Impaired skeletal muscle fat oxidation as a mechanism for insulin resistance in South Asians

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Abstract

The impending global pandemic of obesity, type 2 diabetes and vascular disease suggests an urgent need for both prevention strategies and effective treatment. Of all the common ethnic groups South Asians, who make up a fifth of the world's population, have the highest prevalence of both diabetes and vascular disease. The high rates of diabetes, in particular, occur with lower average adiposity levels, suggesting that South Asians are more susceptible to the effects of obesity. Differences in insulin sensitivity and diabetes prevalence between South Asians and Europeans cannot be fully explained by differences in adiposity alone. The aim of this thesis was to investigate whether differences in oxidative capacity and capacity for fatty acid utilisation in South Asians might contribute, using a range of whole-body and skeletal muscle measures.

Twenty South Asian men and 20 age and BMI-matched white European men underwent exercise and metabolic testing and muscle biopsy to determine expression of oxidative and lipid metabolism genes and of insulin signalling proteins. In fully adjusted analyses, South Asians, compared to Europeans, exhibited significantly reduced insulin sensitivity; lower VO_{2max} and reduced fat oxidation during submaximal exercise at the same exercise intensities. South Asians exhibited significantly higher skeletal muscle gene expression of CPT1A and FASN and significantly lower skeletal muscle protein expression of PI3K and PKB Ser473 phosphorylation. Fat oxidation during submaximal exercise and VO_{2max} both correlated significantly with insulin sensitivity index and PKB Ser473 phosphorylation, with VO_{2max} or fat oxidation during exercise explaining 10–13% of

the variance in insulin sensitivity index, independent of age, adiposity and physical activity.

These data suggest that reduced oxidative capacity and capacity for fatty acid utilisation at the whole body level are key features of the insulin resistant phenotype observed in South Asians, but that this is not the consequence of reduced skeletal muscle expression of oxidative and lipid metabolism genes.

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Author declaration

Other than the published papers referenced or acknowledged in this thesis, the work contained herein is that of the author.

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List of Abbreviations

CHD	Coronary heart disease
VO_{2max}	Maximal oxygen uptake
BMI	Body mass index
NEFA	Non-esterified fatty acid
BP	Blood pressure
HDL	High density lipoprotein
LDL	Low density lipoprotein
VLDL	Very-low density lipoprotein
IMTG	Intramyocellular triglyceride
LCACoA	Long chain acyl CoA
DAG	Diacylglycerol
BMI	Body mass index
HOMA	Homeostatic model assessment
PCOS	Polycystic ovarian syndrome
CRP	C – reactive protein
ATP	Adenosine triphosphate
MODY	Maturity onset diabetes of the young
LADA	Latent autoimmune diabetes of adulthood
VAT	visceral adipose tissue
RMR	Resting metabolic rate
RER	Respiratory exchange ratio
CHO	Carbohydrate
SFA	Saturated fatty acids

MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
RPE	Rate of perceived exertion
IFG	Impaired fasting glycaemia
IGT	Impaired glucose tolerance
PPAR	Peroxisome proliferator-activated receptor
NAFLD	Non-alcoholic fatty liver disease
MRI	Magnetic resonance imaging

Chapter 1

Introduction and Literature Review

1.1 Epidemiology of cardiovascular disease and associated risk factors

Cardiovascular disease (CVD), encompassing coronary heart disease (CHD) and cerebrovascular disease, is the leading cause of death in the UK, accounting for around a third of all deaths (Capewell et al, 2010). Associated with this is the rising prevalence of obesity and type 2 diabetes, with the majority of the UK population now being overweight or obese, and an estimated diabetes prevalence of 4.3%, equating to 2.8 million people (diabetes.org.uk).

1.2 Ethnic differences in cardiovascular risk.

It is well recognised that risk of morbidity and mortality from CVD and type 2 diabetes is influenced by ethnicity. In particular, groups of West African (such as African-Americans and Afro-Caribbeans), Hispanic and South Asian descent are at increased risk compared to populations of white European descent, but while these populations experience an increased risk of vascular and metabolic disease, the prevalence of specific risk factors for these conditions varies considerably between the groups.

Afro-Caribbeans in the UK have an increased prevalence of diabetes, hypertension and stroke compared to their white European counterparts, but a more favourable lipid profile with higher HDL cholesterol and lower triglycerides (TG), and lower mortality from coronary heart disease (McKeigue et al. 1991). Classical cardiovascular risk factor prevalence in the African-American population is similar to UK Afro-

Caribbeans (and they have a similar paradoxically healthy lipid profile) but they experience higher rates of CHD than white North Americans, possibly due to an increased prevalence of obesity (Ogden et al. 2006; US Bureau of the Census 2000). North American native Indians have a higher prevalence of diabetes, hypertension and obesity than white North Americans. They have less healthy lipid profiles, with lower HDL cholesterol and higher triglyceride, and almost double the incidence of cardiovascular disease (Anand et al. 2001; Howard et al.1999).

1.3 Cardiovascular risk in South Asian populations

South Asians (comprising people from India, Pakistan, Sri Lanka and Bangladesh) constitute 20% of the world's population and 4% of the UK population (Office for National Statistics 2003). Of the common ethnic groups, they have the highest risk of cardiovascular disease and diabetes (Lee et al. 2001; McKeigue et al.1993), so provide an ideal model to study the interactions between ethnicity and metabolic and vascular disease risk.

South Asians were one of the first ethnic groups recognized to have higher risk for cardiovascular disease. Over 50 years ago, migrant Asian Indian males in Singapore were reported to have seven times the prevalence of cardiovascular disease of Chinese men, in a large series of autopsies (Danaraj et al. 1959). The increased risk of myocardial infarction and cardiovascular death in South Asians has since been confirmed by a number of reports indicating that migrant South Asian populations in the UK, Singapore, Fiji, South Africa, Trinidad and North America have greater risk of cardiovascular disease than other populations in these countries (Enas et al. 1996; Grundy et al. 2005; McKeigue et al 1993; McKeigue et al. 1991; Wild & McKeigue 1997). More recently, the Interheart study, a global case control study (encompassing

52 countries) found that myocardial infarction occurs on average 10 years earlier in South Asians than in white populations (Yusuf et al. 2004) and South Asians have also been found to require treatment of heart failure at a significantly younger age than European Whites (Singh & Bhattarai 2003).

South Asian men and women in the UK experience approximately 50% higher age-standardised CHD mortality than European whites (Wild & McKeigue 1997) and the CHD mortality in young South Asian men (aged less than 40) is over double that of Europeans (Balarajan et al. 1984; McKeigue et al. 1993) Furthermore, a narrower sex difference in CHD risk between men and women is observed in South Asians, such that South Asian women may have a particularly elevated CHD risk: South Asian women in the UK experience proportionally greater risk than South Asian men, such that their CHD mortality rate is 46% greater than that of women in the general UK population, compared to 36% increased CHD mortality in South Asian men compared with men in the overall UK population (Balarajan et al. 1984; McKeigue et al. 1992). This finding is mirrored by the observed narrower sex-difference in HDL-cholesterol in South Asians in the United States (Chandalia et al. 2008).

1.4 Excess insulin resistance and diabetes in South Asians

The increased CHD mortality in the migrant South Asian population is not entirely explained by classical cardiovascular risk factors, such as cholesterol, blood pressure and smoking (Forouhi et al. 2006; McKeigue et al 1989; Miller et al. 1988; Miller et al.1989). In fact South Asians living in the UK have, on average, a similar blood pressure to matched white Europeans (Agyemang et al. 2007) and a lower rate of cigarette smoking, although there are subgroup differences such as Bangladeshis

tending to have high rates of smoking. However, there is some evidence that increased insulin resistance, and its consequent effects, play a central role in the increased CHD risk seen in South Asians. The Southall study (McKeigue et al. 1991) was the first large cross-sectional study to compare metabolic variables and anthropometric data from Indian and Pakistani subjects with that from age and BMI-matched white European subjects. This confirmed that Asians have a higher prevalence of diabetes (19% versus 4%), and allied to this they also had hyperinsulinaemia (both fasting and following an oral glucose load), central obesity (as measured by waist-hip ratio), hypertension and a dyslipidaemia characterised by higher triglyceride and lower HDL-cholesterol concentrations, but interestingly, not higher total cholesterol concentrations (although migrant South Asians have been found to have significantly higher total cholesterol concentrations than those of South Asians living in India (Bhatnagar et al. 1995)).

The Southall study also found comparable LDL-cholesterol levels in South Asians and white Europeans, but South Asians have subsequently been found to have more small LDL particles, which are more atherogenic than large LDL particles (Kulkarni et al. 1999). Even more recently South Asians have also been found to have more small HDL particles (Bhalodkar et al. 2004), which may also be related to increased CHD risk, as opposed to large HDL particles, which are thought to be protective.

Additional studies have shown that even after adjustment for BMI, waist-hip-ratio, and skin fold thickness, insulin concentrations (both fasting and post glucose-load) remain significantly higher in South Asians (Davey G.J.G et al. 2000; Forouhi et al. 1999), and further studies comparing South Asians with European whites matched for

total body fat (measured by hydrodensitometry) revealed increased insulin resistance in the South Asians (Chandalia et al. 1999)**.** Thus greater insulin resistance is not simply due to excess adiposity. Since the publication of the Southall study findings, there have been many further reports verifying its findings of greatly increased prevalence of type 2 diabetes in migrant South Asian populations (Chandalia et al. 1999; Hughes et al. 1989; Knight et al. 1992; Laws et al. 1994; McKeigue et al 1991) and this is likely to be a key factor mediating their increased cardiovascular disease risk.

The mean age of diabetes onset is over 10 years younger in both migrant South Asians and in those living in India compared to white Europeans (Mukhopadhyay et al. 2006; Ramachandran et al. 2002) and South Asians develop diabetes and metabolic disturbances associated with insulin resistance at lower BMIs than Europeans. Data from the SHARE study (Razak et al. 2007) (Study of Health Assessment and Risk in Ethnic groups) indicates that South Asians living in Canada with a BMI of only 21 $kg.m^{-2}$ experience an equivalent risk of diabetes to white Canadians with a BMI of 30 $kg.m^{-2.}$ (i.e. a 25.5 kg difference in body mass for somebody 170 cm tall). Thus South Asians are likely to experience exponential increases in diabetes risk with increasing adiposity at much lower BMI values than those from white European backgrounds. This concept is illustrated in Figure 1.1.

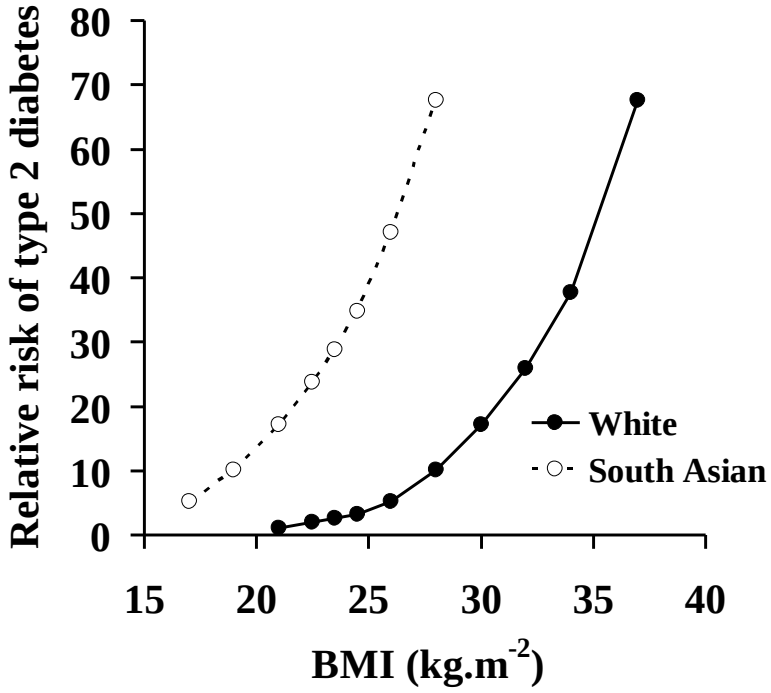

Figure 1.1 BMI and diabetes risk in South Asian and white populations.

Diabetes risk for South Asian populations with increasing BMI is shifted to the left by 9kg.m^{-2}. Data for white populations are based on mean values from (Colditz et al. 1995; Venkataraman et al. 2004). Data for South Asians are based on findings of the SHARE study (Razak, Anand, Shannon, Vuksan, Davis, Jacobs, Teo, McQueen, & Yusuf 2007).

In addition, South Asians with type 2 diabetes also experience increased incidence of microvascular complications, such as diabetic retinopathy and nephropathy, even after adjustment for glycaemic control (Burden et al. 1992; Mather et al 1998). The reasons for this are, as yet, unclear but may include higher triglyceride levels, which have been linked to greater microvascular complications.

1.5 When do metabolic and vascular differences manifest?

In some (Yajnik et al. 2002; Yajnik et al. 2003) but not all (Simmons 1994) reports, lower birth weights, increased adiposity, hyperinsulinaemia and high triglycerides have been observed in South Asian neonates compared with European babies. These findings, if corroborated in larger studies, suggest that the insulin resistant phenotype with high body fat observed in South Asians is evident form a very young age and, thus, at least in part, reflects some combination of genetic and/ or "early origins" (thrifty phenotype) effect. There is conflicting evidence regarding the generational effect on birth weight. One small study found that babies born to second generation migrant Indians living in the UK were larger than those born to first generation Asian women (Dhawan 1995), but two subsequent larger studies have shown that low birth weight persists in the second generation (Draper et al. 1995; Margetts et al. 2002). The extent to which generational birth weight differences might influence diabetes and CHD risk in early *versus* later generation South Asian migrants is not yet known. Such data might help to disentangle the relative importance of thrifty phenotype effects in mediating South Asians' increased cardiometabolic risk.

In a cross-sectional comparison of children aged 8-11 years living in the UK, it was reported that those of South Asian origin had higher fasting and post-glucose insulin concentrations than children of European origin (despite similar waist and hip circumferences and lower ponderal indices) and a steeper rate of increase in insulin concentrations with increasing adiposity (Whincup et al. 2002). Similarly, 13-16 year-old British South Asian children had higher fasting glucose and insulin concentrations and HOMA-estimated insulin resistance than European boys, even after adjustment for differences in adiposity (Whincup et al. 2005). In another report

based on data from the 1999 Health Survey for England, Indian and Pakistani boys had an increased risk of being overweight and Pakistani girls had an increased risk of being obese compared to the general population (Saxena et al. 2004). Postpubertal Asian Indian children living in urban India have also been found to have a high prevalence of insulin resistance, associated with increased central adiposity (Misra et al. 2004).

