are all non-coding, non-splice junction intronic polymorphisms. The potential functional effect of UCSNP43 variant has been investigated by using EMSA with human pancreatic islet and human heptoma nuclear extracts [Horikawa

et al 2000]. Competition experiments demonstrated that the A allele did have an increased affinity for an unknown binding factor, presumably a *trans*-acting factor compared to the G allele, which had little to no binding. UCSNP44 11 bp upstream to UCSNP43 implicated in the European studies [Evans et al 2001] was also included in the intron 3 test fragment, but the presence of either C or T allele made no difference to factor binding. Further studies found chimeric reporter gene constructs with cloned CAPN10 intronic fragments with the UCSNP43 A allele had a 1.6 fold greater transcriptional activity than fragments with the G allele, and this was also modulated by the C/T alleles at UCSNP44. This suggested that these variants could be involved in the regulation of expression of CAPN10 or a nearby gene. Regulatory elements can inflict their effect over considerable distances demonstrated by regulatory element in Chromosome 5 controlling at least three genes extending over 120kb [Loots et al 2000]. In two other studies it was found that subjects homozygous for UCSNP43 G-allele associated with a reduction in CAPN 10 mRNA levels in skeletal muscle biopsies with a specific reduction in the two predominantly expressed splice variants [Baier et al 2000; Yang et al 2001]. CAPN10 intronic variants may influence abundance of the alternate splice variants, some of which may lack protease activity [Ma et al 2001], and individually may have varying biological functions. Alternatively these variants may influence transcription initiation rates and /or mRNA stability [Yang et al 2001]. The UCSNP44 is also in perfect LD with a missense mutation in exon 10, UCSNP110, a coding region of as yet unknown function. This mutation causes a conversion from a Threonine 504 residue to an Alanine. However, in mice this amino acid residue is a Serine suggesting it is not highly conserved. Furthermore, UCSNP19 is in near perfect LD with a group of four CAPN10 intronic SNP's (UCSNP 56, -59, -30, and –65) and also a silent coding mutation in exon 11, UCSNP 48 at codon A620, but again the function of this coding region is unknown.

One important question that stills remains to be elucidated is how a heterozygous haplotype combination is involved in the aetiology of the disease. The effect of combinations of haplotypes on the penetrance of disease phenotypes is not without precedence. In type 1 diabetes (T1DM) several HLA haplotypes determine susceptibility [Dorman & Bunker 2000; Undlien et al 2001]. These include the DR4/DQ8 and DR3/DQ2 haplotypes that determine susceptibility and the DR2/DQ6 haplotype determining dominant protection even in the presence of DR4/DQ8 or DR3/DQ2. Individuals homozygous for DR3/DQ2 or

DR4/DQ8 are not at increased risk of disease, whereas subjects with a combination of DR3 and DR4 haplotypes do have an increased risk of disease. The analogy can be further extended as there are likely to be several T1DM associated susceptibility determinants carried on multiple HLA haplotypes over a 3500kb distance and covering a large number of genes in the HLA Class I, II, III and IV regions. Furthermore, in different ethnic groups different susceptibility haplotypes are found. Whilst there is some evidence that UCSNP43/-44 polymorphisms might have an effect on gene expression, it does not exclude other susceptibility determinants on disease associated CAPN10 haplotypes nor indeed with a closely linked locus as yet unidentified. The analogy supports the importance of the haplotype combination rather than individual haplotype or SNP, although the strength of the overall association in T1DM with HLA is far greater than with CAPN10 and T2DM. This might also explain why preliminary data suggests varying associations with different SNP's. Thus, for instance UCSNP63 associates with T2DM in Botnian Finns and South Indians but not in Mexican Americans. UCSNP44 associates with T2DM in British/Irish subjects [Evans et al 2001] but not confirmed in other ethnic groups and the UCSNP43 association with T2DM appears so far to be unique to Mexican Americans [Horikawa et al 2000].

Further effects on how haplotype combinations can modulate the phenotype can be drawn by analogy with the most common genetic lipid disorder in Caucasians, namely familial combined hyperlipidaemia (FCH). Five polymorphic variants in the apoAI-CIII-AIV gene cluster have been implicated in the disease in FCH families. These variants have no primary causal effect and individually there was no excess transmission of any variant allele to affected offspring by TDT analysis [Groenendijk et al 1999]. However, in association studies certain high-risk haplotype combinations associated with a more severe hyperlipidaemic phenotype. These findings are not unlike those observed with the CAPN10 variants and AGT and T2DM in South Indians. In addition there are other similarities with combinations of haplotypes demonstrated to confer varying degrees of susceptibility or neutrality with homozygousity for haplotypes also not associated with an increased susceptibility to disease. In the FCH study the degree of susceptibility conferred by a haplotype combinations appeared to be the result of a synergistic effect of both haplotypes, influenced by both dominant and permissive effects of individual haplotypes. Furthermore, specific combinations were shown to associate with underlying sub-phenotypic traits that

could contribute to the overt FCH clinical phenotype, such as plasma concentrations of apoC-III, cholesterol, and triglycerides.

A similar situation could also exist with the CAPN10 haplotype combinations. Associations were found with a haplotype combination and abnormal glucose tolerance in the South Indians studied in this thesis. The data also suggests that the 'at risk' 1112 haplotype is dominant in certain haplotype combinations, but its effect is possibly neutralised in other combinations. In the MA study the (1)112 in combination with the (1)221 haplotype reduced risk toT2DM (OR 0.36 95% CI 0.15-0.86), and with (1/2)111 it had a neutral effect. There was an eight-fold difference in risk between highest and lowest risk haplotype combinations [Horikawa et al 2000]. Similarly in the South Indians the haplotype 1121 in combination with 2111 reduced risk to T2DM (OR 0.49, 95% CI 0.25-0.94, p=0.03) in diabetic parents. Whilst in combination with either 1112 or 1221 it increased risk to AGT and/or T2DM. In the FCH studies specific alleles of the individual variants also influenced the phenotypic effect of haplotypes, and due to strong linkage disequilibrium between alleles of some variants, some variant alleles were invariably never found on the same chromosome with specific allele combinations of the other variants. This was also observed with CAPN10 with the uncommon allele 2 of UCSNP63 virtually never found on the same chromosome with allele 2 of either UCSNP44, -43,or –19 in the South Indians, and this was also the case in other populations [Evans et al 2001; Horikawa et al 2000]. Whether, the predominance of only a few CAPN10 haplotypes as a result of strong LD within populations globally reflects an evolutionary selective advantage or results from phenomenia such as population 'bottlenecks', remains to be seen [Nordborg & Tavare 2001].

7.4.7 Conclusion

There have been a number of studies relating CAPN10 genotype to effects on insulin secretion and insulin action, these have been inconsistent, and none include UCSNP63, few used extended variant haplotypes and none assessed haplotype combinations. In normoglycaemic Chinese, subjects who were either heterozygous or homozygous for the A-allele of UCSNP43 had increased insulin levels under glucose load [Xiang et al 2001]. Furthermore in agreement with these findings, in four other studies analysing Chinese non-diabetics and non-diabetic first degree relatives of diabetics [Xiang et al 2001; Ng et al 2001], non-diabetic PCOS subjects [Ehrmann et al 2000] and in non-diabetic North

Europeans [Lynn et al 2002] UCSNP43 G/G homozygotes were associated with reduced insulin secretion in response to glucose load with the latter study also showing a decreased early insulin response in G/G UCSNP43-UCSNP44 haplotypes. In contrast, Germans subjects with the UCSNP43 G allele had higher glucose stimulated insulin secretion and more efficient proinsulin conversion to insulin [Stumvoll et al 2001]. Similar inconsistencies have also been observed between ethnic groups with insulin resistance, with UCSNP43 G and A allele associated with decreased and increased insulin resistance respectively in non-diabetic Chinese, whereas in normoglycaemic Pima Indians G/G homozygotes had increased insulin resistance [Baier et al 2000; Xiang et al 2001; Ng et al 2001]. These findings have been particularly confusing in respect of the Chinese studies where subjects with the UCSNP43 G allele appears to have both reduced insulin secretion and insulin resistance, and yet the G allele was increased in T2DM patients only in one study [Ji et al 2001], whereas in the two other contemporary studies, UCSNP43,-19, -63 variants were either not associated with either T2DM or IGT [Xiang et al 2001] or the G allele frequency was actually significantly lower in the T2DM subjects [Ng et al 2001]. However, one constant aspect of many of these studies although there was inconsistent association with the T2DM phenotype, evidence was detected for an underlying relationship with CAPN10 genotype and insulin secretion or action only in normal glucose tolerant or prediabetics subjects. This possibly suggests that the true CAPN10 clinical phenotype is masked by deterioration to IGT and diabetes with the induction of secondary changes in insulin secretory function and glucose metabolism due to increased plasma glucose levels.