Thus, the combined effect of greater insulin resistance for a given adiposity level in South Asians allied to greater levels of obesity in this ethnic group, which is increasing at a rapid rate in children (Reilly & Dorosty 1999), suggests this group is in greater need of interventions to reduce risk of metabolic diseases. Such interventions may also need to start before adulthood to maximise potential efficacy. Indeed, there is emerging evidence of cases of type 2 diabetes in obese South Asian (more so than European) children living in the UK (Drake et al. 2002; Ehtisham et al. 2000), mirroring the observations in the US in certain populations at increased risk of type 2 diabetes, such as Hispanic Americans and Pima Indians, where the prevalence of type 2 diabetes in the 10-14 age group is estimated to be 22.3 per 1,000 (Fagot-Campagna et al. 2000).

In early adulthood clinical manifestations of insulin resistance, for example polycystic ovarian syndrome (PCOS) in women, begin to present. South Asian women with PCOS present at a younger age than European white women, and with more severe clinical signs of insulin resistance, such as hirsutism, acne, acanthosis nigricans and infertility (Wijeyaratne et al. 2002).

At this early stage in life, South Asians also exhibit various signs of vascular endothelial dysfunction, and increased arterial stiffness, which is a predictor of cardiovascular events. Several small studies have demonstrated ethnic differences in endothelial function, including a study which reported reduced flow-mediated brachial artery vasodilation (Chambers et al. 1999) in young South Asians aged in their 20s to early 30s compared to matched Europeans, a study which found increased augmentation index , which is a non-invasive measure of arterial stiffness, in young South Asians (Din et al. 2006) and a further study which demonstrated reduced maximum vasodilatory response to the beta-2 agonist isoproterenol (Kapoor et al. 1996).

More recently, a larger study comparing non-diabetic US South Asians and Whites, with mean age 35, showed impaired vasodilatory response to sublingual nitroglycerin and following brachial artery occlusion during hyperinsulinaemic euglycaemic clamp in the South Asian group; a finding which correlated with degree of insulin resistance. Further evidence of endothelial dysfunction was demonstrated by another study which found a reduction in the number and impaired function of circulating endothelial progenitor cells (which repair damaged endothelium) in non-diabetic UK South Asians compared to Whites, with a mean age of 26 (Murphy et al. 2007).

These signs of endothelial dysfunction are accompanied by higher inflammatory (e.g. CRP, homocysteine) and thrombotic (lipoprotein a, PAI-1, fibrinogen) markers, and lower adiponectin levels (Anand et al. 2000; Forouhi et al. 2001; Valsamakis et al 2003), all factors associated with higher risk of type 2 diabetes in South Asians.

However, whether any of these parameters/ pathways is causally linked to excess diabetes risk remains uncertain.

1.6 Potential mechanisms for increased insulin resistance in South Asians

1.6.1 Genetic factors

The mechanisms underlying differences in the metabolism of South Asians leading to increased insulin resistance and risk of diabetes and CHD have not been fully elucidated. It is possible that in evolutionary terms, a survival advantage was conferred to populations most at risk of famine and/or infectious diseases, by the ability to store visceral fat when food sources were scarce and sporadic, as postulated by Neel's "thrifty genotype" hypothesis (Neel 1962). Of the three fat compartments visceral fat is most easily mobilized so could have acted as a rapid supply of energy for the immune system when required. The pro-inflammatory cytokines produced by adipose tissue may have been beneficial in the context of, for example, chronic infection with parasites or other pathogens, and could have increased the likelihood of survival throughout childhood in the context of the threat of severe infection. These so-called "thrifty genes" and the storing of visceral adipose tissue have now become disadvantageous to populations who live in a sedentary state with abundant food supplies.

In support of the thrifty genotype hypothesis, South Asians have been found to have a higher prevalence of some genetic defects of insulin signaling, compared to white Europeans. Several polymorphisms of the ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) gene, which increase insulin resistance (Abate et al. 2003; Bacci et al. 2005) are found more frequently in South Asians. The K121Q

polymorphism has been found to inhibit the insulin receptor in vitro, and is significantly associated with measures of insulin resistance (higher insulin AUC during oral glucose tolerance test, lower insulin sensitivity during hyperinsulinaemic euglycaemic clamp *in vivo* (Abate et al. 2003)).

In addition, certain polymorphisms of the peroxisome proliferator-activated receptor (PPAR)-γ gene are thought to confer protection against type 2 diabetes (Altshuler et al. 2000; Deeb et al. 1998; Hara et al. 2000), but a study examining the effect of this genetic variance in South Asians, showed a lack of this protective effect, which is evident in European white populations, despite similar prevalence of the polymorphism in the two groups (Radha et al. 2006). Certain variants of the transcription factor 7-like 2 gene (TCF7L2) have also been found to be major determinants of type 2 diabetes risk in South Asians and in European white populations, but it remains unclear whether there are differences in prevalence of these gene variants between ethnic groups (Humphries et al. 2006).

1.6.2 Maternal and intra-uterine factors

An alternative and complementary hypothesis is the "early origins" or "thrifty phenotype" hypothesis, which proposes that metabolic disturbances *in utero*, which often manifest in small birth size, can lead to predisposition to CHD, type 2 diabetes and other metabolic disorders in later life. This was based on the observation that low birth weight increases risk of impaired glucose tolerance, type 2 diabetes and CHD in adulthood (Hales et al. 1991). The most common form of the hypothesis postulates that poor maternal nutrition or impairments in maternal metabolism result in fetal undernutrition, leading to a programmed tendency towards nutritional thrift,

combined with impaired fetal pancreatic β cell growth, and when these factors are combined with accelerated growth and weight gain in childhood, result in disturbances to glucose-insulin metabolism, a tendency towards adverse patterns of fat storage (i.e. in visceral compartments) and increased risk of diabetes (Hales & Barker 1992), particularly when food supplies are abundant and physical activity levels are low (Laaksonen et al. 2003).

While both the thrifty genotype and thrifty phenotype hypotheses can help explain why moving from less to more obesogenic environments lead to increased risk of CHD and diabetes, it is not immediately clear why certain ethnic populations are more affected by insulin resistance and diabetes than others, as all populations have been subject to fairly similar changes in environment over the past few centuries. One possibility is that historically white European populations moved away from agriculture and became "urbanised" much earlier, so their genes adapted to this change in lifestyle, while South Asians and other ethnic populations maintained agricultural, rural lifestyles for longer, and have only recently begun to move towards urbanisation. There could also, conceivably be interactions between genes and climate or prevalence of environmental pathogens, which may have provided selection advantages for a thrifty genotype for longer in South Asia than in Europe.

It is possible that the "thrifty phenotype" particularly affects South Asians, as many have recently undergone a transition from 'traditional' to 'westernised' environments, such that first generation migrant South Asian mothers (or mothers who have moved from rural to urban environments) are likely to be thin and undernourished antenatally, but their children will have grown up in a "westernised" urban

environment, where they will have been more likely to experience accelerated growth during childhood and consequent increased adiposity and insulin resistance. A "thrifty phenotype" effect could conceivably then persist in the subsequent generation as maternal insulin resistance and obesity can also have "programming" effects on the fetus leading to increased risk of obesity and metabolic disease later in life (Hales et al. 1991), but the size of this effect may diminish as increasing numbers of generations are exposed to Westernised lifestyles. Data indicating lower birthweights in South Asian compared to European babies (Yajnik et al. 2002; Yajnik et al. 2003) and an increase in birthweights between the offspring of first and second generation Indian migrants to the UK (Dhawan 1995) would be consistent with a thrifty phenotype effect, but examination of birthweights and future metabolic disease risk in future generations of migrant South Asians is probably necessary before any final conclusions about the effect of the "thrifty phenotype" can be drawn.

1.6.3 Mitochondrial efficiency?

A further, recent hypothesis attempting to explain the tendency of South Asians to develop central obesity and its metabolic consequences, is the "mitochondrial efficiency hypothesis" (Bhopal & Rafnsson 2009). This theory postulates that genetic mutations of mitochondrial uncoupling protein genes (in response to climactic, nutritional and infectious triggers) have resulted in South Asians having a higher capacity to generate ATP, a chemical energy source, which allows energy within cells to be stored and released as required for metabolism. This is thought to be a consequence of the more efficient conversion, of dietary calories to ATP, rather than heat, by "tightly coupled" mitochondria (i.e. mitochondria which will generate the maximum amount of ATP and the minimum amount of heat per calorie), through

oxidative phosphorylation. This hypothesis was based, however on one small study by Nair *et al* (Nair et al. 2008), who reported that middle-aged, non-diabetic Asian Indian men had increased skeletal muscle expression of genes involved with oxidative phosphorylation and the citrate cycle and increased capacity for mitochondrial ATP production than age, gender, and BMI matched Northern European Americans, despite being more insulin resistant. The investigators concluded that mitochondrial dysfunction could not account for the South Asians' greater insulin resistance, and further, that their results suggested increased mitochondrial efficiency in South Asians.

This finding is somewhat controversial, and does not tell the whole story. In contrast to this data suggesting increased mitochondrial efficiency, existing evidence indicates that skeletal muscle oxidative capacity is characteristically reduced in insulin resistance and obesity (He et al. 2001; Kelley & Simoneau 1994; Simoneau et al. 1999). Evidence also suggests that there is no difference in basal metabolic rate (when adjusted for fat-free mass) between South Asians and Europeans (Soares et al. 1998). It is not known whether capacity for lipid oxidation is reduced in South Asians compared to Europeans, or whether these factors contribute to South Asians' increased insulin resistance, and as the study by Nair *et al* was small, further studies looking at ethnic differences in mitochondrial enzymes, uncoupling proteins and skeletal muscle lipid oxidation are required.

1.6.4 Epigenetics

In addition to these potential genetic and "early origins" effects, it is possible that epigenetic differences between South Asians and other populations could contribute to their increased metabolic disease risk. A "thrifty epigenotype" hypothesis has, in

fact, recently been proposed, which suggests that epigenetic changes rather than DNA sequence polymorphisms explain most of the heritability of obesity and type 2 diabetes (Stoger 2008). Epigenetic effects describe heritable changes in gene expression, and thus function, which are stable over rounds of cell division, but which do not involve changes in the underlying DNA sequence, so can occur over shorter periods of time than gene-mutations (Bird 2002). These changes can result from environmental stimuli and are, for example, seen in the effects of diet on chronic disease (Issa 2000). Epigenetic changes, perhaps resulting from a change in diet or climate, may have a role to play in the metabolic differences which have developed in South Asian populations over, what is in evolutionary terms, an apparently short time-frame. This is an area which has not yet been investigated in South Asians and clearly warrants further study.

1.6.5 Disorders of insulin secretion

It is also worth considering additional rare causes of diabetes in South Asians which may manifest as type 2 diabetes, but which, in fact, result from impaired insulin secretion. These potential causes of diabetes have not been described in South Asians. They include monogenic diabetes, particularly MODY3, caused by hepatic nuclear factor (HNF) 1α mutations, which usually results in onset of diabetes in adolescence or early twenties, and latent autoimmune diabetes (LADA), which presents sub-acutely without ketoacidosis. A recent study examined 96 young patients in Lucknow, India, who had been diagnosed with "type 2" diabetes before the age of 30 (Sahu et al. 2007). They found that these young patients were markedly heterogeneous, with only 60% being overweight or obese, but despite this a low frequency of defects resulting in impaired insulin secretion was observed. HNF 1α mutations were only discovered in 3% of a target sub-group tested and islet

autoimmunity (assessed by glutamic acid decarboxylase and insulinoma antigen 2 antibodies) was present in 3% of the total study population. This study suggests that such defects are likely to represent a very small proportion of all Asians who are diagnosed as having type 2 diabetes.

1.6.6 Migration and urbanisation

The observed differences in insulin sensitivity between South Asians and white neonates and children suggest a constitutional or innate degree of insulin resistance which is likely to have a genetic and/ or "early origins" effect. However it is also clear that environmental factors throughout life are important and postulated environmental causes, which may account for at least some of the increase in insulin resistance in South Asians, include urbanisation, diet and, perhaps in particular, lower levels of physical activity in this population. It seems likely that there is a gene-environment interaction, as South Asians seem to experience greater adverse consequences as a result of living in the same environment as white Europeans, for example, greater increases in insulin resistance with increasing obesity (Razak et al. 2007; Whincup et al. 2002; Whincup et al. 2005).

The prevalence of type 2 diabetes in urban India is around five times as high as that in rural areas (Ramachandran et al. 1992; Ramachandran et al. 1997) and this observation has been mirrored in Pakistan (Ramachandran et al. 2001; Shera et al. 1999; Shera et al. 2007), Bangladesh (Abu et al. 1997) and Nepal (Singh & Bhattarai 2003). This could be related to the change from a traditional subsistence lifestyle involving physically active occupations with heavy manual labour in rural areas to sedentary jobs in the city, combined with increased income, more readily available

food with high energy and fat content and better public transport (Ramachandran et al. 1992; Ramachandran et al. 1999; Ramaiya et al. 1990). Indeed, mean BMI in urban India is 22.3 $kg.m^{-2}$ (Ramachandran et al 1997), compared to 19.6 $kg.m^{-2}$ in rural India (Lubree et al. 2002; Ramachandran et al. 1992; Reddy 1999), a difference of 8 kg in a person 170 cm tall, suggesting that decreased physical activity and/ or increased energy intake leading to increased adiposity is likely to be a key driver of the increase in type 2 diabetes associated with urbanisation in South Asia. It is also known that when South Asians migrate further, to developed countries (i.e. Northern Europe, the United States and Australia/ New Zealand), they experience increased rates of diabetes compared to not only the white population, but also compared with their siblings and original communities in South Asia (Bhatnagar et al. 1995; Simmons et al. 1989) (see Figure 1.2). Thus, urbanisation appears to have a disproportionately large adverse effect on diabetes risk in those of South Asian origin.