The observed associations with CAPN10 genotype and insulin resistance may be related to the reduction in skeletal muscle CAPN10 mRNA, specifically a decrease in the two predominant splice transcripts observed in Pima Indian UCSNP43 G/G homozygotes [Baier et al 2000; Yang et al 2001]. The homozygous UCSNP43 G allele subjects also appeared to preferentially oxidise lipids, rather than protein or carbohydrates when supplied exogenously, indicating significant differences in nutrient partitioning between genotype groups [Baier et al 2000].

Despite the evidence of linkage and association between CAPN10 and diabetes, the biology of CAPN10 and its role in the pathogenesis of diabetes is still not understood. Studies using cysteine protease inhibitors have implicated members of the calpain family as being involved in either the promotion [Croce et al 1999] or inhibition of protein secretion

[Yamazaki et al 1997], depending upon the cell type. A role of calpains in insulin secretion from mouse pancreatic islets has been demonstrated with protease inhibitors acting on exocytosis of insulin [Sreenan et al 2001; Zhou et al 2000; Yun-Ping et al 2000]. Inhibition of calpain has also been found to modify insulin mediated glucose transport in both muscle and adipocytes [Zhou et al 2000; Yun-Ping et al 2000]. However, these studies have not specifically targeted CAPN10. Finally, calpains have been implicated in influencing signalling pathways that control differentiation of myoblasts [Ueda et al 1998], osteoblasts [Murray et al 1997], chondrocytes [Yasuda et al 1995] and preadipocytes to adipocytes [Patel et al 1999]. Effects on the differentiation of tissues such as pancreas, adipose and muscle tissues could have major consequences on insulin secretion and action and progression to T2DM. The knockout of the HMGIC gene in mice prevents adipocyte differentiation [Anand & Chada 2000] and the resulting inadequacy of the tissue to accommodate excess energy promotes storage of calories in the liver and muscles. This was proposed to be in part responsible for the development of diabetes in these mice. Based on this evidence Danforth [2000] suggested that too few adipocytes could predispose to T2DM. Furthermore, factors that influence the development and differentiation of cell types within the pancreas will have crucial influences on its eventual endocrine function [St-Onge 1999]. It is therefore clear that future work should now be directed at understanding the biology of CAPN10.

In summary: CAPN10 appears to be a minor gene in the susceptibility to T2DM in South Indians. Contemporary studies have provided evidence that this gene may influence glucose tolerance status in a subset of individuals, and hence progression to overt T2DM. Observations in this thesis that an 'at risk' haplotype combination of this gene conferred a substantial increased risk to both IGT/IFG and T2DM supports this view. In addition, CAPN10 genotype appeared to be associated with physiological factors that lead to impaired glucose tolerance which are separate from factors that influence progression to T2DM such as weight gain, as subjects with the predisposing haplotypes tended to be thin. Alternatively, the observation of association of CAPN10 variants with disease may be the result of linkage disequilbrium with other causative mutations either within CAPN10 or in another gene in close proximity.

CHAPTER 8:
OVERALL DISCUSSION

8.1 RETROSPECTIVE REVIEW OF STUDY DESIGN

8.1.1 Linkage Study

The non-parametric linkage study performed on the *NIDDM1* region of chromosome 2q in this thesis did not provide evidence for a T2DM disease susceptibility gene in British/Irish subjects, as previously found in a genome scan for T2DM in Mexican Americans [Hanis et al 1996]. Indeed, these findings are in keeping with a majority of the other linkage studies (table 1.4.3) that have failed to find even nominal linkage to 2q. However, the replication of linkage results although extremely important can be problematic. Simulations have demonstrated that to attain sufficient power in replication studies sample sizes must be substantially greater than those used in the study that detected the original linkage [Suarez et al 1994]. If this was applied to the linkage study in this thesis study and contemporary studies, most lacked sufficient power to replicate the linkage results. Nonetheless, subsequent analysis of a larger dataset from the same study group used in this thesis has confirmed the negative linkage result in North Europeans [Evans et al 2001].

There have been remarkable successes in positionally cloning disease susceptibility loci by linkage analysis when applied to simple Mendelian disorders. Moreover, linkage analysis has proven to be robust with low false positive rates even when stringent levels of significance have been applied in these studies [Rao et al 1978; Morton et al 1998a]. However, in the case of non-mendelian complex disorders there has been a notable lack of success in identifying genes by linkage analysis, with the possible exception of Crohn's disease [Ogura et al 2001].

One potential reason for the hindered progress may be the underlying genetic architecture of the complex disease undermining the ability to detect linkage. For example, non-allelic heterogeneity is rare in Mendelian diseases but in complex non-mendelian disorders this may not be the case. Linkage would be difficult to detect if there were a large number of distinct loci with alleles at different loci each independently capable of increasing susceptibility to the same disease phenotype. Moreover, if any single gene only accounted for a small proportion of the disease segregating in families and with the high allelic diversity expected in most populations, even the most significant risk alleles would account

for only a few percent of the total variance in disease liability. Extremely large families or huge numbers of ASP's would be necessary to obtain robust evidence of linkage. The expected allelic diversity combined with high false positive rates and lack of replication, suggests that linkage analysis overall might have only a limited potential in mapping disease susceptibility genes in non-mendelian complex diseases [Risch 2000].

The complexity and magnitude of the problem that may well be encountered in the hunt for genes in complex diseases has been alluded to in a recent review discussing the mapping of complex diseases [Wright et al 1999]. It was postulated that at least 100 loci might affect susceptibility to coronary artery disease, and less than one third of cases of pigmentary retinopathy are caused by more than 600 mostly rare disease alleles at more than 55 loci. However, if a disease susceptibility gene has common alleles that cause sufficiently large displacement (ie. has a high relative risk) then detection by linkage analysis is possible. In T1DM, susceptibility genes have been found using linkage studies including the phylogenetically ancient polymorphic risk alleles of HLA and Insulin genes [Concannon et al 1998, Cucca et al 2001]. Furthermore, in contrast to the negative predictions, a T2DM susceptibility gene was found as a result of a genome scan, namely CAPN10 in the *NIDDM1* region [Horikawa et al 2000]. The identification of the CAPN10 was even more remarkable when considering that based on the eventual observed association of the CAPN10 variant UCSNP43 with increased risk to T2DM in Mexican Americans, theoretically 100,000 sib pairs would have been required to have detected the original level of linkage [Altshuler et al 2000]. However, the final location of the gene relied heavily on the utilisation of methods based on linkage disequilibrium.

8.1.2 Candidate gene approach

Association studies can be a much more effective genetic tool than linkage as they can provide adequate power to identify genes with low to moderate relative risks (<1.5), even with stringent levels of significance and in diseases that are both aetiologically and genetically heterogeneous [Risch & Merikangas 1996]. Simulation experiments have shown that LD has an increased power to detect susceptibility genes in complex disorders [Genin & Clerget-Darpoux 1996]. Consequently, candidate gene approaches using association studies have been widely used to study complex disorders. However, association studies are also only in reality statistical arguments that are dependent on generating correlations between

two or more events, characteristics or variables. The statistical outcome of these studies could be entirely due to unknown underlying confounding factors and could be influenced by a number of other statistical, clinical and genetic factors. Caution therefore has to be applied in association studies. In this respect as part of a retrospective overview of the study design a number of aspects were considered:

(1) Sample Size: The population/sample size used must be adequate to provide sufficient power to detect an association, especially if the disease related alleles have a low frequency and/or relative risk. Calculation of power for the loci studied in this thesis found that the South Indian urban survey in most cases did provide adequate power. In contrast the South Indian families and obese/slim cohort in some circumstances were underpowered. In epidemiological studies statistical power based on sample size can often be determined prospectively. Unfortunately *a priori* knowledge of a candidate locus such as relative risk, disease related alleles, allele frequencies and regional patterns of linkage disequilibrium (for haplotype construction) are not usually available within the same ethnic group. Hence most genetic studies must be performed before power of the study can be determined.

(2) Misclassification: The associations statistically generated in this thesis relied entirely on the correct classification of both genotype and phenotype. Laboratory methods were instigated to guard against genotype misclassification. However, all the statistical analyses were made under the assumption that there was no phenotypic misclassification, as there was no provision to do otherwise.

(3) Population Dynamics: The South Indian population appeared to be ethnically homogeneous and had no obvious evidence of a high degree of consanguinity or skewed population dynamics (observed genotype frequencies for all loci studied did not deviate from expected Hardy-Weinberg values, bar one [CAPN10 UCSNP19] that had increased heterozygosity suggestive of genotyping error or possible 'outbreeding']. However, even if some 'inbreeding', is present in this South Indian Dravidian population it may not be detrimental as some geneticists believe this to be advantageous as it can increase the rates of fixation for risk alleles, cause polarisation of phenotypic effects, and reduce non-allelic heterogeneity [Wright et al 1999].