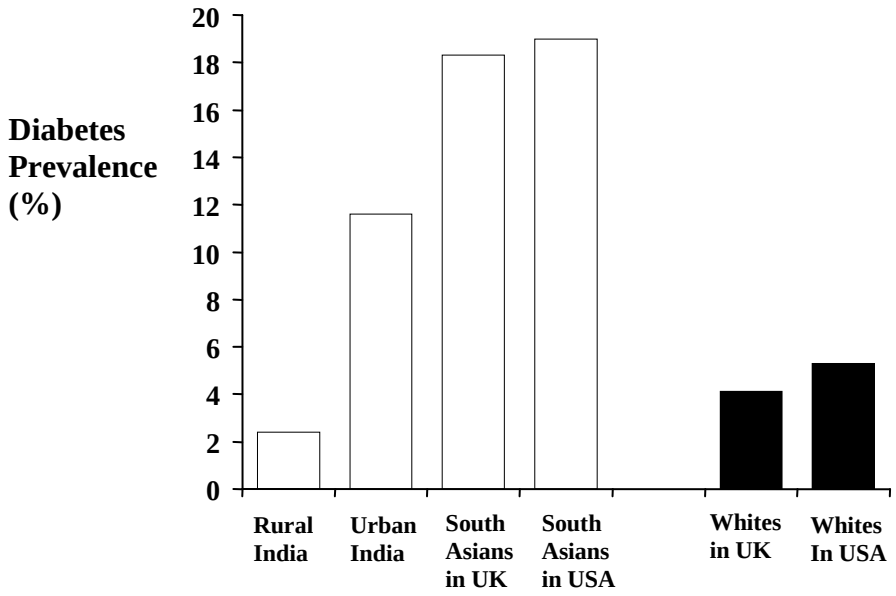

Figure 1.2 Diabetes prevalence in South Asian and white populations by country and area of residence. Data for rural and urban India from (Razak, Anand, Shannon, Vuksan, Davis, Jacobs, Teo, McQueen, & Yusuf 2007); data for South Asians and whites in the UK from the Southall study (McKeigue, Shah, & Marmot 1991); data for South Asians in the USA from (Venkataraman, Nanda, Baweja, Parikh, & Bhatia 2004) and data for whites in the USA from (Harris et al. 1998).

1.6.7 Diet

Globally, the consumption of dietary fat and sugar has increased dramatically over the last 20 years (Drewnowski & Popkin 1997; Popkin 2001) and studies looking at dietary intake in urban Asian Indians have found low intake of monounsaturated and polyunsaturated fatty acids (PUFA) and fibre, but high intake of saturated fats, carbohydrate and trans-fatty acids (largely related to the use of a hydrogenated oil called vanaspati, which is used to make ghee) (Misra et al. 2009). Other reports show that over time, the dietary intake of migrant South Asians living in the United States changes to closely resemble that of the white American population (Raj et al. 1999),

but as their diets are no less healthy than those of white Americans, this in itself cannot explain why they are more insulin resistant than the background population. Diets of South Asians in the UK have been found to be generally healthier than that of the UK population as a whole, with lower total energy intakes, higher intakes of complex carbohydrate, vegetable fibre and PUFA (McKeigue et al. 1985; Miller et al. 1988; Sevak et al. 1994). Although UK South Asians' diets have been found to be low in *n*-3 PUFA, and to have a low *n*-3 to *n*-6 PUFA ratio (Lovegrove et al. 2004), an intervention trial examining the effect of supplementation of *n*-3 PUFA showed no improvement in insulin sensitivity in South Asians (Lovegrove et al. 2004). Thus, there is no real evidence to suggest that less healthy eating habits can explain South Asians' disproportionately high insulin resistance and risk of diabetes.

1.6.8 Physical activity

Levels of physical activity have been found to be related to glycaemia, in UK South Asians (Gill et al. 2011) and in Australians (Dunstan et al. 2005), with strong independent associations found between sitting time and glucose concentrations 2 hours post OGTT in South Asians (Gill et al. 2011), and associations between hours of television viewing and insulin resistance in Australians (Dunstan et al. 2005). The latter study also demonstrated an inverse relationship between hours of physical activity per week and insulin resistance.

Unfortunately, a number of studies have reported significantly lower physical activity levels in South Asians living in the UK, compared with the general population (Hayes et al 1994; Barnett et al. 2005; Dhawan & Bray 1997; Fischbacher et al. 2004; Hayes et al. 2002; Health Education Authority 2000). Notably the 2004 Health survey for

England reported that Indians, Pakistanis and Bangladeshis are respectively 14%, 30% and 45% less likely to meet the recommended target for physical activity than the general population.

The reasons for this are unclear but some studies have shown that South Asian women in particular experience many barriers to increasing physical activity, including other illnesses, cultural norms and social expectations (Lawton et al. 2006). Qualitative research has suggested that cultural attitudes to changes in lifestyle, particularly increasing exercise, can be difficult to overcome (Vyas et al. 2003), and that conflicts of interest may often exist, between the desire to exercise and fear of social disapproval (Grace et al. 2008).

It should be noted that most of the published studies investigating ethnic differences in physical activity in adults have been reliant on self-report questionnaires, which are known to be of limited validity, reliability and sensitivity, compared to objective measures of physical activity (Shephard 2003). In addition, in the context of different ethnic groups, the same questions may be subject to different cultural interpretations, which will also have an impact on the data obtained (Fischbacher et al. 2004). Only one recent study has provided objective evidence, using accelerometers, that South Asian children aged 9-10 years old have lower levels of physical activity than white European children of the same age (Owen et al. 2009). This finding requires confirmation in adults, with a view to examining the relationship between physical activity and insulin resistance, but it does seem to be the case that South Asians may adopt particularly sedentary lifestyles when they migrate to Westernised countries, which could exacerbate their underlying predisposition to insulin resistance.

The Indian Diabetes Prevention Programme demonstrated that increasing physical activity can prevent type 2 diabetes in South Asians. The lifestyle intervention used was modest, compared with those used in the US Diabetes Prevention Programme (Knowler et al. 2002) and in the Finnish Diabetes Prevention Study (Laaksonen et al. 2005), and unlike the US and Finnish studies, did not produce any weight loss. Despite this, the investigators found that 30 minutes of brisk walking per day (with ~ 60% adherence) reduced the incidence of diabetes by around 30% in Indian men and women with impaired glucose tolerance (Ramachandran et al. 2006). Additional data from prospective cohort studies (Helmrich et al. 1991; Lynch et al. 1996) indicate that the benefits of increasing physical activity in reducing insulin resistance and risk of diabetes are likely to be most marked in groups who are most insulin resistant and most at risk of developing diabetes at baseline. This evidence suggests that sedentary South Asians should increase their physical activity levels to reduce their diabetes risk.

1.7 The role of adipose tissue in insulin resistance

Increased adiposity and an adverse pattern of fat partitioning in South Asians is likely to play a key role in insulin resistance and development of the metabolic syndrome. Adipose tissue is now recognised to be an endocrine organ and not merely an inert storage depot for triglyceride. It is exquisitely sensitive to the antilipolytic effect of insulin, a response which is blunted in insulin resistance, type 2 diabetes (Karpe & Tan 2005) and obesity (Coppack et al. 1992). This impaired response to insulin, in combination with expanded fat mass, results in increased release of NEFA into the circulation, ultimately contributing to ectopic fat accumulation and in turn reduced

muscle and liver glucose uptake and utilisation with resultant hyperglycaemia and hyperinsulinaemia (see Figure 1.3).

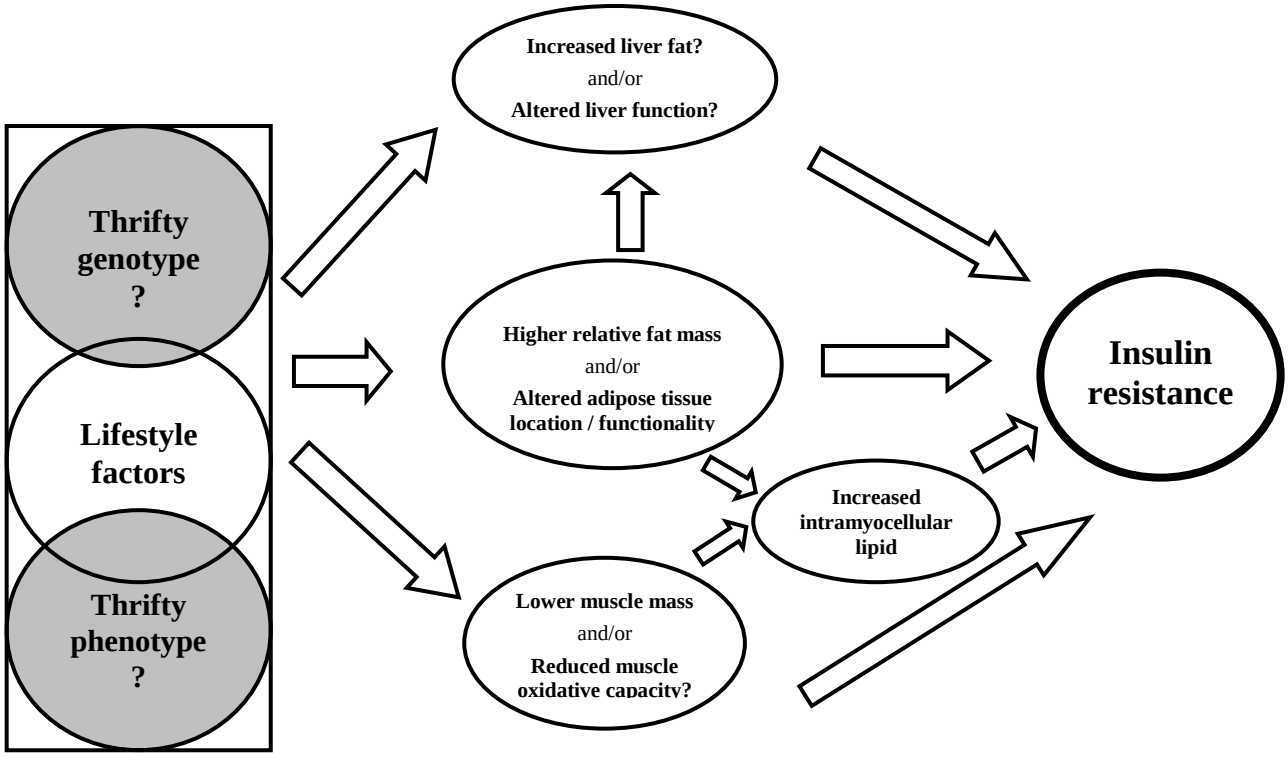

Figure 1.3 Hypotheses attempting to explain the increased insulin resistance in South Asian populations

It is now well accepted that abdominal adiposity is associated with insulin resistance and its associated cardiovascular consequences (Kissebah & Krakower 1994; Wajchenberg 2000). The concept that different abdominal adipose tissue compartments differentially influence risk of insulin resistance and diabetes is an area of great current research interest. For example there is evidence from several studies to suggest that visceral adipose tissue (VAT) in particular is implicated in insulin resistance and metabolic syndrome (particularly in hyperinsulinaemia and

dyslipidaemia) (Despres 1993; Thorne et al. 2002; Vohl et al. 2004), including a study which looked at surgical resection of visceral adipose tissue (omentectomy) in obese patients undergoing gastric banding (Thorne et al. 2002). This latter study reported that, in comparison to patients who had gastric banding alone, those who had had visceral adipose tissue removed experienced similar weight loss but a 2 to 3-fold greater improvement in insulin sensitivity and fasting glucose levels. In contrast, loss of subcutaneous adipose tissue via liposuction has been shown to have no metabolic effect (Klein et al. 2004).

In contrast, recent research comparing body fat distribution and insulin resistance in South Asians with that in white North Americans found insulin resistance (assessed by hyperinsulinaemic euglycaemic clamp) to be associated with increased total body fat and subcutaneous abdominal fat, and also with increased adipocyte size in the South Asian group, but investigators found no difference in intra-peritoneal fat (VAT) between the two groups (Chandalia et al. 2007). This intriguing finding suggests that, at least in the South Asian population, visceral adipose tissue may not be the clear culprit linked to greater insulin resistance, but rather excess subcutaneous adipose tissue, and is consistent with prior studies which report deep subcutaneous adipose tissue to be particularly detrimental to insulin sensitivity (Kelley et al. 2000; Misra et al. 1997).

The finding of similar quantities of visceral adipose tissue in the two populations may also suggest a problem with adipose tissue functionality rather than quantity in South Asians. Chandalia *et al* reported that abdominal subcutaneous adipocytes were larger in South Asians, compared to men of white European descent and that these larger, perhaps dysfunctional adipocytes released less adiponectin and more NEFAs

(Chandalia et al. 2007) in to the circulation. This finding has recently been corroborated by Anand and colleagues (Anand et al 2011), who found that adjustment for adipocyte size ameliorated differences in insulin sensitivity between a larger groups of South Asians (n = 56) and Europeans (n = 52) accross a range of BMIs. Further evidence of dysfunctional adipose tissue exists in the literature, with 2 studies having found large adipocytes to be predictive of insulin resistance and type 2 diabetes independent of total adiposity (Drolet et al. 2008; Weyer et al. 2000). The recent observation that South Asians with normal BMI have higher levels of NEFA and leptin, lower levels of adiponectin and impaired suppression of NEFA during an oral glucose tolerance test, compared with European whites matched for total fat mass (Abate et al. 2004), also supports the hypothesis of dysfunctional, insulin resistant adipose tissue.

It has also been suggested that deep compartments of subcutaneous adipose tissue may contribute more to insulin resistance than more superficial subcutaneous adipose tissue compartments. Indeed, a recent report proposed the "adipose tissue overflow hypothesis" (Sniderman et al. 2007) as an explanation for increased insulin resistance in South Asians. Its authors suggested that adipose tissue is divided into primary and secondary compartments, based on sequence of development, the primary compartment being superficial subcutaneous adipose tissue and the secondary compartment comprising deep subcutaneous adipose tissue and visceral adipose tissue, which are related to dyslipidaemia and insulin resistance. With surplus energy intake the primary compartment becomes "full" and secondary compartments develop and expand, giving rise to their associated adverse metabolic consequences. They hypothesised that South Asians have smaller primary compartments than white Europeans and that therefore a more rapid deposition of fat occurs into "adverse" fat

compartments. This hypothesis would be consistent with a more rapid onset of dyslipidaemia and insulin resistance in South Asians at lower BMI and lower total percentage body fat. Clearly, this hypothesis requires more formal testing.

A further possibility is that South Asians may have a relatively smaller proportion of brown adipose tissue making up total fat mass than Europeans. This type of fat is now known to persist beyond the neonatal period and to generate heat from dietary energy, thus is responsible for some contribution toward glucose disposal (Cannon & Nedergaard). There have been no studies investigating brown adipose tissue in South Asians, or indeed in any ethnic population.

Adipose tissue is also responsible for the production and release of hormones adiponectin, visfatin, leptin, resistin and active cortisol (via the enzyme 11-β-hydroxysteroid dehydrogenase type 1) as well as the pro-inflammatory cytokines tumour necrosis factor-α (TNF α), interleukin 6 (IL-6), the anti-inflammatory cytokine adiponectin (which correlates to insulin sensitivity) (Yamauchi et al. 2002), and the pro-coagulant factors plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA). The inflammatory response is reflected by high sensitivity CRP, which has been shown to predict cardiovascular events (Ridker et al. 1998).

Studies looking at these risk markers in South Asian populations have found higher levels of high sensitivity CRP (Chambers et al. 2001; Forouhi et al. 2001) , IL-6 (Petersen et al. 2006), t-PA (Forouhi et al. 2003) leptin (Kalhan et al. 2001; Yajnik et al. 2002) and PAI-1 (Anand et al. 2000; Kain et al. 2001), together with significantly lower adiponectin levels (Mohan et al. 2005; Ryo et al. 2004; Snehalatha et al. 2003) compared with white populations. Many of these differences have been found to

persist after adjustment for total or abdominal fat mass (Abate et al. 2004; Chandalia et al. 2007). Low adiponectin concentrations and increased leptin, IL-6 and TNFα concentrations are associated with an increased risk of type 2 diabetes (Hu et al. 2004; Li et al. 2009; Wannamethee et al. 2007) and these markers are mechanistically implicated in insulin resistance. It is, therefore possible that differences in adipose tissue function which result in dysregulation of adipokines within adipose tissue, independently of adipose tissue mass, contribute to the altered adipokine profile and greater insulin resistance observed on South Asians. Further investigation of gene and protein expression of adipokines within adipose tissue in South Asians is required to test this hypothesis.

Homocysteine, lipoprotein (a) and fibrinogen, which are novel risk factors for insulin resistance and vascular disease, are also reported to be elevated in South Asians (Bhatnagar et al. 1995; Chambers & Kooner 2001).

Whilst all these differences are of interest, it should be appreciated that there are currently no prospective studies which determine to what extent (if any) these risk factor perturbances explain excess diabetes or vascular risk in South Asians. Moreover, it is entirely possible that such differences, and excess risk of metabolic diseases in South Asians, may be largely accounted for by differences in fat location and reduced physical activity. On the other hand it is possible, that differences in adipose tissue function which result in dysregulation of adipokines within adipose tissue, independently of adipose tissue mass, contribute to the altered adipokine profile and greater insulin resistance observed on South Asians. Further investigation of gene and protein expression of adipokines within adipose tissue in South Asians is

required to test this hypothesis, as are future prospective studies with better baseline phenotyping to better disentangle independent risk factors for incident diabetes and vascular events.

1.8 The role of skeletal muscle in insulin resistance

Skeletal muscle is responsible for 75% of all insulin stimulated glucose uptake, and most of this glucose (i.e. that which is not oxidized) is directed towards glycogen synthesis. Defects in muscle intracellular glucose transport are, therefore, a key determinant of whole-body insulin resistance (Petersen & Shulman 2002). A relative oversupply of lipid to skeletal muscle due to increased fatty acid delivery or decreased fatty acid oxidation may precipitate insulin resistance in muscle by inducing an accumulation of intramyocellular lipid: triglyceride (IMTG) and the fatty acid metabolites, long-chain acyl-CoA (LCACoA), diacylglycerol (DAG) and ceramide (Goodpaster & Brown 2005). Studies have shown that infusion of lipid induces acute insulin resistance in muscle, probably due to increased DAG mass and PKC activity (Itani et al. 2002) and it is known that type 2 diabetes and insulin-resistant states are associated with chronically elevated circulating levels of NEFA.

While previous studies have reported a correlation between intramyocellular triglyceride (IMTG) and insulin resistance (Krssak et al. 1999, Pan et al. 1997), it is now not thought to be a causal factor, and at least one study (which examined IMTG in South Asians) has shown no correlation between IMTG and insulin resistance (Forouhi et al. 1999). IMTG is known to be increased in athletes (Goodpaster et al. 2001) and has also been found to increase by over 50% in response to acute exercise, an effect which is accompanied by improved insulin sensitivity and reductions in

intramyocellular ceramide and DAG (Schenk & Horowitz 2007) suggesting separate mechanisms for IMTG accretion in athletes and insulin resistant states. More recently, beneficial metabolic changes in skeletal muscle have been found to occur in response to chronic exercise in obese older adults, also accompanied by increases in IMTG, glycogen storage, capillary density and percentage of type 1 oxidative muscle fibers (Dube et al. 2008).

There is some recent evidence that the relationship between IMTG and insulin resistance may be specific to muscle fibre type, with one study reporting greater IMTG content in type 1, but not type 2 myocytes in insulin resistance, associated with a lower proportion of type 1 myocytes (Coen et al. 2010). No studies to date have investigated differences in skeletal muscle fibre type between South Asians and Europeans.

Pattern of distribution of lipid within muscle could also be a determinant of skeletal muscle insulin sensitivity, however at least one study found similar patterns of IMCL storage in patients with type 2 diabetes, normoglycaemic controls and trained endurance athletes (van Loon et al. 2004)

It is, therefore thought that in obesity and insulin resistance, excessive fatty acid delivery leads to an adverse pattern of fat partitioning in myocytes, with more fatty acids being directed towards a build-up of DAG, ceramide and LCACoA, and less towards stable triglyceride (Horowitz 2007).

The insulin signalling pathway in normal skeletal muscle is illustrated in Figure 1.4. When excess LCACoA, DAG and ceramide are present, they have been proposed to inhibit insulin action through the activation of protein kinase C (PKC) and c-Jun-terminal kinase (JNK) which inhibit insulin receptor (IR) tyrosine kinase activity, tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), and protein kinase B (PKB) activity (Hegarty et al. 2003; Schmitz-Peiffer 2000). In addition, PKC inhibits insulin-stimulated phosphatidylinositol 3 kinase (PI3K) activity and may also result in oxidative stress through activation of the IKK/ NFK-β pathway (which is a pro-inflammatory pathway) (Itani et al.. 2002).

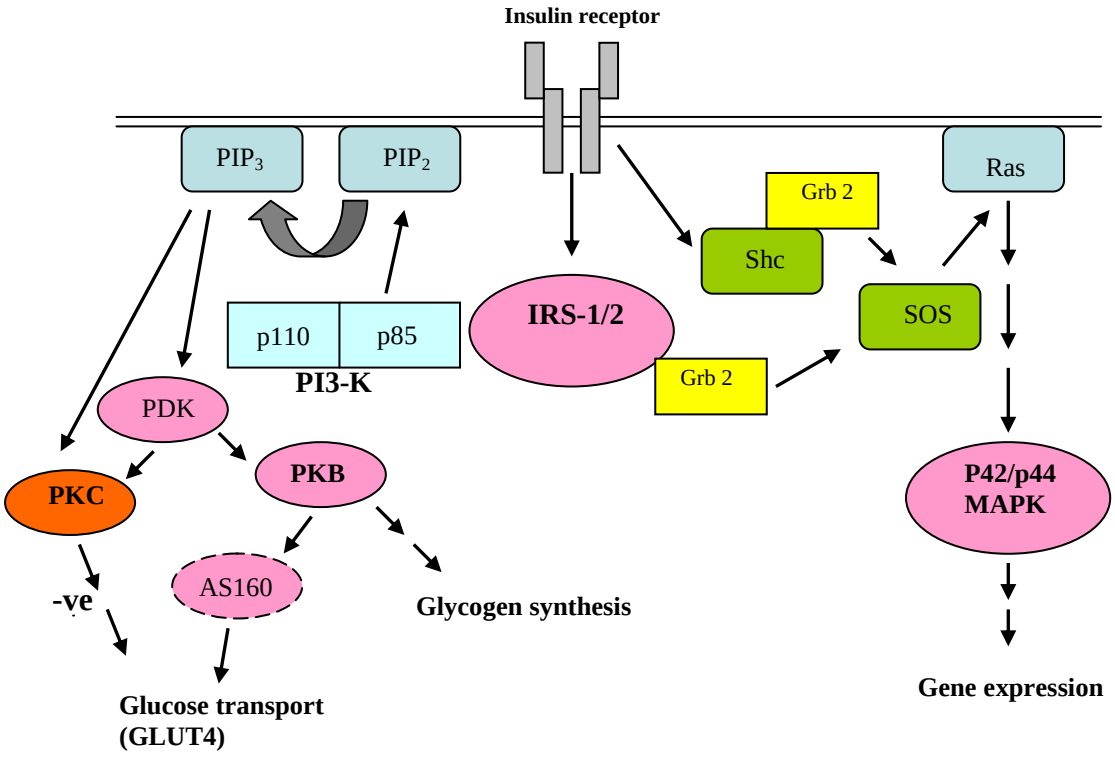

Figure 1.4 The insulin signalling pathway in skeletal muscle

Altered insulin signalling at various stages in the insulin signalling pathway has been described in patients with type 2 diabetes, obesity, PCOS and other insulin resistant states, although defects which are absolutely characteristic of insulin resistance have yet to be described, as there are a large number of studies with apparently contradictory findings. IRS-1 expression has been found to be both reduced (Brozinick et al. 2003; Stentz & Kitabchi 2007) and unchanged (Hammarstedt et al. 2003; Krook et al. 2000; Rajkhowa et al. 2009) in insulin resistant states, and there are also conflicting results from studies examining the expression of p85, the regulatory subunit of P13K, in insulin resistance (Adochio et al. 2009; Bandyopadhyay et al. 2005; Barbour et al. 2005; Colomiere et al. 2009; Friedman et al. 1999; Hammarstedt et al. 2005b), and of PKB (Bandyopadhyay et al. 2005; Hojlund et al. 2008; Karlsson et al. 2005; Kim et al. 1999; Krook et al. 1998; Meyer et al. 2002). This is clearly an area where further studies are required to resolve this issue. To the best of the author's knowledge, there have been no previously published data comparing insulin signalling molecule expression in South Asians and Europeans, or indeed between any ethnic groups, so this is also an area which requires investigation.

The sphingolipid ceramide was first implicated in insulin resistance in 1990, when its levels were found to be high in muscle of the obese Zucker rat (Turinsky et al. 1990). More recently the inhibition of ceramide synthesis has been found to markedly improve glucose tolerance and delay the onset of frank diabetes in these obese rats (Holland et al. 2007).

There is some indirect evidence to suggest that these mechanisms may contribute to the insulin resistant phenotype of South Asians. For a given level of adiposity, South

Asians exhibit higher circulating concentrations of NEFA than matched individuals of European descent (Abate et al. 2004) and fatty acid uptake into muscle, although regulated by fatty acid transporters such as fatty acid translocase (FAT/CD36), is largely dependent on circulating fatty acid concentrations (Bonen et al. 2004). In addition, one study found over 30% higher IMTG in South Asians compared with BMI-matched Europeans, although intra-muscular concentrations of fatty acid intermediates were not specifically quantified in this study (Forouhi et al. 1999).

It is hypothesised that the accumulation of fatty acid intermediates in insulin resistant skeletal muscle is not only due to lipid oversupply (as a result of high circulating levels of NEFA) but is also a consequence of impaired fatty acid oxidation by skeletal muscle. There is an increasing body of evidence to suggest that impaired skeletal muscle oxidation of fatty acids, represented by reduced mitochondrial enzyme activity (e.g. citrate synthase and β-hydroxoacyl CoA dehydrogenase) is strongly associated with insulin resistance (Bruce et al. 2003). In addition activity of the mitochondrial enzyme carnitine palmityltransferase (CPT I), the rate-limiting enzyme for entry of long-chain acyl-CoA (LCACoA) into mitochondria, is reduced in insulin resistant muscle (Kim et al. 2000; Simoneau et al. 1999). Furthermore, expression of skeletal muscle acetyl-CoA carboxylase β (ACC β) – which regulates fatty acid oxidation by catalysing the carboxylation of acetyl CoA to form malonyl CoA, which in turn inhibits CPT 1 (Ruderman et al. 1999) – is reduced following weight loss in obese subjects and these reductions correlate strongly with reductions in fasting insulin concentrations (Rosa et al. 2003).

Exercise tests can provide a number of indices of *in vivo* skeletal muscle and whole body oxidative capacity. It has been reported that maximal oxygen uptake, or VO_2 max (i.e. a measure of whole-body oxidative capacity, which correlates strongly with skeletal muscle oxidative enzyme activities in heterogeneous populations (Bekedam et al. 2003; Maughan & Gleeson M 2004)) is a strong predictor of whole-body insulin sensitivity in white Europeans and this is independent of visceral adiposity and family history of type 2 diabetes (Bekedam et al. 2003; Nyholm et al. 2004; Thamer et al. 2003). Furthermore, a recent study reported that in rats selectively bred for high or low aerobic capacity from the same founder population, the animals bred for low aerobic capacity had 58% lower VO_{2max} (by definition) but also exhibited 131% higher fasting insulin and 20% higher fasting glucose than their counterparts bred for high aerobic capacity (Wisloff et al. 2005), consistent with substantially greater insulin resistance.

Limited data are available, which indicates that differences in whole body oxidative capacity are evident when comparing men of South Asian descent with those of Europeans. VO_{2max} values in sedentary South Asians are typically around 10-15% lower than comparable sedentary European groups (Davey et al. 2000; Hardy & Eston 1985; Verma et al. 1979). Interestingly, the relatives of patients with type 2 diabetes, who, like South Asians, have increased insulin resistance (Barwell et al. 2008; Ezenwaka et al. 2001; Humphriss et al. 1997; Perseghin et al. 1997) and risk of type 2 diabetes (Kobberling J & Tillil H 1982; Ohlson et al. 1988) also have significantly lower values for VO_2 max (~10-15% lower) than age, BMI and body-fat matched controls (Nyholm et al. 2004; Thamer et al. 2003). It is therefore tempting to speculate that increased insulin resistance in South Asians compared to Europeans

may be, at least in part, a consequence of reduced skeletal muscle oxidative capacity, particularly with respect to fatty acid oxidation, leading to a mismatch between fatty acid delivery and utilization within muscle. This suggestion warrants further investigation.

1.9 Does excess liver fat play a role in insulin resistance in South Asians?

Ectopic fat can accumulate in the liver as well as in skeletal muscle, partly as a result of excess of circulating NEFA, delivered via the portal vein (which drains VAT), and via the systemic circulation. A further mechanism for the development of ectopic liver fat is increased *de novo* hepatic lipogenesis, due to the hyperinsulinaemia seen in insulin resistance, and because of altered patterns of storage of dietary carbohydrate, which is diverted away from skeletal muscle glycogen synthesis and towards hepatic lipogenesis. The latter mechanism suggests that excess liver fat secondary to *de novo* hepatic lipogenesis occurs, partly as a result of skeletal muscle insulin resistance. Recent research verifies this by demonstating that acute exercise can ameliorate this effect in young, lean, insulin-resistant men by redressing the balance towards skeletal muscle glycogen synthesis and away from hepatic lipogenesis, resulting in a 30% reduction in hepatic lipogenesis following a single bout of exercise (Rabol et al. 2011).