The relative merits of the South Indian urban population survey compared to a conventional case-control study could be questionable. Populations that are derived from a peripheral part of a larger epidemiological study that were not originally designed for examination of genetic factors, such is the case for the South Indian urban survey have been criticised for a 'lack of thoroughness' [Tabor et al 2002]. However, an across the board random collection of subjects within an ethnic population would go some way in avoiding the selection bias possible in a conventional case-control study design.

(4) Replication: Candidate gene approaches have frequently suffered from a lack of reproducibility both within and between different ethnic groups [Tabor etal 2002]. Non-replication of association of specific locus alleles between populations of different ethnic background could be due to a number of reasons including both allelic and non-allelic heterogeneity giving rise to the same disease phenotype. In addition, if the variant is merely a disease marker and not causative then differences in chromosomal linkage disequilibrium due to demographic variation between ethnic populations could produce different results. Alternatively association with the same variant allele may be undetectable or different between ethnic groups due to differences in relative risks or pleiotropic genotype effects on phenotypic traits. In this respect replication studies within the same ethnic group would be more robust. In this thesis a second data set using South Indian families not only allowed the opportunity to replicate findings within the same ethnic group but also facilitated the application of a different study design to complement the population association study. In addition it provided an enriched resource for case-control studies, with recruitment selection increasing the probability of an individual carrying a disease allele.

Family based TDT analysis where both linkage and association must be simultaneously present to attain a positive correlation has certain advantages over population association study designs. Firstly, the TDT test utilises non-disease but related individuals from within the families as 'pseudocontrols', therefore theoretically avoiding the confounding effects of population stratification. Power studies have also shown that the detection of linkage in complex traits by TDT has much greater power than allele sharing methods used by non-parametric linkage analysis [Risch & Merikangas 1996].

However, a TDT approach can also be fraught with problems and the traditional case-control approach even with its confounding problems of population stratification and high

false positive rates still offers distinct advantages [Morton & Collins 1998b]. For example, TDT tests fail to take into account an 'inbreeding coefficient' which reduces its efficiency [Morton & Collins 1998b], whereas extensive studies in large population samples found this to be a negligible problem [Morton et al 1991]. Morton also contends that the stratification argument in favour of TDT is purely mathematical and not based on genetic evidence and is only actually true under certain population conditions [Morton & Collins1998b]. Consequently failure for a TDT to replicate a positive population association result might mean a classical Type I error in the population study, but could also equally reflect a type II error in an inefficient family based test.

In the studies in this thesis TDT analysis of disease phenotypes did not always support associations demonstrated in the population sample. Certainly in the case of the CAPN10 gene this was due to the low disease allele frequency of UCSNP63, lack of heterozygosity, too few families and hence lack of statistical power. The strength of the TDT test is determined by the degree of linkage disequilibrium between the polymorphic sites examined. This is at its most efficient when the disease concerned is rare and the allele frequency differential between the disease and the normal chromosome is at its greatest [Xiong & Guo 1997].

Furthermore, during the course of my studies on CAPN10 an additional limitation of the TDT test was exposed. The observations in the population association studies that heterozygous haplotype combinations of CAPN10 were more important than individual haplotypes for risk to disease would not have been detected solely by using a family based approach. As a consequence of the positive association in the population sample the TDT test had to be modified to allow analysis of the simultaneous transmission of two risk haplotypes in families. Using this adaptation the positive result in the population association study was confirmed.

Overall this argues in favour of the simultaneous use of both family and population sample resources if available.

(5) Enhancement of detection: In complex disorders such as T2DM and obesity with potentially large number of susceptibility loci (and alleles), the disease state is complex and an insensitive indicator of the underlying pathogenic process. Unless variation in a candidate gene has a major impact on susceptibility to the idiopathetic phenotype then false negative

studies are possible. In this respect the analysis of intermediate quantitative traits can prove to be more sensitive especially if an effect on sub-phenotypic traits can be predicted based on possible gene function. However, such studies are limited to available sub-phenotypic data that may not always be comprehrensive or the most appropiate. For example, studies in South Indians in this thesis on UCP2 and UCP3 found gene variants associated with available data on variation in body mass (BMI) and fat distribution (WHR) which would not be incongruous with the potential role of uncoupling proteins in body weight homeostasis. Importantly an increase in risk to T2DM and related metabolic syndromes in South Indians appears to be strongly influenced by progessive central obesity [Davey et al 2000]. Even relatively small gains in abdominal adiposity can increase propensity to disease. However, in South Indians the measurement of sagittal diameter rather than BMI and WHR would have been a much more sensitive indicator of variation in central obesity in this ethnic group [Davey et al 2000].

In contrast other intermediate trait data ie. IFG/IGT in the South Indian population provided additional information that many other studies have missed through omission of clinical diagnosis of this sub-clinical grouping. The association in this thesis with CAPN10 was found in both subjects with IFG/IGT and T2DM. However, only a proportion of the IFG/IGT go on to become overtly diabetic, which suggests that CAPN10 could be one of the genes that broadly underlie glucose intolerance within a population.

The power to detect an association is not only enhanced by clinical sub-grouping but also if the ethnic group has a reduced environmental risk [Wright et al 1999]. The South Indian population presumably have not been fully subjected to the impact of environmental factors that 'westernised' populations have. Therefore in this respect this resource is potentially more useful in studying subtle genotype-phenotype interactions. Feasably, the lack of replication of the significant South Indian association with the UCP2 variants and BMI in White European obese subjects (notably only negative after statistical correction for multiple comparisons) might be indicative of a masking effect. Alternatively, genotype-subphenotype relationships have often been more readily detectable in non-disease or pre-disease subjects rather than in the overt disease state. For example, studies on intermediate traits such as insulin sensitivity and secretion with CAPN10 gene variants were only correlated in normoglycaemic subjects and not in diabetics [Lynn et al 2002; Baier et al 2000]. The South Indian study population were recruited cross-sectionally without prior selection criteria apart

from ethnicity. Therefore, it would be expected that the whole broad spectrum of the non, pre, and disease state are represented possibly making it more sensitive in studying the effect of the genetic variation on phenotype.

(6) Haplotypes: The generation of haplotype data is proving to be increasingly important in respect of providing information on linkage disequilibrium patterns particularly between different ethnic populations. In the context of population association studies it is believed that analysis of haplotypes can be statistically advantageous in terms of providing increased power to detect association, but only under certain circumstances [Bader 2001]. Simulation studies have suggested that there is a 'crossover' of the statistical usefulness of analysing individual SNP's verses haplotypes and this is determined by both the number of possible haplotypes in a given population and the number of 'causative' SNP's within the haplotype [Bader 2001]. If this model had been applied to the CAPN10 study, then haplotype analysis should have been more powerful than individual SNP analysis. In contrast, there would not have been any great benefit in analysing haplotypes of the UCP2-UCP3 variants. However these simulations did not take into account scenario where it would be necessary to investigate the effects of haplotype combinations as in the CAPN10 gene.

In pedigrees haplotype phase can be readily determined but in unrelated subjects this usually has to be estimated. Many of the current statistical methods that estimate haplotype frequency in population samples rely entirely on the expectation-maximisation (EM) algorithm, such as the EH program [Ott 2000]. It has been suggested that this method is inefficient and prone to bias and ideally experimentally derived haplotype phase data is preferable [Hodge et al 1999; Douglas et al 2001]. The practicalities in producing experimentally derived haplotype data in the South Indian survey subjects would have involved a monumental task based on the techniques available at the time. Fortunately, yet again having both a family and population resource from the same ethnic group proved to be useful. Invaluable knowledge of patterns of linkage and disequilibrium and hence haplotype frequencies derived from the families allowed the unambiguous assignment of haplotype phase to a majority of the urban survey subjects. Certainly alternative methods using estimations would have been less reliable in downstream analyses.

However, it has also been suggested that haplotypes should not be analysed alone, because if selection processes are present, haplotypes conferring an extreme phenotype are eliminated

at a faster rate than the underlying SNPs, with this negative selection tending to eliminate the extreme multi-hit haplotypes from the population. However, the individual causative alleles may still have considerable population frequency [Bader 2001]. This supports the view that analysis in the thesis of both individual variants and as haplotypes was fully warranted.

8.2 DISSECTION OF A COMPLEX TRAIT

Rich [1990] suggested that the best-fit genetic model for T2DM involved only a few genes with a moderate effect, superimposed on a polygenic background. However, Risch [2000] intimates that this actually reflects geneticist's preference for a oligogenic/multigenic model, as it has a tractable degree of complexity. Complex disease models that have infinite genetic complexity, resulting from numerous effector genes, but each only contributing a modest effect, would represent a monumental task in the identification of susceptibility loci. However, whatever the genetic model of the disease it will be necessary to break it down into its simplest components. Theoretically, the identification of all the susceptibility genes and an understanding of their individual underlying biological contribution of each gene to the overall disease pathophysiology would eventually provide a means to determine an individuals risk to disease. The work in this thesis using a candidate gene approach has attempted to address this paradigm.

8.2.1 Uncoupling Protein Genes

There is reasonable certainty based on the evidence presented in chapter 2 that uncoupling proteins UCP1, UCP2 and UCP3 uncouple oxidative phosphorylation at the inner mitochondrial membrane. However, the exact physiological role of either UCP2 or UCP3 in either thermoregulation, modulation of ATP synthesis, control of oxidative stress, or fatty acid/glucose oxidation still remains to be fully elucidated [Boss et al 2000; Adams 2000; Simonyan & Skulachev 1998; Richard et al 1998; Rippe et al 2000; Samec et al 1998a].

Studies in this thesis found associations with variants of UCP2 and UCP3 with variation in fat mass (BMI) and in fat distribution respectively. These findings suggests that variations in these genes in humans may predispose an individual to either a reduced efficiency in modulating body weight or may be instrumental in influencing regional fat deposition.

In the previous section one highlighted important aspect of association studies was the need for replication studies to further substantiate positive findings. To date five studies, including the study in this thesis, have reported positive associations with BMI and the UCP2 Ins/Del variant in exon 8 (and also A55V in some instances) with p values ranging from 0.001-0.007 [Cassell et al 1999; Evans et al 2000; Yanovski et al 2000; Zheng et al 2000; Buemann et al 2001]. Moreover, these associations have been found across a number of diverse ethnic populations including Caucasians, Africans, Chinese and Asians. In addition, the UCP2 exon 8 variant has been correlated with variation in sleeping metabolic rate in Pima Indians [Walder et al 1998]. Recently a promoter polymorphism at position –866 in UCP2 has been identified that appears to control the transcription of the UCP2 gene and may determine the preferential expression of the exon 8 insertion or deletion allele [Esterbauer et al 2001]. The variant was attributed to causing 15% of the variation in adiposity in the population group studied. However, this association was not confirmed in one other population studied [Dalgaard et al 2001a].

Similarly variants of UCP3, particularly the promoter –55 variant have also been associated with variation in BMI/obesity in three studies although this has not been as consistent as the UCP2 exon 8 variant studies [Cassell et al 2000; Otabe et al 2000; Halsall et al 2001]. Although the –55 promoter variant was not associated with BMI in the South Indians in this thesis instead it was positively associated with variation in WHR. The observed BMI associations could reflect linkage disequilibrium with the UCP2 gene. However, the -55 variant has also been significantly associated with higher LDL-cholesterol levels in French and fasting blood glucose in the Western Pacific Ocean Palauan islanders [Meirhaeghe et al 2000; Yanagisawa et al 2001]. In addition, other UCP3 variants have correlated with basal fat oxidation (exon 6 splice variant) and levels of resting energy expenditure (the exon 5 Tyr210Tyr) in African-American populations [Argyropoulos et al 1998; Kimm et al 2002]. In addition, a newly identified UCP3 intronic variant (GAIVS6) that has not been screened in any other population including the South Indians was found to be significantly associated with BMI, fat mass, percentage fat, skinfold thickness, and leptin levels in French Canadians [Lanouette et al 2001].

Overall the findings from genetic studies provide compelling evidence that UCP2 and UCP3 could be involved in the regulation of body-weight homeostasis in humans. Whether UCP2/UCP3 have roles in mediating fuel partitioning and/or adaptive energy expenditure,

an inherited inability to efficiently remove surplus calories by thermogenic wastage, or a deficiency in skeletal muscle to utilise lipids as fuel substrates would both promote increased fat deposition. Even only a small persistent discrepancy between daily intake and energy output could result in substantial weight gain over time.

However, there have been a number of negative studies for both UCP2 and UCP3, which must be considered especially in light of the widely perceived view that there tends to be positive publication bias [Klannemark et al 1998; Urhammer et al 1997 & 1998; Chung et al 1999; Kubota et al 1998; Otabe et al 1998; Dalgaard et al 1999]. It is possible that either the positive or negative associations represent statistical error as a result of underlying confounding factors. Alternatively, the differences observed between the studies may reflect both or either differences in the gender or ethnicity of the study populations. A number of factors that could determine a studies ability to detect an association including those dependent on the ethnic background of the population were discussed in the previous section (8.2.1). Certainly genetically determined differences in fat distribution patterns can be influenced by both ethnicity and gender [Park et al 2001; Snehalatha et al 1997; Bouchard et al 1990].

The South Indian subjects studied in this thesis possess a high degree of heritability for intra-abdominal fat deposition compared to other ethnic groups [Davey et al 2000]. This tendency in South Asians to accumulate visceral fat is likely to have a central role in associated metabolic disturbances with even small increases shown to be predictive of an increased risk to T2DM [Snehalatha et al 1997; McKeigue et al 1991& 1992; Ramachandran et al 1997a & 1998]. Furthermore, a recent study that tested the hypothesis that an effect on resting energy expenditure by variation in UCP1, UCP2 and UCP3 may be different across racial groups found that variation in UCP3 (strongest effect exon 5 Tyr210Tyr) had a more pronounced effect in African-Americans than in White Europeans [Kimm et al 2002]. Interestingly, most of the negative studies with variants of UCP2 and UCP3 have been in White Europeans.

In respect of gender it is widely recognised that men and women differ in fat deposition (discussed in chapter 1). In the discussion of chapter 4 and 5 attention was drawn to the fact that the subjects used in most of the negative studies were either predominantly or entirely male. In contrast, many of the positive findings including those in this thesis were either with females or in study groups with an increased proportion of females. Accumulating

evidence suggests that gender determined differences in regional fat distribution could have important ramifications in respect of physiological processes. Men under weight reduction regimes have increased mobilisation and loss of intra-abdominal fats compared to women who predominantly lose subcutaneous fat (SC) [Wirth & Steinmetz 1998]. This loss of intra-abdominal fat rather than SC appears to be associated with a pronounced improvement in metabolic profile, in respect of lipids, lipoproteins and blood pressure. Observations that human omental adipocytes display a range of biochemical properties that distinguish them from subcutaneous adipocytes combined with the existence of fat depot specific effects on gene expression infers that different fat deposits may function very differently [Minocci et al 2000; Montague et al 1998]. Important examples include the significantly increased expression of leptin in SC fat compared to omental fat deposits, and fat depot variation in the expression Alanine Transaminase, and the apoptosisprotein-2 (cIAP2) genes [Montague et al 1998; Thulsrup et al 1999].

Gender and ethnically determined differences in adipose tissue deposition could exacerbate the phenotypic effect of UCP2, hence influencing the ability to detect an association. However, although UCP3 is not transcribed in adipose tissue it may also be indirectly affected by fat deposition. UCP3 expression varies considerably depending on the type of muscle fibres [Hesselink et al 2001; Schrwauen et al 2001]. UCP3 activity in different muscle fibres may be modulated by variation in muscle cell sympathetic nerve activity and β3-adrenergic stimulation, which has also been correlated with gender differences in fat distribution [Jones et al 1996; Rodriguez et al 2001]. In addition, it has been postulated that reduced muscle sympathetic nervous activity is a potential mechanism in predisposing humans to body weight gain and obesity possibly as a result of diminished thermogenic response to stimuli which is particularly evident in older individuals [Spraul et al 1993; Kerckhoffs et al 1998].

One interesting aspect of the thesis study was the observed relationship of UCP2 genotype with plasma leptin levels. Although, there are no equivalent replicate studies to support these findings, an intronic variant in UCP3 (GAIVS6) has been significantly associated with leptin levels in French-Canadians [Lanouette et al 2001]. These findings might indicate the existence of a biological pathway whereby uncoupling proteins influence body weight regulation via the action of leptin.

Leptin, in addition to its anorectic effect has a stimulatory effect on energy expenditure, affects several neuroendocrine mechanisms and regulates multiple hypothalamic–pituitary axes [Tritos & Mantzoros 1997]. Pre-obese subjects tend to have low leptin levels but most obese humans paradoxically have over expression of leptin, suggesting the presence of a 'leptin resistance' [Ravussin & Gautier 1999; Lonnquist et al 1995]. In this thesis it was demonstrated that lower leptin levels than would be expected by weight (BMI) were associated with variation in UCP2 genotype in the British Caucasian cohort. This might suggest that dysfunctional UCP2 expression could also have possible effects on either leptin action or secretion in peripheral tissues.

However, whereas Leptin modulates UCP expression in a number of tissues in rodents but at present there is no evidence to suggest the existence of a direct feedback pathway with UCP's influencing leptin action [Zhou et al 1997; Gimeno et al 1997; Larrouy et al 1997; Scarpace et al 1998; Chavin et al 1999; Melia et al 1999; Commins et al 1999; Gomez-Amrosi et al 1999; Sivitz et al 1999; Ceddia et al 2000; Villarroya et al 2001]. If UCP2 genotype does influence leptin action then several possible mechanisms might be postulated to support the association observed.