Ectopic fat results in hepatic insulin resistance, nonalcoholic fatty liver disease (NAFLD) and eventually cirrhosis and portal hypertension. Although the molecular mechanisms underlying this are not fully understood, it is hypothesized that increased fatty acid delivery to hepatocytes results in hepatic steatosis (i.e. an increase in intrahepatic triclyceride and diacylglcerol) which, similar to the accumulation of fatty

acid intermediates in skeletal muscle, activates protein kinase C and JNK 1. These enzymes interfere with insulin signalling by reducing tyrosine phosphorylation of IRS-1 and IRS-2. Ultimately, the ability of insulin to activate hepatic glycogen synthase is reduced, and this stimulates gluconeogenesis (Samuel et al. 2004) . Hepatic steatosis strongly correlates with insulin resistance, and indeed the liver enzyme alanine aminotransferase, which is elevated in around 70% of patients with fatty liver, has been shown to be predictive of type 2 diabetes (Sattar et al. 2004).

Limited data suggest that the prevalence of NAFLD in South Asians is high, and strongly associated with diabetes and IGT (Amarapurkar et al. 2007; Mohan et al. 2009), and that excess hepatic lipid may contribute to insulin resistance in South Asians. One such report found that South Asian men exhibited a 3 to 4-fold increase in the prevalence of insulin resistance (measured by HOMA) compared to White, East Asian, Black and Hispanic groups. This was accompanied by a 2-fold increase in hepatic triglyceride content and IL-6, in the South Asians compared to the other ethnic groups (Petersen et al. 2006), although the groups were only matched for BMI and not for body-fat percentage, which may have confounded the results. While it is not possible to infer a causal relationship between hepatic lipid accumulation and hepatic insulin resistance in South Asians from these data, they are consistent with the hypothesis that increased circulating NEFA in South Asians leads to ectopic fat accumulation culminating in "end-organ" insulin resistance. Again, this is an area where further study is needed.

1.10 Future Research Needs

As the reasons behind the increased prevalence of insulin resistance and diabetes in South Asians remain elusive, further research is required. Imaging studies, using MRI, to corroborate the location of excess adipose tissue and its relationship to adverse metabolic profiles are needed, as well as muscle phenotyping studies, examining muscle lipid oxidation and insulin signalling, and more prospective studies relating traditional and, in particular, novel risk factors to event risks. Studies with objective ascertainment of real levels of physical activity in South Asians, and controlled exercise intervention trials, would also be beneficial to determine the extent to which physical activity level can modulate insulin sensitivity and diabetes risk in this group.

It is clear that there is a higher prevalence of type 2 diabetes and CHD in South Asians, compared with white European populations, and it has become increasingly clear that risk factors related to these manifest early in life, so genetic or programming factors are relevant. In line with the latter observation, South Asians are more sensitive to the effects of weight gain and may move fat more quickly to adverse fat locations (centrally, either to deep subcutaneous abdominal stores or to visceral compartments) and some preliminary evidence suggests that they may also move fat more quickly to skeletal muscle and the liver, with associated metabolic consequences.

The low prevalence of diabetes in rural India (Mohan et al. 2008) suggests that increased diabetes risk can be avoided by management of diet and lifestyle to incorporate more physical activity, and to maintain a lean body weight. Clinically,

therefore South Asians have a greater need to be physically active and to remain lean than their white European counterparts but unfortunately, there is evidence that South Asians are in fact less active, and in some developed countries their obesity levels are greater than those seen in the indigenous population. Thus, in parallel with the need for further research, there is an urgent need for a worldwide public health policy targeting South Asians from a young age, to encourage and enable them to remain lean and become more physically active. Cultural and religious factors should be taken into account and there should be an emphasis on producing culturally appropriate, targeted information and on working with existing South Asian organisations within communities. The pilot UK Asian Diabetes Study (UKADS) is an example of how a culturally-specific approach can improve outcomes (O'Hare et al. 2004). This looked at differences in blood pressure, lipids and diabetes control in 361 South Asian patients with diabetes, who had been randomised to receive either conventional care or enhanced care (with Asian link workers supporting patients, organising education and explaining the purpose of medication). The patients who had enhanced care had significantly greater blood pressure reductions and total cholesterol reductions after a year than those in the conventional group. Although the target population in this study was patients with diabetes, a similar sort of scheme could be used to educate and encourage families of South Asian diabetes patients or schoolchildren about increasing physical activity levels and improving diets.

1.11 Summary

In conclusion, the preceding literature review will have made the reader aware of the magnitude of the problem of increased insulin resistance in South Asians, along with the resultant increased morbidity and mortality associated with type 2 diabetes and cardiovascular disease in this population. The review also examined current hypotheses speculating about the underlying mechanisms for South Asians' increased insulin resistance. Genetic differences which result in alterations in skeletal muscle and adipose tissue function, combined with an increasingly obesogenic environment which promotes sedentary lifestyles and "Westernised diets" are likely to be important. Given the major influence that skeletal muscle has on whole-body insulin sensitivity, and the fact that South Asians have been found to have differences in skeletal muscle structure (i.e. increased intramyocellular lipid), investigating skeletal muscle function in South Asian populations is a logical step towards providing insight into the mechanisms underlying insulin reistance in this population.

This thesis aims to test the hypothesis that insulin resistance in South Asians is caused, at least in part, by a defect in skeletal muscle oxidative capacity, resulting in reduced oxidation of fatty acids. Increased circulating NEFA, which has been observed in South Asians, up-regulates fatty acid uptake into the skeletal muscle and this increased uptake combined with reduced oxidation produces a mismatch between fatty acid uptake and oxidation, resulting in the accumulation of intramyocellular lipid and subsequent impairment of insulin signalling.

The studies described in Chapter 3 aim to investigate this hypothesis by examining lifestyle, physical and metabolic characteristics of sedentary, normoglycaemic South

Asian men compared to matched European controls, to determine the influence of lifestyle and adiposity as potential mediators of insulin resistance in South Asians. Additionally, it aims to identify differences in skeletal muscle function between the two groups, through measurement of cardiovascular fitness, of whole-body fat oxidation during incremental exercise, and at rest, and of skeletal muscle oxidative and lipid metabolism genes expression.

The tests performed in Chapter 4 will examine insulin signalling, with an aim of discovering ethnic differences in protein expression of various components of the insulin signalling pathway in skeletal muscle samples, both fasting and following insulin stimulation.

Chapter 2

General Methods

2.1 Introduction

This chapter describes the methods employed to recruit volunteers for the study, for the measurement of anthropometric, metabolic and cardiorespiratory variables and for all laboratory analyses. This study was conducted with the ethical approval of the North Glasgow NHS Trust Research Ethics Committee and was conducted according to the principles expressed in the Declaration of Helsinki. All participants gave written informed consent (appendix A2).

2.2 Subject recruitment

All subjects were Glasgow residents, and were apparently healthy. They were recruited via local advertising using newspapers and radio broadcasts. Recruitment posters were displayed around the Glasgow University main campus, Garscube campus and and Strathclyde University campus. A number of local general practitioners agreed to display recruitment posters in their surgery waiting rooms. In addition posters were displayed in outpatient clinic areas of the Western Infirmary, Gartnavel General Hospital and Glasgow Royal Infirmary. Potential volunteers could access information about the study through a weblink sited on the University Sports and Recreation website. In addition to this, volunteers were able to provide basic contact details and note their interest by submission of a webform which was emailed to the principal investigators. Information about the study was disseminated by word-of-mouth, by both the investigators and by volunteers themselves, and was responsible for a small proportion of the total number of subjects recruited.

Volunteers were given an information sheet, which described in detail the purposes of the study, the study protocol, and any potential risks and discomforts associated with participation (appendix A1). A confidential questionnaire about past medical history and family history of disease was completed by all subjects (appendix A3), as were questionnaires about physical activity (appendix A4) and diet (appendix A5). Resting blood pressure was measured using a mercury sphygmomanometer (Accoson, Essex, UK) and a fasting blood sample was collected to test glucose, cholesterol and lipid profile in addition to liver, renal and thyroid function. The following criteria were used for subject recruitment:

- Male aged 18 – 40 years
- South Asian (i.e. Pakistani, Indian, Bangladeshi or Sri Lankan) or Northern European (i.e. British, Irish, French, German, Dutch, Scandinavian)
- Non-smoking
- Relatively sedentary (i.e. taking part in less than 2 hours of planned aerobic exercise per week and working in sedentary job)
- Blood pressure < 160/90 mmHg
- Plasma glucose concentration < 7.0 $mmol.l^{-1}$ and no known diabetes
- Normal liver, renal and thyroid function

Volunteers taking prescribed medications were not excluded from the study, provided that their prescription was stable and did not include medications which are recognised to modulate carbohydrate or lipid homeostasis. Figure 2.1 illustrates the recruitment process, and the number of volunteers involved in the study, including

drop-outs and those who were excluded from the study. The reasons given for volunteers dropping out of the study were:

- Too much of a time commitment (1 European)
- Financial compensation perceived to be too little (1 South Asian)
- Fear of blood tests/ muscle biopsy (2 Europeans, 1 South Asian)
- No reason given and contact lost (4 Europeans, 2 South Asians)

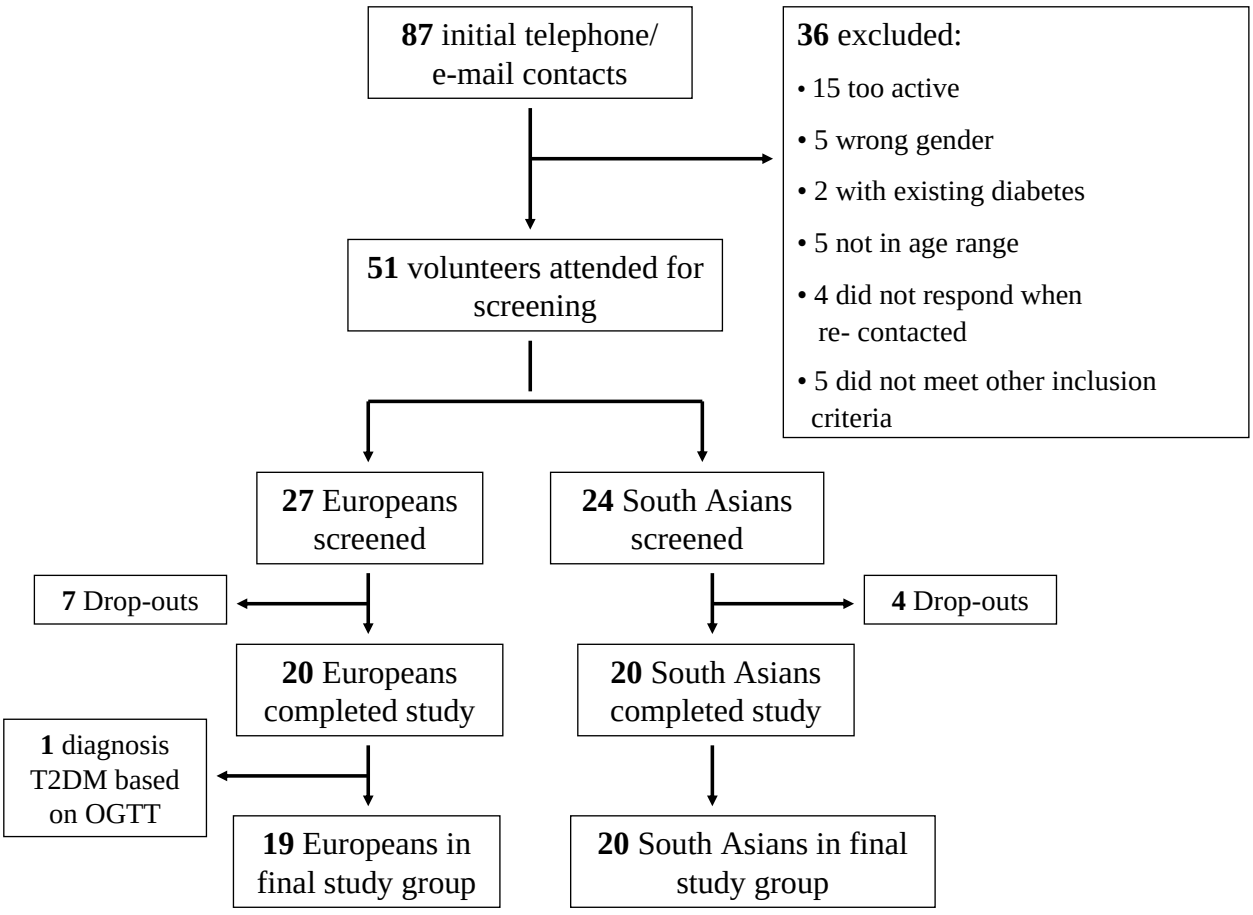

Figure 2.1 Flow chart demonstrating volunteer recruitment with details of dropouts and exclusions at each stage of the study.

2.3 Exercise stress test

All subjects underwent a Bruce Protocol Exercise Tolerance Test (BPETT) (Bruce, Kusumi, & Hosmer 1973) to ensure that there were no cardiovascular contraindications to their participation in the study. This consists of an incremental exercise test with 3-minute stages, during which workload increases in a stepwise fashion with every stage, due to increases in both the speed and the gradient of the treadmill. In the study 12-lead electrocardiogram (ECG) tracings were obtained using a Quinton Q710 ECG machine (Quinton instruments Co, Bothwell, WA, USA), at rest, during each of the 3 minute stages of the Bruce protocol, and during recovery. Blood pressure was measured at rest, prior to the exercise test and then 5 minutes post-exercise. The BPETTs were all performed and interpreted by the author (a registered medical practitioner with Advanced Life Support (ALS) accreditation). All volunteers achieved an adequate workload on BPETT and remained free of symptoms and electrocardiographical signs of coronary artery insufficiency. One volunteer, however, developed ECG signs suggestive of abnormal cardiac conduction during the ETT. He was referred to the cardiology clinic at Glasgow Royal Infirmary for further evaluation, and underwent echocardiography, which did not reveal any structural or functional cardiac abnormalities.

2.4 Anthropometric measurements

All measurements were performed using standardised methods according to the International Society for the Advancement of Kinanthropometrics (ISAK) protocol (Marfell-Jones M et al. 2006). The same ISAK-accredited experimenter performed all measurements to abolish any possibility of inter-observer variability. All measurements were taken twice, and a mean value was taken for each.

2.4.1 Height and body mass

Height was measured to the nearest 0.1 cm using a free standing stadiometer (Invicta Plastics Ltd, Leicester, UK). Subjects stood barefoot with their heels together, their backs against a fixed backboard and their heads orientated in the Frankfurt plane (i.e. the position where the inferior margin of the orbit is in line with the upper margin of the ear canal). The stretch technique was used, such that subjects were asked to inspire, at which point gentle upward pressure was applied to the lower jaw, and a sliding headboard was lowered to the top of the head, with enough pressure to compress the hair. Body mass was measured to the nearest 0.05 kg using a manual balance weighing scale (Avery Industrial Ltd, Leicester, UK). Subjects were weighed and measured barefoot and wearing light clothing (typically shorts and a t-shirt).

2.4.2 Waist and hip circumferences

Waist and hip circumferences were measured to the nearest 0.1 cm using a steel measuring tape (Hoechstmass Balzer GmbH, Frankfurt, Germany)**,** with the subject wearing light clothing (typically shorts only) and standing with their feet together. Waist circumference was measured at the narrowest point between the costal margin and the iliac crests (or if this could not be identified, at the level midway between the costal margin and the iliac crests), with the abdominal muscles relaxed. Hip circumference was measured around the maximal circumference of the buttocks, at approximately the level of the pubic symphysis. Waist-hip ratio was determined by dividing the mean waist circumference by the mean hip circumference.

2.4.3 Skinfold measurement.

Figure 2.2 Suprailiac and biceps measurement sites

Skinfold measurements were taken at the biceps, triceps, subscapular and suprailiac sites, on the right hand side of the body. Suprailiac sites and biceps sites are illustrated in Figure 2.2 above. They were measured using Harpenden skinfold callipers (Holtain Ltd, Crymych,UK) to the nearest 0.1 mm. The same set of callipers was used for all subjects. All readings were taken three seconds after closure of the calliper jaws, to avoid excess compression of adipose tissue. Sites for skinfold measurement were located using bony landmarks as follows:

- **Biceps** – midway between the acromial process and the superior head of the radius on the midline, anteriorly, with the forearm supinated
- **Tricep** – midway between the acromial process and the superior head of the radius on the midline, posteriorly, with the forearm supinated
- **Subscapular** – 20 mm below the inferior angle of the scapula and 45 degrees to the lateral side of the body
- **Suprailiac** – immediately superior to the iliac crest in the mid-axillary line

2.5 Assessment of Body Composition

Dual X-ray absorptiometry (DEXA), which is well recognised as an accurate method for assessing body composition and body fat distribution (Gallagher & Song 2003) was used for this purpose in the study. Lean body mass and fat mass were calculated for each subject, and all scans were performed by the same operator who was an experienced radiographer, and with a GE Medical LUNAR Prodigy DEXA scanner (GE Healthcare Diagnostic Imaging, Slough, Berkshire, UK). Scan data were interpreted using the scanner's generic software. Scans were performed in the Radiology Department of the Royal Hospital for Sick Children, Yorkhill, Glasgow and all subjects were informed that DEXA scanning necessitates the delivery of a small dose of radiation (figure quoted 0.0007 mSV, or one fiftieth of the radiation dose delivered during a standard chest X-ray).

2.6 Expired air measurements

Expired air collections were made using two separate techniques. Douglas bags were used to collect expired air at rest immediately prior to all exercise tests and during both treadmill exercise protocols. The ventilated hood method was used during the final study visit, for collection of expired air and measurement of metabolic rate at rest, both in the fasted state and post glucose ingestion.

2.6.1 Expired air measurements using Douglas bags

At rest, prior to exercise, and throughout all exercise sessions, Douglas bags were used to collect expired air samples. Immediately prior to resting measurements, subjects completed a 10-minute run-in period, sitting in a chair, to ensure that they were adequately rested. A nose clip and a mouthpiece connected to a lightweight 2-

way valve (2700 Series, Hans Rudolf Inc, USA) were then introduced to the subject for 3 minutes before any air collection, for subject comfort and familiarisation. Thereafter, expired air samples were collected into 100 or 150 litre Douglas bags (Cranlea and Co, Birmingham, UK), via the mouthpiece valve and a length of plastic tubing connected to the Douglas bag by a further 2-way valve. Resting measurements were taken over two 5-minute periods.

During the maximal exercise test, the mouthpiece and nose clip were worn continuously, the mouthpiece being supported by an adjustable headset. During the submaximal test, however, the mouthpiece and nose clip were passed to the subject two and a half minutes into each four minute exercise stage, allowing 30 seconds before the expired air collections were taken at minutes 3 to 4. They were then removed until two and a half minutes into the next stage.

Following the collection of expired air into a Douglas bag, a small volume of gas was passed through a flow meter and into a gas analyser (Servomex 4000 Series, Servomex Group Ltd, East Sussex, UK) where the percentage of oxygen and carbon dioxide was measured. The remaining expired air was extracted by a vacuum and passed through s dry gas meter (Harvard Apparatus Ltd, Kent, UK), which also measured the temperature of the air. VO_2 and VCO_2 were calculated and the respiratory exchange ratio was calculated using indirect calorimetry.

Prior to all measurements, Douglas bags were evacuated and the gas analyser was calibrated against reference gases of known oxygen and carbon dioxide content (BOC Gases, BOC Ltd, Surrey, UK)

2.6.2 Resting expired air measurements using ventilated hood

Basal metabolic rate and energy substrate utilisation were measured in all subjects after an overnight fast of at least 12 hours. Subjects attended the metabolic suite, where they were asked to lie on a couch in a supine position. After 10 minutes of lying down, the ventilated hood with surrounding plastic skirt connected to an Oxycon-Pro (Viasys Healthcare, Warwick, UK) analyser was placed over the subject's head. During the measurement, room air was drawn into the hood, and expired air was extracted at the same rate in to the Oxycon-Pro system for analysis. Expired oxygen and carbon dioxide were determined, allowing calculation of oxygen consumption (VO_2) and carbon dioxide production (VCO_2). Measurements were taken for 25 minutes, with VO_2 and VCO_2 results averaged over 1 minute, and an average of the final 15 minutes of data was used for the calculation of respiratory exchange ration, energy expenditure and rate of energy substrate utilisation using indirect calorimetry.

This resting measurement was also repeated for all subjects 2 hours after a glucose load (standard 75 gram oral glucose tolerance test).

Prior to use, the Oxycon-Pro system was calibrated using reference gases (Viasys Healthcare, Warwick, UK) of a known oxygen and carbon dioxide content, and corrections were made for barometric pressure and ambient temperature.

2.7 Exercise tests

All exercise tests were performed in a purpose built laboratory, using a motorised treadmill (Woodway GmbH, Weil am Rhein, Germany). Prior to both the VO_2 max and the submaximal test protocols, and in addition to the resting measurement, subjects underwent a warm-up phase, which consisted of a 10 minute walk on the treadmill at 5 $km.h^{-1}$ on a 0% gradient. Expired air was collected at minutes 8 until 9 and 9 until 10.

2.7.1 Maximal exercise test

Prior to the maximal exercise test, all subjects had undergone a familiarisation session, which took place in conjunction with the screening exercise stress test. The purpose of this was to allow all subjects the opportunity to practise walking on a treadmill, to experience walking with the nose clip and mouthpiece in place, and therefore, to minimise any tendency towards hyperventilation during the exercise tests. After the warm-up phase the treadmill speed was increased to 5.5 $km.h^{-1}$, and the gradient was increased to 3%. Thereafter, every 2 minutes the gradient was increased by a further 3%, until a gradient of 21% was reached, after which the gradient remained constant at 21% but the speed was increased by 3 $km.h^{-1}$ every 2 minutes. All subjects were verbally encouraged to continue until exhaustion, and expired air samples were collected over one minute during the last 3 or 4 stages of the test. Immediately after the VO_2 max test, subjects stepped back onto the treadmill and completed a cool-down phase for 5 minutes at 4 $km.h^{-1}$ and 0% gradient. This is illustrated in Figure 2.3.

The achievement of VO_2 max was confirmed by an RER of 1.00 and a heart rate which was at least 85% of age-predicted maximum heart rate. In addition, a supra-maximal test was performed at 115% of peak work rate to confirm a plateau in VO_2. This test lasted for 2.5 minutes with expired air samples collected every 30 seconds.

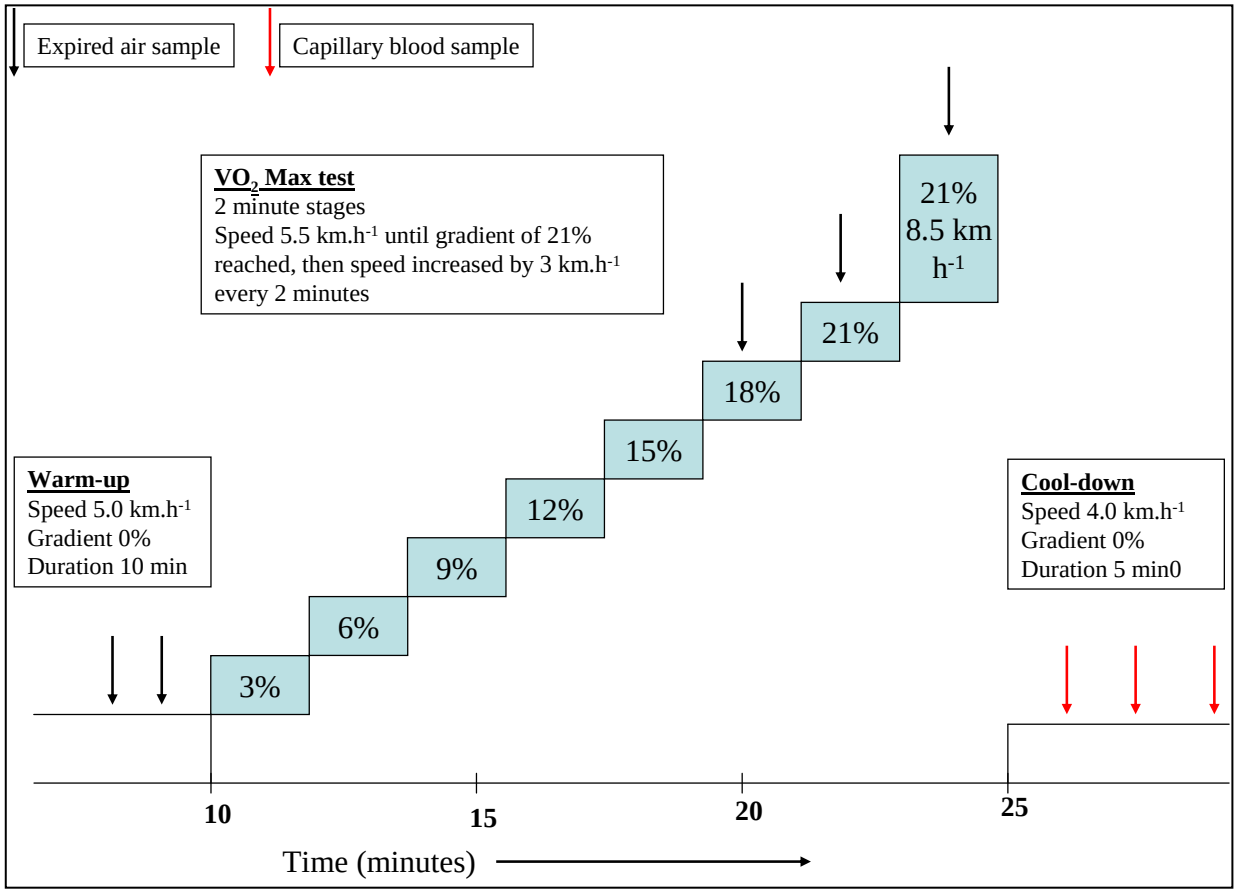

Figure 2.3 VO_{2max} test protocol

2.7.2 Submaximal exercise test to determine lactate threshold

For this test, individual protocols, specifically tailored for each subject, based on their VO_2 max were created**.** Following the warm-up phase, the treadmill speed was increased to 5.5 $km.h^{-1}$, and the gradient was increased up to a pre-determined value, based on VO_{2max}. Each stage of the sub-maximal exercise test lasted for 4 minutes and expired air collections were taken at minutes 3-4 of each stage. Thereafter, heart

rate and RPE score were recorded and a capillary blood sample was taken for later analysis to determine lactate concentration. The treadmill gradient was then increased by 1% and this was repeated for a total of 8 stages. Thereafter subjects again completed a cool-down phase for 5 minutes at 4 km.h^{-1} and 0% gradient. This is illustrated in Figure 2.4

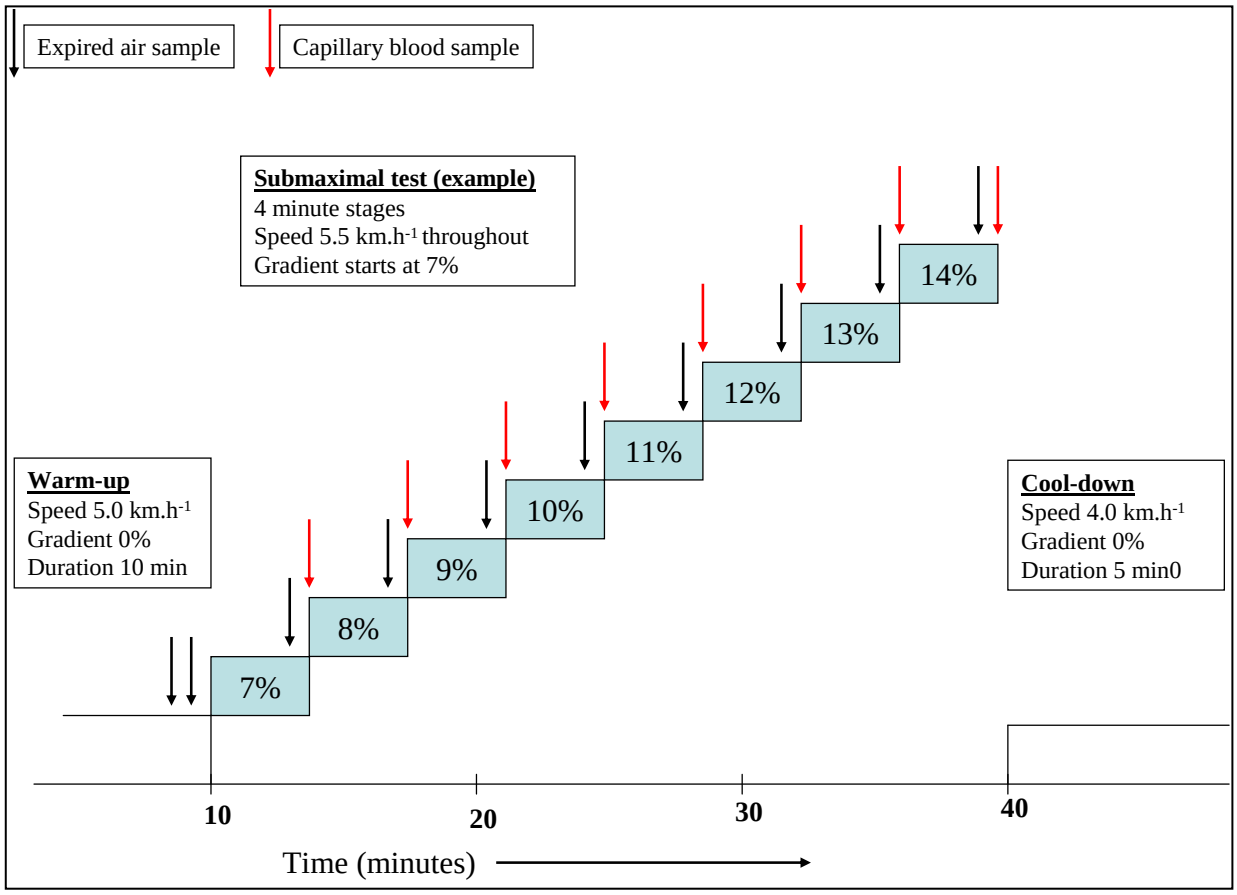

Figure 2.4 Typical submaximal exercise test protocol

2.8 Measurement of heart rate and rate of perceived exertion

Heart rate was measured using short-range telemetry (Polar S610i Heart Rate Monitor, Polar Electro, Oy, Kempele, Finland). Heart rate was monitored throughout all exercise sessions and during the recovery phase of each session. Rate of perceived

exertion was recorded at 4 minute intervals, at the end of each exercise stage during the lactate profile exercise test, using the Borg Scale (Borg 1982).