In several rodent studies involving the individual knockout of UCP1, UCP2 or UCP3 genes, mice do not become obese, and alternative physiological roles have been suggested for both UCP2 and UCP3 [Enerback; Gong 2000; Vidal-Puig2000; Arsenijic]. Two alternative physiological roles have been widely proposed, but are still speculative as all three uncoupling genes have not be knocked out in unison, therefore compensatory interactions between these genes cannot be entirely eliminated and may account for some of their findings. Firstly, a role in limiting the overproduction of apoptotic ROS to maintain cellular integrity, with the main source of ROS being from mitochondria due to increased proton availability from fat oxidation.

Secondly the reduction in the effects of lipotoxicity on tissues by altering fuel utilization within the cells, with this probable action of UCP2 having been already demonstrated in adipocytes [Wang et al 1999; Chavin et al 1999; Arsenijic et al 2000; Boss et al 2000]. Hepatic steatosis and excess lipid in muscle and pancreas are characteristic in obese diabetics. It has been hypothesised that excess calorie intake beyond the capacity of the adipose tissue, itself becoming insulin resistant when filled to capacity ($\cong 3\mu$ g lipid/cell), increases the abnormal deposition of lipids in liver, muscle and blood [McGarry & Dobbins

1999; Danforth et al 2000]. In skeletal muscle an increase in tissue FFA content interferes with insulin transport via blood across endothelium [Baron 1994; Steinberg et al 1997]. This can stem the access of insulin to insulin sensitive tissues causing insulin resistance and also disrupt insulin stimulated glucose utilisation [Bergman 1997; Hamilton-Wessler et al 2000; Randle et al 1994; Baldeweg et al 2000; Boden et al 1997]. Muscle insulin resistance combined with the other deleterious effects of lipotoxicity, such as over-production of glucose by liver, and blunted secretion in pancreas, are characteristic of T2DM [McGarry & Dobbins 1999; Unger et al 1999]. Uncoupling activity could modulate either the levels of ROS production or degree of lipotoxicity and hence directly protect tissues such as pancreatic β-cells, skeletal muscle and liver from their deleterious effects [Wang et al 1999; McGarry & Dobbins 1999; Unger et al 1999; Mohanty et al 2000; Chen et al 2001; Pessayre et al 2001].

The control of either ROS or lipotoxicity by UCP2 in adipocytes and other target tissues could have effects on both leptin secretion and post leptin receptor transduction pathways. Leptin acts at least in part by mediating the release of the orexigenic neuropeptide Y (NPY), propiomelanocortin (POMC), and melanocyte stimulating hormone (α-MSH) in the hypothalamic arcuate nuclei. UCP2 is abundantly expressed in these hypothalamic nuclei [Richard et al 1999; Diano et al 2000]. Therefore UCP2 could be crucial in preventing neural tissues becoming leptin insensitive from the deleterious effects of increased ROS levels or lipotoxicity, with the latter affecting either the passage of leptin across the blood brain barrier or on cell surface leptin receptors [Caro et al 1996; Kalra et al 1999]. Ageing in rodents causes an impairment of hypothalamic nuclei uptake of leptin independently of increasing adiposity, which is commensurate with elevated mitochondrial ROS production augmented by ageing and a decline in cellular UCP2 activity [Wallace 2001; Fernandez-Galaz et al 2001; Yamashita et al 1999]. Studies in rodents have suggested that ageing induced reduction in UCP2 expression and physical activity are responsible for observed increased cold sensitivity and obesity [Yamashita et al 1999]. ROS also appears to be able to modulate both gene expression and signal transduction pathways within cells, although these mechanisms are still not completely known [Shih et al 2001]. UCP2 could control expression of other genes by influencing levels of ROS and hence brain tissue function. Further studies on the neuronal expression of UCP2 also suggest that heat generated by axonal UCP2 can modulate neurotransmission in homeostatic centres and thereby coordinate

the activity of the brain circuits that regulate daily energy balance and related autonomic and endocrine processes [Horvath et al 1999].

Interestingly, studies in humans have shown that adipose tissue UCP2 expression is inversely correlated with both leptin and increasing adiposity [Pinkney et al 2000]. Therefore, increasing adiposity in humans would suppress the very mechanisms that may be designed to correct the damage caused by lipid saturation of tissues, this would be exacerbated by the reduced control of tissue ROS production that increases with age and thus opening the way to further progression to the obese and diabetic state [Bergman & Ader 2000; Wang et al 1999].

However, the arguments for these alternative physiological roles for uncoupling protein functions are largely pivotal on experimental findings in rodents. Although, the regulation of human and rodent UCP1, UCP2, and UCP3 expression does appear to be mediated by a variety of similar stimuli, there are evidently differences in tissue specific response between species. This is illustrated by differences observed in the induction of UCP2 expression in WAT between rodents and humans. In rodents, whereas food intake, plasma insulin, PPAR agonists, fatty acids and leptin overall appear to induce UCP2 expression, although not always entirely consistently [Qian et al 1998; Zhou et al 1997; Aubert et al 1997; Villarroya et al 2001]. In humans, plasma leptin, insulin and fatty acids suppress UCP2 expression in WAT, with food intake having no effect at all [Pinkney et al 2000].

The uncoupling protein family are highly conserved with orthologs discovered in many species of mammal and also in fish, birds, insects and even plants suggesting an evolutional importance in their metabolic function [Argyropoulos& Harper 2002]. However, there are fundamentally considerable differences in the environmental conditions and requirements for adaptive mechanisms between animal species, suggesting that the physiological roles of these genes could be quite different. The function of non-shivering thermogenesis for UCP1 in BAT is an important adaptive response essential for the survival of hibernating rodents, penguins, cold adapted and possibly new-born mammals [Rothwell & Stock 1981; Duchamp et al 1991; Bouchard et al 1997]. However, its role in adult humans existing under very different thermoneutral-environmental conditions appears to be largely redundant. Although there is a high degree of homology between the UCP3 proteins, the promoter region of UCP3 in humans is very different to that in rodents, [Esterbauer et al 2000; Yoshitomi et al 1998b]. This would not only suggest that there has been major functional diversification of

uncoupling proteins between species as a result of rapid phylogenetic evolution of these genes, but in addition the trans-acting factors and hence other downstream mechanisms controlling expression of this gene are also very different. Furthermore, UCP2 and UCP3 genes are both in close chromosomal proximity and have a high degree of cross homology in both humans and rodents, suggesting that they may be the result of an evolutionary gene duplication event. Evidence of functional differences and the differential tissue expression of UCP2 and UCP3 suggest they might have also developed very different physiological roles.

However, a similarity in function between species cannot be totally disregarded. A 17 month-old girl that had profound isolated episodes of hypothermia had both deficiencies in skeletal muscle mitochondrial respiratory chain complexes III and IV and also demonstrated decreased muscle UCP3 function, inferring that UCP3 may also still be important in cold adaptation processes in humans [Cholley et al 2001].

In conclusion the findings in this thesis and contemporary genetic studies provide substantial evidence that the uncoupling protein homologues UCP2 and UCP3 influence underlying physiological processes that alter an individual's propensity to weight gain. However, more research is necessary to establish the true functional roles of both UCP2 and UCP3 in humans.

8.2.1 CAPN10 gene

The findings in thesis that sequence variation of the CAPN10 gene can increase risk to the development of T2DM are in agreement with both the seminal and some contemporary studies [Horikawa et al 2000; Evans et al 2001; Schwarz et al 2001; Baier et al 2000]. However, it is evident from all these studies that the resulting disease phenotype is not determined by a single gene variant, but by a culmination of the combined effects of a cluster of mainly intronic polymorphisms. These variants at present have not been linked anyway to causality. These CAPN10 gene variants are all in considerable linkage disequilibrium and hence globally generate only a few haplotypes [Fullerton et al 2002]. However, the varying degree of individual risk to disease is conferred not only by certain haplotypes but is also dependent on interactions or synergistic effects between the haplotypes.

The importance of the CAPN10 gene in susceptibility to disease varies between different ethnic groups and appears to be determined by the frequencies of 'risk' haplotypes. The low frequency of one of the main 'risk' haplotypes in South Indians suggests that CAPN10 is only a minor T2DM susceptibility gene in this population. Geographic and evolutional studies on the distribution of CAPN10 polymorphisms/haplotypes in humans and non-human primates have to some degree illustrated the global diversity that may be expected [Fullerton et al 2002]. For example these investigations found that the alleles of UCSNP19 and UCSNP63 (C) may have had a selective advantage in non-African populations and because of this they have persisted in these populations. In contrast, one of the 'at risk' haplotypes (121) that is common in Europeans and Asians was found to be rare to completely absent in Africans suggesting other 'at risk' haplotype combinations would be responsible for susceptibility at this locus in subjects in this ethnic group.