2.9 Indirect Calorimetry

Expired air samples were collected at rest and during the exercise tests, as described in section 2.4. For each exercise stage and rest period (fasting or post glucose), mean VO_2 and VCO_2 were used to calculate energy expenditure, by indirect calorimetry, using equations derived by Frayn (Frayn 1983).

Urinary nitrogen was not measured directly in the study, however, as a constant rate of nitrogen excretion has previously been described as $0.00011 g.kg^{-1}$ (Flatt et al. 1985; Melanson et al. 2005), and as there is no evidence to suggest ethnic differences in nitrogen excretion, this value was applied to the equations below, for all resting measurements, in all subjects with the constant N calculated as:

$$N\ (g.min^{-1}) = 0.00011 \times \text{body mass (kg)} \qquad \textbf{(Equation 2.1)}$$

Using Frayn's equations, VO_2 and VCO_2 were calculated as below:

$$VO_2\ (l.min^{-1}) = 0.746\ CHO + 2.03\ fat + 6.04\ N \qquad \textbf{(Equation 2.2)}$$

$$VCO_2\ (l.min^{-1}) = 0.746\ CHO + 1.43\ fat + 4.89\ N \qquad \textbf{(Equation 2.3)}$$

where CHO is carbohydrate and N is nitrogen excretion. Using the above equations, it was possible to calculate VO_2 and VCO_2, corrected for protein oxidation. Using equations 2.2 and 2.3 VO_2 and VCO_2 were calculated, also corrected for protein

oxidation, so it was possible to calculate non-protein oxygen consumption ($NPVO_2$) and non-protein carbon dioxide consumption ($NPVCO_2$). Non-protein respiratory quotient (NPRQ) was calculated using the following equation:

$NPVO_2$ ($l.min^{-1}$) = 0.746 CHO + 2.03 fat - 6.04 N **(Equation 2.4)**

$NPVCO_2$ ($l.min^{-1}$) = 0.746 CHO + 1.43 fat - 4.89 N **(Equation 2.5)**

NPRQ = $NPVCO_2$ / $NPVO_2$ **(Equation 2.6)**

Energy substrate oxidation was calculated as follows:

Fat oxidation ($g.min^{-1}$) = ($NPVO_2$ – $NPVCO_2$) / 0.6 **(Equation 2.7)**

Carbohydrate oxidation ($g.min^{-1}$) = (4.55 x $NPVCO_2$)

– (3.21 x $NPVO_2$) **(Equation 2.8)**

Protein oxidation ($g.min^{-1}$) = n x 6.25 **(Equation 2.9)**

Energy expenditure (EE) was calculated by multiplying the amount of substrate oxidised by the energy density value appropriate to each substrate according to the methods of Brody (Brody 1999) and Mottram (Mottram 1979):

EE (kJ) = (fat x 39) + carbohydrate x 1.55) + (protein x 17.0) **(Equation 2.10)**

Net energy expenditure and substrate utilisation were calculated by subtracting the baseline rate from the total energy expenditure/ substrate utilisation to give the rise above resting values.

2.10 Blood sampling

Three different techniques were used for blood sampling. Screening blood tests were collected by venepuncture, from an antecubital vein. During the metabolic assessment visit, regular venous blood samples were taken from a cannula, prior to adipose tissue and muscle biopsies, and at minutes 0, 30, 60, 90 and 120 post oral glucose tolerance test (75 grams of anhydrous glucose diluted in water and lemon juice). During exercise tests capillary blood samples were taken.

2.10.1 Venous blood

Venous blood was collected from an indwelling cannula (Biovalve, 18G/ 1.2 mm, Vygon, France), placed in a large antecubital vein, via a three-way tap with a 10 cm length of tubing (Connecta Plus, BD, Sweeden). Venous cannulae were flushed with non-heparinised saline (0.9% NaCl) to maintain patency, and blood samples were collected into 10 ml tubes containing K_3EDTA (BD Vacutainer systems, Plymouth UK). In instances where blood could not be successfully collected in this manner, venepuncture was used to take blood from an antecubital or forearm vein. Blood samples were immediately placed on ice and then into a refrigerated centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) within 15 minutes of collection and plasma and red blood cells were separated. Plasma was then pipetted into screwcap microtubes (2.0 ml, Alpha Laboratories, Hampshire, UK) in 0.5 ml aliquots and stored at -80^{o}C.

On the baseline blood sample (i.e. the very first sample taken before the biopsies), remaining red cells were washed with 5 mls of normal saline (0.9% NaCl) and

centrifuged at 2500 rpm for 10 minutes, and this was repeated twice. Red cells were pipetted into 2 ml screwcap microtubes in 1 ml aliquots, and stored at -80^{o}C.

2.10.2 Capillary blood

Capillary blood was collected during both the maximal and submaximal exercise tests in order to determine lactic acid concentration. During the maximal exercise test samples were taken at minutes one, 3 and 5 post-maximal effort, and during the submaximal exercise test samples were taken at the end of each four-minute stage (8 samples in total). Each sample was taken using a contact-activated lancet (Becton Dickinson & Co, Plymouth, UK) to pierce the skin on the right thumb, and blood was collected into a 20 µl micropipette (Blaubrand GmbH, Germany). The capillary blood was then dispensed into an Eppendorf tube containing 200 µl of 0.4 mol perchloric acid, mixed and immediately placed on ice. Within 1 hour of collection samples were centrifuged for 1 minute at 140,000 rpm. The supernatant was separated and stored at -80^{o}C.

2.11 Blood sample analysis

2.11.1 Screening blood tests

All study subjects had blood tests taken at screening for the following analyses:

- Urea and electrolytes
- Liver function tests
- Glucose
- Total cholesterol, HDL-cholesterol (LDL-cholesterol – calculated using Freidwald equation), Triglyceride.
- TSH, free T4

These analyses were performed in the biochemistry laboratory at Gartnavel General Hospital.

2.11.2 Insulin

Plasma insulin (appendix B1) was measured in the Vascular Biochemistry department at Glasgow Royal Infirmary using a commercially available ELISA kit which utilises a solid phase two-site enzyme immunoassay with <0.01% cross-reactivity with pro-insulin (Mercodia AB, Uppsala, Sweden). Coefficient of variation was <4% and all samples were analysed in duplicate by colleagues at Glasgow Royal Infirmary.

2.11.3 Spectrophotometric procedures

Plasma glucose (appendix B2), NEFA (appendix B3), triglyceride (appendix B4) and lactic acid (appendix B5) analysis were carried out at the University of Glasgow on defrosted fasting plasma samples (Roche diagnostics GmbH, Mannheim, Germany. Wako Chemicals GmbH, Germany). LDL-cholesterol concentrations were calculated using the Friedwald equation (Friedewald, Levy, & Fredrickson 1972). All measurements of glucose, TG and NEFA were performed on a Cobas Mira Plus (ABX Diagnostics, France). Lactic acid was measured on an Ascent flourometer (Thermo scientific, Surrey UK). Coefficient of variation for spectrophotometric analyses was <3.1%. These analyses were all carried out by the author and colleagues at the University of Glasgow.

2.11.4 Radioimmunoassay procedures

Leptin (appendix B6) was measured by colleagues at Glasgow Royal Infirmary using a radioimmunoassay method developed internally.

2.11.5 Inflammatory markers

Adiponectin, IL-6 and TNF-α concentrations were also measured by colleagues at Glasgow Royal Infirmary and were determined by ELISA (R+D systems Europe, Abingdon, UK)

2.11.6 Accuracy of blood anaylsis procedures

Quality control sera were used to ensure accuracy of all of the above analyses (Mercodia AB, Uppsala, Sweden. Phoenix Europe GmbH, Karlsruhe Germany. Roche Diagnostics GmbH, Mannheim, Germany, Randox Laboratories Ltd, Co Antrim, Ireland. Wako Chemicals GmbH, Germany). For all analyses, samples were tested in 2 separate batches, each containing samples from an equal number of South Asian and European subjects.

2.12 Metabolic Assessment

During the final study visit, subjects attended fasting and had a resting expired air collection taken, using the ventilated hood system. Following this, a cannula was inserted and after 10 minutes the first blood sample was taken. Skeletal muscle biopsies were then performed. A further blood sample was taken and then a drink containing 75 grams of glucose was taken over a 3 minute period. Further blood samples were taken at 30 minute intervals thereafter for 2 hours, and then the resting expired air collection was repeated. Because of a concern about the possibility of stress-induced hyperglycaemia following muscle biopsy, pre and post biopsy blood samples were taken for measurement of baseline glucose and insulin.

2.12.1 Percutaneous skeletal muscle biopsy using semi-open technique

With the subject still lying supine on the examination couch, the biopsy site was identified by measuring 20 cm above the right patella and projecting a line laterally. The skin was marked at this site, which overlies the lateral aspect of the *vastus lateralis* muscle.

A sterile drape was then tucked under the subject's leg and the skin surrounding the marked site was cleaned with sterile swabs and iodine solution. The skin was infiltrated with approximately 10ml of 2% lidocaine, and 10 minutes were allowed for the anaesthetic effect to occur.

An incision was made to the skin at the biopsy site with a sterile scalpel approximately 1 centimetre wide and deep, and biopsy forceps (Weil-Blakesley forceps, Gowlands Ltd, Croydon, UK) were inserted. Subcutaneous tissue was blunt - dissected using the forceps until the muscle layer was reached, and a small amount of muscle tissue, approximately 400mg, was removed and dispensed onto a piece of muslin stretched over a plastic box.

A sterile swab and pressure were applied and then a further sample was taken. Pressure was then applied for 10 minutes, after which the wound was closed with paper stitches (Luekostrip skin closures, Smith+Nephew, London, UK) and covered with a sterile dressing (Mepore dressing, Molnlyke healthcare, Gothenburg, Sweden).

All subjects were advised to avoid vigorous exercise for 24 hours.

2.13 Analysis of muscle samples

2.13.1 Determination of skeletal muscle insulin signaling protein expression

Immediately following biopsy, samples were divided into 6 separate pieces. Four of the 6 pieces were snap frozen in liquid N_2 and 2 pieces were allowed to recover for 30 minutes in 10 ml of Krebs-Ringer-Hepes buffer (118 mM NaCl, 25 mM Hepes-NaOH, pH 7.4, 5 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 5mM glucose, 0.1% (w/v) bovine serum albumin) at 37^{o}C, gassed with 100% O_2 prior to incubation for 10 min. They were then incubated in the presence or absence of human soluble insulin (Actrapid, NovoNordisk, Bagsvaerd, Denmark) at a concentration of 10 nmol/l for 10 minutes, after which both samples were placed into screw cap microcentrifuge tubes, snap frozen in liquid N_2 and then stored at -80^{o}C.

Muscle samples were placed in a Dounce homogenizer with homogenisation buffer (50mM Tris (hydroxymethyl) aminomethane (pH 7.5 at 4^{o}C), 250mM sucrose, 1mM EDTA, 1mM EGTA, 5mM NaF, 5mM NaPPi, 250mM mannitol, 1mM DTT, 0.1mM benzamidine, 0.1mM PMSF, 1mM vanadate, 5µg/ml SBTI) at a concentration of 1:8 volumes. Lysates were created by passing the glass pestle up and down the tube 20 times, until the samples were fully homogenized.

Samples were then transferred into microcentrifuge tubes and centrifuged at 350,000g for 30 minutes to separate the cytosolic fraction. The supernatant was pipetted into separate tubes and stored at -80^{o}C. The pellet was then resuspended in the same homogenisation buffer (at a concentration of 1:3 volumes) with 1% (v/v) NP-40 and

centrifuged at 100,000g for 60 minutes, after which the supernatant, containing the membrane (particulate) fraction, was pipetted into separate tubes.

Protein content of each sample was calculated according to the method of Bradford (Bradford 1976). Samples were loaded (10-20µg / lane) onto an 8% SDS-polyacrylamide gel, resolved by SDS-PAGE and transferred to nitrocellulose prior to Western Blotting. Blots were probed with commercially available antibodies raised against insulin receptor (insulin receptor (β subunit), insulin receptor substrate (IRS) proteins –1 and –2, phosphatidylinositol 3'-kinase (p85 and p110 subunits), PTEN, PKB (α and β), GLUT4 and PKC isoforms. Resultant bands were quantified by densitometry and compared with an internal standard to assess expression levels.

Phosphorylation of PKB at Ser473 was assessed in the muscle homogenates described above by Western blotting using phosphorylation site-specific antibodies.