Studies on the function of the CAPN10 protein are presently scarce to non-existent. However, preliminary studies correlating phenotypic variation to genetic variation for CAPN10 support a role for the protein in both influencing insulin action and secretion [Baier et al 2000; Sreenan et al 2001; Elbein et al 2002; Lynn et al 2002]. There are again clearly ethnic differences in the effect of the variant loci on phenotype. Whereas UCSNP43 associates with insulin sensitivity in Pima Indians, the UCSNP19 and UCSNP63 variants have a more significant effect in Caucasians [Baier et al 2000; Elbein et al 2002]. The observed decreases in skeletal muscle insulin sensitivity and reduced insulin mediated glucose transport could be due to variation in expression of specific splice variants, with some isoforms possibly being biologically inactive [Yang et al 2001; Ma et al 2001]. Diabetogenic effects by the CAPN10 protein have also been proposed through modulation of insulin exocytosis in the pancreatic β-cells and hence possible effects on insulin secretion or by regulation of glucose metabolism in peripheral tissues such as adipocytes [Sreenan et al 200; Hoffstedt et al 2002].

Although little is known of the function and pathophysiological effect of CAPN10, other members of the calpain superfamily have been investigated and associated with disease. Members of the calpain superfamily are important proteases that have been demonstrated to be downstream molecules of hormone receptors, membrane-type tyrosine kinases and adhesion molecules [Sato & Kawashima 2001]. Calpains proteolyse and regulate the activity of many signalling related substrates, such as protein kinase (PKC), alpha subunit of G-

proteins and protein tyrosine phosphatases. Consequently, calpains may modulate signal transduction pathways in a number of molecules including growth factors, T-cell receptors and integrins [Sato & Kawashima 2001].

Calpain proteases have already been implicated in several disease processes. In limb-girdle muscular dystrophy a dysfunctional skeletal muscle specific CAPN3 appears to have direct effects on the progressive atrophy of limb and trunk muscles [Huang et al 2001]. In addition, over-activation of calpains have also been implicated in a number of other pathological processes, with CAPN1/ CAPN2 associated with stroke, traumatic brain injury and Alzheimer's disease and CAPN9 with gastric cancer [Huang et al 2001]. One possible 'disease causing' factor that has been implicated in these degenerative disorders, are the increased amounts of the 'unusually stable' calpain-mediated cleavage break-down products (BDPs). BDP's appear to be related to apoptotic processes mediated by the caspase family of proteases and are increased in normal ageing and Alzheimer's disease [Vanderklish & Bahr 2000].

The study on CAPN10 in this thesis provides preliminary evidence supporting a role for this gene in contributing to susceptibility to T2DM. Ultimately extensive functional work is required to prove conclusively whether the CAPN10 gene actually play's a primary role in the development of T2DM or is secondary in nature by facilitating the progression to the overt disease state.

The positive findings in this thesis from a 'hypothesis-driven' candidate gene approach have contributed to another step forward in the knowledge of which genes might influence susceptibility to T2DM and obesity. Moreover, the studies in this thesis still endorse Risch's definition for a complex trait by confirming the degree of complexity and genetic heterogeneity that had been expected [Risch 2000]. CAPN10, UCP2 and UCP3 are only minor genes for disease susceptibility, each individually insufficient to give rise to either the overt T2DM or obesity phenotype. Thus, in both multifactorial disorders to be able to eventually generate a genetic risk profile of an individuals disease susceptibility would require the knowledge of a large proportion of these minor contributing genes, combined also with the relative contribution of each individual gene, and any gene-gene, and gene-environmental interactions. This is unfortunately further complicated in that unlike mendelian disease phenotypes where a culmination of " a gene with a large displacement,

giving rise to a (near) one-to-one correspondence between genotype at a single locus and phenotype". In complex non-mendelian disorders "genes are without large displacement, giving rise to significant overlap of genotype distributions with a lack of one-to one correspondence between genotype and phenotype" [Risch 2000]. Considerable overlap of phenotypic traits were observable just in the three genes studied in this thesis, with correlations in common in other studies for insulin secretion, insulin action, fuel oxidation / fuel partitioning and sleeping metabolic rate [Baier et al 2000; Walder et al 1998; Chan et al 2000; Screenan et al 2001; Stumvoll et al 2001; Argyopopoulos et al 1998ab; Willi et al 1998]. Consequently, for complex diseases there will never be simple cause-and-effect/genotype-phenotype interactions that are evident in simple Mendelian disorders with any T2DM phenotype or sub-phenotypes an aggregate of multiple gene effects. This overall suggests that the task ahead will be a challenging one.

8.3 GENE DISCOVERY AND DISEASE PREVENTION

The ultimate rationale for studying a disease is to manage, treat and eventually prevent disorders. The identification of these susceptibility loci that are responsible for the genetic component of T2DM and /or obesity could greatly assist in the elucidation of the underlying pathophysiological mechanisms leading to the diseases and is central to the development of more effective preventative and therapeutic strategies for this condition.

An understanding of an individual genetic risk profiles in combination with established clinically defined predisposing factors could be used as part of a primary prevention in reducing risk of onset of disease. The identification of the genes responsible for MODY that accounts for 1% of T2DM in the UK (approximately 20,000 people) have allowed the development of diagnostic and predictive genetic tests for 80% of MODY families [Shepherd et al 2001]. Families with a history of T2DM would presumably be enriched in specific T2DM susceptibility alleles/genes and information on the sharing and transmission of a majority of the susceptibility polygenes could feasibly allow an overall determination of risk and early preventive measures. In the future, the risk of first-degree relatives may be determined in families with a history of T2DM similar to that of MODY. In terms of both primary and secondary prevention the identification of susceptibility genes could not only identify new biological pathway's that may permit specific intervention, but also facilitate the individual tuning of treatments.

The field of pharmacogenomics is emerging as a relatively new sub-discipline of molecular genetics. Future developments in this field will be largely dependent on the information made available through analysis of the Human Genome. Drug response is under the control of multiple genetic factors, and a better understanding of complex traits would permit stratification of patient populations presenting a single disease phenotype into subclasses whose disorders might have differing genetic components or different responses to particular therapeutics. Clinical trials to associate genotypes in relation to individual variations in therapeutic drug response and in the occurrence of adverse drug reactions are becoming more common with the eventual view to produce customised drugs and treatments [Marshall 1997].

Whether following the elucidation of true functional roles of either UCP2, UCP3 or CAPN10 they can be exploited to develop treatments for obesity or T2DM remains to be seen. No susceptibility genes identified so far relating to either of these multifactorial diseases have permitted the development of preventive treatments.

A recent study was designed using transgenic mice over-expressing UCP1 in skeletal muscle to determine whether controlling mitochondrial uncoupling oxidative phosphorylation in skeletal muscle could possibly be a suitable treatment for obesity and T2DM [Li et al 2000]. In these studies skeletal muscle oxygen consumption was increased by 98% in low expressing mice, and 246% higher in high expressing mice compared to the wild type strains on the same dietary regime. The transgenic mice also weighed less, had lower levels of glucose and triglycerides combined with better glucose tolerance than the control mice. In addition, low UCP1 expressing mice were resistant to obesity under two different high fat diets and had lower glucose, insulin and cholesterol levels. There was also a concomitant increase in metabolic rate both at rest and during exercise. Furthermore, the mice were also more responsive to insulin, and had enhanced glucose transport in skeletal muscle, despite an increased muscle triglyceride content.

Whether modulation of UCP2/UCP3 in humans could have a similar impact remains to be seen. In the 1930's agents that increased mitochondrial proton conductance were clinically administered in humans as a treatment for obesity. In 1934 it was estimated that 100,000 people in the USA were using a classic uncoupling agent, dinitrophenol, with dramatic success [Tainter et al 1934]. However, due to the occasional problems with cataracts and

some fatal overdoses its popularity declined and it was eventually withdrawn from use [Parascandola et al 1974].

How useful the uncoupling protein homologues could be in disease prevention in humans all depends on the eventual elucidation of their true physiological roles. The manipulation of UCP2 gene expression in two recent studies in rodents produces conflicting observations resulting in it being referred to both as an 'adiposity angel and diabetes devil' [O'Rahilly 2001]. Rodents over-expressing uncoupling proteins made mice resistant to diet induced obesity and improved pancreatic β-cell function in Zucker diabetic rats [Clapham et al 2000; Wang et al 1999]. Whereas, in other studies over-expression of UCP2 in rat and mice pancreatic islets caused impaired cellular function, causing a reduction in insulin secretion and contributed to increased obesity [Chan et al 1999; Zhang et al 2001]. Indirectly the UCP2 and UCP3 proteins may already be involved some treatment processes. UCP2 and UCP3 both have putative PPAR sites in their promoter regions [Tu et al 1999; Solanes et al 2001]. Two classes of drugs presently used in humans, fibrates and thiazolidinediones control hyperlipidaemic and insulin insensitivity respectively. These drugs exert their effect as synthetic ligands activating or up-regulating PPAR's and modulating the expression of other genes [Park et al 1998; Michalik & Wahli 2001; Schoonjans et al 1996; Spiegelman 1998].

8.4 EXTENSION OF STUDIES

8.4.1 UCP2 and UCP3 genes

In respect of future research on the human uncoupling protein homologues, this will primarily involve functional studies on the UCP2 exon8 insertion/deletion and the UCP3 -55 promoter polymorphisms. Preliminary data in chapter 6 suggested that the UCP3 promoter variant might modify trans-acting factor binding. These findings need to be verified with further studies to examine whether this polymorphism actually disrupts *cis*-elements and thus influences transcription rates of the gene using gene reporter studies in skeletal muscle cell lines.

The effect of the 45bp insertion/deletion in the 3'UTR of UCP2 also requires functional investigation. Evidence is accumulating that variation in the 3'UTR sequences of genes can also have major effects beyond influencing mRNA stability. This can take the form of either disruption of 3'UTR *cis*-acting elements and cleavage sites or by determining the length of

polyadenylated tails [Ross 1995]. Simple mutations in the 3'UTR can cause enhanced mRNA 3'end formation leading to increased cleavage site recognition, enhanced 3' end processing and elevated mRNA accumulation and protein synthesis. A single base mutation in the 3'UTR of the prothrombin gene (coagulation factor II gene) causes a deleterious 'gain-of-function' and is thought to be central in the pathophysiology of the genetic disorder hereditary thromobphilia [Gehring et al 2001]. Furthermore, myotonic dystrophy (DM) is caused by a trinucleotide repeat expansion in the 3'UTR of the DM protein kinase gene, with the increased nuclear accumulation of the expanded allele thought to contribute significantly to the pathogenesis of the disease [Savkur et al 2001].

In addition two novel polymorphisms identified in the promoter of the UCP2 gene and in an intronic region of UCP3 (GAIVS6) have been both positively associated with obesity and intermediate traits leading to obesity in White Europeans [Esterbauer et al 2000; Lanouette et al 2001] Clearly, both these novel variants deserve further investigation in other ethnic populations such as the South Indians.

8.4.2 CAPN10 gene

The association of the CAPN10 'at risk' haplotype combination in South Indians will be further confirmed by investigations in other available population groups. The positive associations with T2DM observed with the CAPN10 gene variants identified in this thesis and other studies could quite conceivably be the result of linkage disequilibrium with causative mutations in a gene nearby [Horikawa et al 2000; Schwarz et al 2001]. In the seminal study the SNP UCSNP22 also had significant 'evidence of linkage' and localized inter-genetically 3' to CAPN10 and 5' to a G-protein receptor 35 gene (GPR35) identified in close proximity to the CAPN10 gene [Horikawa et al 2000]. Analysis of the degree of linkage disequilibrium to determine the point of decay and pairwise analysis of all available polymorphisms across this whole region in different ethnic groups should indicate whether CAPN10 is the true susceptibility gene. Furthermore, determination of the ethnic variation in LD relationships in this chromosomal region may clarify some of the observed differences in association studies.

There is also accumulating evidence supporting potential mechanisms near the chromosomal telomeric regions that alter the expression of genes in sub-telomeric regions. These effects appear to be the result of a reduction of telomere length through ageing, and could have a

physiological impact in humans as it does in yeast [Baur et al 2001]. CAPN10 gene localizes to the sub-telomeric region of chromosome 2q and investigations in this respect might therefore be warranted.

Functional studies are still lacking for CAPN10 and presently studies on whether a dysfunctional protein could affect insulin production and secretion in rat pancreatic β-cells are currently under way in our research group.

8.4.3 Future Directions

As previously discussed linkage analysis has a limited potential for mapping complex non-mendelian diseases, it will only succeed if an aetiological variant contributes a moderate to large effects to disease predisposition [Risch & Merikangas 1996; Collins et al 1997]. Risch succinctly summarises the problem " the low hanging fruit were easy to harvest, but we are now left with a great deal of fruit at top of tree with no obvious way to reach it" [Risch 2000]. Genome scan strategies utilising large scale SNP genotyping and LD for mapping genes of complex diseases have been proposed using a number of different approaches [Roses 2000; Risch & Merikangas 1996; Collins et al 1997]. The number of SNP's required for entire genome searches would be dependent on the extent of LD in the human genome. In humans LD may extend 5kb-100kb [Krugliyak 1999; Collins 1999, Abecasis et al 2001] with varying irregularly across the whole genome [Abecasis et al 2001]. Some genetic statisticians contend that it is as little as 3kb [Kugliyak & McAllister 1998] and 500,000 SNPs would be necessary to achieve adequate power for mapping even in isolated populations. Contrarily others have predicted LD extending 60kb [Ott 2000; Lee 2001].

Potentially genome scans would still require hundreds of thousands of SNP's and study populations numbered in the thousands to obtain adequate power to detect association in complex diseases [Fulker et al 1999; Abecasis et al 2000; Sham et al 2000]. Therefore, Risch [2000] argues in favour of a strategy involving selection of SNP's that are more likely to be causative or located in close proximity to causative mutations, ie coding mutations, and non-coding 3'UTR, 5'UTR variants. It is estimated there are at least 60,000 non-conservative coding mutations in the human genome in the entire world population, of which a substantial fraction are already known. This approach could however potentially miss mutations that have other effects, such as those that cause alteration in the gene environment in relation to the more tightly wound heterochromatic structure and less

condensed euchromatic states ie Position Effect Variegation (PEV) [Kleinjan & Van Heyningen 1998].

The selection of a study population group would also be important with founder populations that have remained small over demographic history requiring fewer markers and would be more adept to detect rare disease alleles with a greater LD per base pair [Wright et al 1999; Laan & Paabo 1997; Lonjou et al 1999]. However they would not necessarily have a greater advantage when common alleles are related to disease [Wright et al 1999]. There could also be problems in regions of chromosomes where there is little or no LD, unless the causative mutation is selected in the analysis purely by chance, in contrast to linkage analysis, evidence of no association would not allow exclusion of the region for the absence of a disease gene [Nickerson et al 1998].

The recent publication of the first comprehensive analysis of human genome with 93% coverage and with 50% in uninterrupted sequence contigs [Nature Feb 2001]] revised the projected total number of human genes from 100,000 to only 32, 000 [Lee 2001]. The ramifications of this projection and the added fact that at least 40% of human genes have alternate splice functional isoforms, suggests that there are other orders of genetic complexity to accommodate all necessary proteome function [Collins 2000]. In other species this appears to be the case with the genome of Drosophila melanogaster having less genes than a simpler organisms such as eg. Caenorhabditis elegans, but this is compensated by having an expanded proteome diversity with alternate splicing generating many transcripts from a single gene [Graveley et al 2001].

In humans alternate splice sites are primarily in protein coding regions and are particularly common in genes for membrane receptors, and genes specific for immune and nervous systems [Dredge et al 2001]. The vascular endothelial growth factor (VEGF) that induces microvascular permeability [Robinson & Stringer 2001], the integrin family of transmembrane proteins [de Melker & Sonnenberg 1999] and a number of cytokines [Atamas et al 1997] all have a number of distinct splice isoforms that differ not only in their expression patterns but also in their biochemical and biological properties. CAPN10 has at least 10 splice variant isoforms that are differentially expressed. In skeletal muscle two splice isoforms predominate, and variation in their levels could be related to the polymorphic variants associated with increased risk to T2DM, particularly as some isoforms might be biologically inactive [Yang et al 2001; Ma et al 2001]. The UCP3 gene also

produces two transcripts with one lacking an important transmembrane domain yet still appears to be functional in terms of proton conductance. Whether any of these splice isoforms have similar or completely different biological functions still remains to be seen. However, even with the complete sequence of the human genome eventually being available, it is by no means the endgame. Downstream studies will still be necessary for all identified transcribed genes to establish tissue specific expression and function. Finally, the roles of genes/proteins in physiological pathways will need to be elucidated to permit genetic variation to be connected with phenotype variation [Nadeau & Dunn 1998].

ACKNOWLEDGEMENTS:

All the research work and analysis was carried out by myself within the laboratories of the Department of Diabetes and Metabolic Medicine (formerly the Medical Unit), Barts and The London Queen Mary's School of Medicine and Dentistry, University of London, London, UK, unless otherwise stated elsewhere below. The supervision of studies throughout the period of this thesis was provided by Professor Graham Hitman, with whom all studies were planned.

The work in this thesis relies on subject samples from several resources. Firstly the South Indian samples that were all originally recruited, collected and clinically characterized at the Diabetes Research Centre, Chennai under the direction of Professor Viswanathan and his colleagues, Dr Mohan, Dr Snehalatha, and Dr Ramachandran. Additional genotype analysis for ex-paternity in these families were gratefully carried out by the Department of Haematology, (The Royal London Hospital), with expertise provided by Dr Denise Syndecombe-Court and Chris Phillips.

The Caucasian Obese/lean samples were collected over many years by a number of colleagues at the Royal London hospital obesity clinic, and thanks are due to the following; Gill Hood, Beverly Mclaughlin, Dr Lorraine Albon, Dr Asjid Qureshi, Dr Salim Janmohamed Dr Philip Beales, Dr Yolanda Weaver, and Professor Peter Kopelman. Additional credit must go to both Gill Hood and Lorraine Albon for an expanded metabolic characterization in the more recent samples. Radio-immune assays for leptin were performed in the Department of Clinical Biochemistry by Dr Kate Noonan and colleagues under the direction of Professor Jackie Burrin (Barts and The London Queen Mary's School of Medicine and Dentistry).

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APPENDICES:

APPENDIX I: BUFFERS AND REAGENTS

Nucleon DNA extraction kit reagents:

Reagent A: (10 mM Tris-HCl, 320 mM sucrose, 5 mM $MgCl_2$, 1% Triton X-100; adjust to pH 8.0 with 2M NaOH.

Reagent B: 400 mM Tris-HCl, adjust to pH to 8.0 with 2 MNaOH, 60 mM EDTA, 150 mM NaCl, 1% SDS).

Sequencing loading Buffer: 5:1 mix of Deionised Formamide: 25mm EDTA pH8.0 with 50mg/ml Blue Dextran.

TEN buffer: 10 mMTris-HCL; 1mM EDTA; 0.1 M NaCl ; pH 8.0

EMSA Reagents:

Binding buffer 5x concentrate: 100mM Hepes, pH7.5, 5mM EDTA, %0mM $(NH_4)_2 SO_4$, 5mM DTT, 1%(w/v) Tween 20, 150mMKCl.

Gel loading Buffer: 1ml volume 60%-0.25xTBE, 40%glycerol, ± 0.2% Bromophenol Blue.

EMSA 10cm x 10cm non-denaturing polyacrylamide gel: 4.1%, 40:1 (acrylamide: bis-acrylamide) in 0.5x TBE.

20ml gel mix

2.0ml	40% Acrylamide
1.0 ml	2% BisAcrylamide
2.0 ml	5x TBE
0.625 ml	80% glycerol
0.010 ml	TEMED
0.150 ml	10% APS

5.785

14.3 ml H20

Chemiluminescent Detection Reagents:

10x SSC : 1.5M NaCl; 0.15M Sodium citrate; pH7.0.

Buffer 1: 0.1M Maleic acid; 0.15M NaCl, pH7.5 adjusted with NaOH, autoclaved

Blocking Solution: Blocking reagent (Roche), 10%w/v in Buffer1 and autoclaved.

Washing Buffer: Buffer 1 + 0.3%v/v Tween 20.

Buffer 2: Blocking solution, 1: 10 diluted in Buffer 1

Buffer 3: 0.1M Tris-HCL; 0.1M NaCl, pH 9.5.

CPSD Substrate Solution: CPSD stock solution (10mg/ml) diluted 1:100 in Buffer 3.

Restriction Enzyme Buffers:

Fok I 10x buffer (New England Biolabs):

200mM Tris-acetate,

100mM magnesium acetate

500mM potassium acetate

10mM Dithiothreitol (pH 7.9)

TthIII1 10x buffer (New England Biolabs):

100mM Bis Tris Propane-HCl

100mM $MgCl_2$

10mM DTT (pH 7.0)

Hae III 10x buffer (Amersham Pharmacia):

100mM Tris –HCl (pH 7.5)

100mM $MgCl_2$

10mM DTT

500mM NaCl

Hha I 10x buffer (Promega UK Ltd):

100mM Tris-HCL (pH 7.9)

500mM NaCl

100mM Dithiothreitol (DTT).

APPENDIX II: QUANTIFICATION OF DNA

Absorbance was read at 260 and 280 nm using a 1in 30 dilution of DNA (10µl in 290µl). The OD_{260} of 1.0 gives an indication of the quantity, corresponding to a concentration of 50ug/ml of double stranded DNA, consequently values were multiplied by 1.5 to get DNA concentration in µg/µl. Quality can be determined by the ratio of the OD at 260nm and 280nm (OD_{260}/OD_{280}). A ratio of 1.8 or greater indicates a pure preparation of DNA, a ratio significantly less than 1.8 indicates contamination with protein.

APPENDIX III: PUBLICATIONS ARISING:

(i) Peer reviewed publications

Cassell.P.G, Jackson A.E, North.B.V, Evans J.C, Syndecombe-Court.D, Phillips.C, Ramachandran A, Snehalatha C, Hattersley A.T, and Hitman.G.A. Haplotype combinations of Calpain 10 gene polymorphisms associate with increased risk to impaired glucose tolerance and Type 2 Diabetes mellitus in South Indians. *Diabetes* 2002 May; 51(5):1622-8.

Evans JC*, Frayling TM*, **Cassell PG***, Saker PJ*, Hitman GA, Walker M, Levy JC, O'Rahilly S, Rao PV, Bennett AJ, Jones EC, Menzel S, Prestwich P, Simecek N, Wishart M, Dhillon R, Fletcher C, Millward A, Demaine A, Wilkin T, Horikawa Y, Cox NJ, Bell GI, Ellard S, McCarthy MI, Hattersley AT. Studies of association between the gene for calpain-10 and type 2 diabetes mellitus in the United Kingdom. ***Am J Hum Genet*** 2001 Sep; 69(3):544-52 (***cited as contributed equally)**

P.G.Cassell, P.J.Saker, S.J.Huxtable, E.Kousta, A.E.Jackson, A.T.Hattersley, T.M.Frayling, M.Walker, P.G.Kopelman, A.Ramachandran, C.Snehelatha, G.A.Hitman and M.I.McCarthy. Evidence that single nucleotide polymorphism in the uncoupling protein 3 (*UCP3*) gene influences fat distribution in women of European and Asian origin. ***Diabetologia*** (2000) 43: 1558-1564

P.Cassell, M. Neverova, S. Janmohamed, N. Uwakwe, A. Qureshi, M.I.McCarthy, P.J.Saker L. Albon, P. Kopelman, K. Noonan, J. Easlick, A. Ramachandran, C. Snehalatha, C. Pecqueur, D. Ricquier, C. Warden and G.A. Hitman. An uncoupling protein 2 gene variant is associated with a raised body mass index but not Type II diabetes. ***Diabetologia*** (1999) 42: 688-692

(ii) Other Published Articles

Cassell PG and Hitman GA

Chapter titled Genetics of Type 2 Diabetes in book *Diabetes: Perspectives and Advances in Diagnosis and Treatment.* Edited by E.Shafrir and I.Raz. Pub. Martin Dunitz 2002.

(iii) Published Abstracts:

P.G.Cassell, A.E.Jackson, J. Evans, A Ramachandran, C Snehalatha, G.Hitman. A Haplotype Combination of the Calpain10 Gene Polymorphisms is a Determinant of Abnormal Glucose Tolerance in South Indians. ***Diabetes*** June 2001, Volume 50, Suppl2, 115-OR

P.G.Cassell, A.E.Jackson, J.C.Evans, A. Ramachandran, C. Snehalatha, and G.A.Hitman. Calpain 10 gene polymorphism associates with Impaired Glucose Tolerance /Type2 Diabetes in a South Indian population. ***Diabetic Med***, April 2001 Vol 18, Suppl 2;P53

AE Jackson, **P.Cassell,** A. Ramachandran, C.Snehalatha, and G.A.Hitman.
Polymorphic Variation of Calpain 10 (NIDDM 1) and its relationship to type 2 diabetes and intermediate traits in a South Indian population. ***Diab.Res.Clin.Prac,*** Vol 50, Suppl 1.P695

P.Cassell, N.C.Uwakwe, P.G.Kopelman, A. Ramachandran, C.Snehalatha, and G.A.Hitman. An association between a novel variant of the UCP3 gene and fat distribution in South Indian females. ***Diabetic Med***, Vol 17, Suppl 1; P87

P.G.Cassell, N.C.Uwakwe, P.G.Kopelman, A. Ramachandran, C.Snehalatha, and G.A.Hitman. A novel UCP3 regulatory region polymorphism is associated with body fat distribution in a South Indian Population. ***Diabetologia*** 1999,42: suppl 1 A117: 431

P.G.Cassell, M.Neverova, S .Janmohammed, N.C.Uwakwe, A.Qureshi, P.G.Kopelman, A. Ramachandran, C.Snehalatha, D.Riquier, C.H.Warden, and G.A.Hitman. An association between UCP2 and body mass index in a south indian population. ***Diabetologia*** 1998 41:430,suppl 1

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APPENDIX 1
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