2.13.2 Determination of skeletal muscle expression of oxidative and lipid metabolism genes

Skeletal muscle RNA was extracted using an E.Z.N.A. Tissue RNA kit (Omega Bio-Tek, Inc., Norcross, GA) according to the manufacturer's protocol using Precellys Ceramic Homogenisation beads (CK14, PEQLAB Ltd, Farnborough, UK) and a Hybaid Ribolyser (Thermo Scientific, Loughborough, United Kingdom). cDNA was synthesised using an Applied BioSystems High Capacity cDNA Reverse Transcription kit (Life Technologies Corporation, Carlsbad, CA) with random hexamers according to the manufacturers protocol, but doubling the volumes to increase yield. Quantitative polymerase chain reaction (qPCR) assays were designed

for each transcript using the Roche Universal Probe Library Assay Design Center (http://www.roches-applied-science.com/)

Messenger RNA (mRNA) expression levels of transcripts were measured in triplicate using the Universal Probe Library Set (Roche diagnostics GmbH, Mannheim, Germany.) and Absolute QPCR Mix (ABGene, Epsom, UK). Each reaction contained 1.35µL of each primer (10 µmol/L stock), 0.15 µL probe, amd 1µL complementary DNA in a final volume of 15 µL. Cycle threshold outliers (C_1) were removed using the median absolute deviation method (0.6745 * absolute difference of data point from median/ median) with a maximum acceptable threshold of 3.5. Average amplification efficiency for each assay was determined using version 7.5 of LinReg PCR (Ramakers et al. 2003) after exclusion of C_t outlier data points and efficiency values lying greater than ± 1.96 standard deviations (SDs) from the mean. Non- C_t outliers were averaged for each triplicate and corrected for amplification efficiency (C_t * log [mean efficiency]/ log). For each assay, reactions on multiple plates were required, so control samples were run on plates with C_t values on subsequent plates to be directly comparable to those on plate 1, using the formula $C_{t\ (\text{on plate X})}$ + "average C_t control samples plate 1" – "average C_t control samples plate X".

Four potential calibrator genes (ACTB, GAPDH, RPLP0 AND RLP10) were compared for stability under experimental conditions using NormFinder (Andersen, Jensen, & Orntoft 2004) RPLP0 was found to be the most stable across all subject groups and intervention conditions. The corrected C_t values for each target gene and RPLP0 were used to calculate the *delta* C_t and the average *delta* C_t of the baseline

control group was used to calculate the *delta delta* C_t. The relative expression values used for statistical analyses were given by $2^{-delta\ delta\ Ct}$.

2.13.3 Determination of skeletal muscle mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratio

DNA was extracted from muscle using a QIAamp DNA Mini Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's protocol (including the RNase treatment) using Precelly's Ceramic Homogenisation beads (CK14, PEQLAB Ltd, Farnborough, United Kingdom, and a Hybaid Ribolyser (Thermo Scientific, Loughborough, United Kingdom). qPCR assays were designed using the Roche Universal ProbeLibrary Assay Design Center, for the mitochondrial genome overlapping two neighbouring genes (tRNA leucine 1 and NADH dehydrogenase 1) and the nuclear genome using the β2-adrenergic receptor gene promoter. Relative DNA levels were determined in a similar fashion to the mRNA expression levels.

All analyses of muscle samples in sections 2.13.1 -2.13.3 were carried out by colleagues at the University of Glasgow (Dr Gillian Milne and Dr Colin Moran).

2.14 Power calculation

Sample size calculation was based on differences in insulin sensitivity and VO_2max between the South Asian and European groups. Based on data from the Southall study, 2-hour post-glucose load insulin concentrations were ~2.1 times higher in South Asians than BMI-matched Europeans (41 *vs* 19 $\mu U.ml^{-1}$) and the population standard deviations (SD) in 2-hour insulin concentrations were 10 $\mu U.ml^{-1}$ (52% of the mean) and 38 $\mu U.ml^{-1}$ (94%), respectively, for the European and South Asian

populations(McKeigue et al. 1991). Assuming a population SD of 100%, 20 subjects in each group would be needed to detect a 90% difference in 2-hour insulin between South Asians and Europeans with 80% power at $\alpha = 0.05$. VO_2max values in sedentary South Asians are typically ~15% lower than comparable sedentary European groups(Hardy & Eston 1985; Verma et al. 1979) and the population SD in VO_2max for healthy young sedentary European/South Asian adults is ~15% (Hardy & Eston 1985; Verma et al. 1979). Groups of 20 subjects would enable us to detect a 13% difference in VO_2max between South Asians and Europeans with 80% power at $\alpha = 0.05$. This number of subjects (i.e. 20 per group) would enable us to detect significant correlations between variables of 0.42 at $\alpha = 0.05$ within each group separately and of 0.30 at $\alpha = 0.05$ for the two subject groups combined. Correlations in the literature between insulin sensitivity and indices of oxidative capacity/fat oxidation rates (e.g. VO_2max, oxidative enzyme activities, fasting fat oxidation) are in the order of 0.6 to 0.8 (Bruce et al. 2003; Nyholm et al. 2004). To allow for 20% dropout the initial plan was to recruit 25 subjects in each group.

2.15 Statistical Methods

Data were analysed using Statistica (version 6.0, StatSoft Inc., Oklahoma) and Minitab (version 14, Minitab Inc., Pennsylvania). Data were tested for normality using the Ryan-Joiner normality test and transformed as appropriate. Box-Cox plots were used to determine the most appropriate transformation for data which did not follow a normal distribution.

General linear models were used to compare data between European and South Asian groups. As age and BMI were not identical between the two groups, statistical

analysis of all variables was undertaken both unadjusted and after adjustment for age and BMI, to confirm that this did not influence the study outcomes. Further adjustment for other variables was undertaken as appropriate to determine whether observed differences between groups in variables of interest were independent of possible confounders.

Univariate regression analysis was used to determine relationships between variables. A homogeneity-of-slopes regression model was used to identify whether the slope of relationships between variables differed between the South Asian and European groups. Where the slopes differed significantly between the groups, univariate regressions were performed for the South Asian and European groups separately, otherwise univariate regressions were performed on the combined group to maximize statistical power.

To determine the extent to which relationships between variables were in independent of confounders, partial correlations were undertaken adjusting for potential confounders (such as such as age, BMI, fat mass and physical activity level). This classic statistical approach has been used previously to determine the independent effects of individual variables within multi-factorial systems on biological outcomes (Speakman et al. 2002; Westerterp & Speakman 2008).

Statistical significance was accepted at the $p < 0.05$ level.

Chapter 3

Effects of lifestyle, adiposity and oxidative capacity on insulin resistance in South Asian and European men

3.1 Introduction

The association of cardiorespiratory fitness with skeletal muscle fat oxidation is evidenced by the strong relationship between maximum oxygen uptake (VO_{2max}) and skeletal muscle oxidative enzymes in Europeans (Helge et al. 2006; Sahlin et al. 2007; Venables et al. 2005). VO_{2max} is a strong predictor of whole-body oxidative capacity and insulin sensitivity in Europeans, independent of visceral adiposity and family history of type 2 diabetes (Bruce et al. 2003; Nyholm et al. 2004).

As described in chapter 1, functional measures of substrate utilisation at the limb or whole-body level have demonstrated this relationship between skeletal muscle fatty acid oxidative capacity and insulin sensitivity, with the discoveries of reduced whole-body fatty acid oxidation in obese individuals (Kelley et al. 1999; Kim et al. 2000) and reduced fat oxidation across the leg in individuals with type 2 diabetes (Kelley & Simoneau 1994), both of which correlate strongly with insulin sensitivity (Kim et al. 1999). In keeping with this finding, low VO_{2max} values are often (Bruce et al. 2003; Melanson et al. 2005; Nyholm et al. 2004) but not always (Ostergard et al. 2006) associated with insulin resistance in white American and European populations.
A low level of cardiorespiratory fitness is an independent predictor of type 2 diabetes (Wei et al. 1999), and is likely that fitness influences insulin sensitivity, at least in part, via effects on muscle lipid metabolism (Goodpaster & Brown 2005; Goodpaster et al. 2003), as trained individuals oxidize more fat during exercise than untrained

individuals at any given exercise intensity (Holloszy 1967). In addition, significantly lower VO_{2max} values (~10-15% lower) have been found in relatives of patients with type 2 diabetes, who are more insulin resistant than matched controls (Ezenwaka et al. 2001; Humphriss et al. 1997; Perseghin et al. 1997), and have about three times the risk of developing diabetes compared to those with no diabetes family history (Kobberling & Tillil 1982; Ohlson et al. 1988), and compared with age, BMI and body-fat matched controls (Nyholm et al. 2004; Thamer et al. 2003).

Further evidence that skeletal muscle fatty-acid oxidation contributes to insulin sensitivity is described in detail in chapter 1. Briefly, activities of the skeletal muscle mitochondrial enzymes responsible for both fat oxidation and transport of fatty acids into the mitochondria (β–HAD, citrate synthase, CPT1) (Bruce et al. 2000; Simoneau et al. 1999), have been found to correlate strongly with whole-body insulin sensitivity in Europeans, and the enzyme ACC β, which down-regulates fat oxidation, is reduced following weight loss in obese subjects, and these reductions in ACC β correlate strongly with reductions in fasting insulin concentrations (Simoneau et al. 1999).

Limited data indicate that a difference in whole body oxidative capacity is also evident when comparing men of South Asian origin with those of European descent – VO_{2max} values in sedentary South Asians are typically ~15% lower than those in comparable sedentary European groups (Hardy & Eston 1985; Verma et al. 1979). One group of investigators (Hardy & Eston 1985) performed measurements of body composition in addition to measurements of cardiorespiratory fitness, and no significant anthropometric differences were observed between the two groups. Neither study measured insulin sensitivity, nor examined the relationship between

insulin sensitivity and cardiorespiratory fitness. This difference in fitness could, at least partly explained by the low levels of physical activity typically seen in South Asians (at least, those living in the UK) compared with their European counterparts (Barnett et al. 2005; Dhawan & Bray 1997; Fischbacher et al. 2004; Hayes et al. 2002; Health Education Authority 2000).

Taking into account the known relationships between VO_{2max}, whole-body fat oxidation (the majority of which takes place in skeletal muscle) and insulin sensitivity, it is possible to speculate that although increased fatty acid delivery to skeletal muscle could contribute to increased skeletal muscle insulin resistance in South Asians, reduced capacity for fatty acid oxidation may also play a role in inducing a mismatch between fatty acid uptake and fatty oxidation in skeletal muscle.

In addition to skeletal muscle function and fitness, dietary factors, such as the quantity and quality of fat and carbohydrate intake, may also influence insulin sensitivity, although until recently there had not been any large intervention studies examining this issue. Previous studies, one cross sectional (Maron et al. 1991), and one intervention involving very small numbers (Lovejoy et al. 1998) suggested that there was a positive correlation between total and saturated dietary fat intake and fasting insulin concentrations, independent of weight. The recent RISCK trial (Jebb et al. 2010), however, was a large controlled intervention trial examining the effect of diets which were high or low in saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) and which had high or low glycaemic index. The investigators found that the type of fat consumed (i.e. replacing SFAs with MUFAs) had no effect on

insulin sensitivity, although there was a non- significant trend towards improved insulin sensitivity with a low fat diet.

Evidence regarding the effect of carbohydrate quantity and type on insulin sensitivity is less well established. The popular "Atkins diet" fuelled interest in the effects of carbohydrate intake on insulin sensitivity, and subsequently some small intervention studies found improvements in insulin sensitivity in non-diabetic individuals on a very low carbohydrate diet (Meckling et al. 2002; Meckling et al. 2004; Volek et al. 2004). Other studies, however, had conflicting results, with at least one small study demonstrating improved insulin sensitivity in patients with type 2 diabetes on a high carbohydrate diet (Sargrad et al. 2005). Clearly, further larger studies are required to clarify the question of whether carbohydrate quantity affects insulin sensitivity. Carbohydrate quality may also exert an effect and it has been suggested that low glycaemic index (GI) diets may improve insulin sensitivity, although study methods and findings are inconsistent. Several small studies seem to support this theory (Aston 2006; Frost et al. 1996; Frost et al. 1999), but the RISCK trial found no effect on insulin sensitivity of a low GI compared to a high GI diet.

The effect of dietary protein intake on insulin sensitivity is also unclear, with some studies finding no effect of high protein intake (Sargrad et al. 2005) and others suggesting beneficial effects on insulin sensitivity of a high protein diet in both animal models (Belobrajdic et al. 2004) and in patients with type 2 diabetes (Linn et al. 1996; Parker et al. 2002).

The fact that diet influences insulin sensitivity suggests that dietary differences between South Asian and European populations are likely to be relevant in determining causes of insulin resistance in South Asians. As mentioned in chapter 1, however, there is no real evidence that UK South Asians have poorer diets than the UK population as a whole (McKeigue et al. 1985; Miller et al. 1988; Sevak et al. 1994), indeed diets of UK South Asians have been reported to be, if anything, generally healthier than those of the general population, with lower total energy intakes, higher intakes of complex carbohydrate, vegetable fibres and polyunsaturated fatty acids (PUFAs) (Sevak et al. 1994), so there is no clear evidence that differences in insulin sensitivity and diabetes risk are explained by unhealthy dietary habits. It could be the case, however, that South Asians are more susceptible to metabolic consequences from the same degree of dietary Westernisation as Europeans.

The association of adipose tissue function, including production of hormones, adipokines and pro-inflammatory cytokines with insulin resistance is also important, and has been discussed fully in chapter 1. Data suggest that differences in circulating concentrations of these hormones and cytokines, indicating possible differences in adipose tissue functionality, may also be associated with insulin resistance in South Asians (Anand et al. 2000; Chambers et al. 2001; Forouhi et al. 2003; Forouhi et al. 2001; Kain et al. 2001; Petersen et al. 2006; Ryo et al. 2004). This hypothesis requires to be tested further by examination of gene and protein expression of adipokines within the adipose tissue of South Asians and Europeans, and studies in this area are in progress.

The first aim of this part of the study was, therefore, to investigate whether there were any phenotypic differences between South Asians and Europeans in body composition, insulin sensitivity and other metabolic variables including fatty acids and adipokines, and to examine any relationships between these variables. The second aim was to investigate whether there were any behavioural factors (e.g. in diet or physical activity levels) which might explain previously described differences in insulin sensitivity.

3.2 Methods

3.2.1 Volunteers

Volunteers were recruited, screened and enrolled in the study as detailed in section 2.2. Specific inclusion criteria are also defined in this section. Further information detailing recruitment response and excluded volunteers can be found in Figure 2.1. The South Asian group was composed of 20 men of Indian, Pakistani and Bangladeshi origin. The European group was composed of 20 men of British, French Scandinavian origin, although one European man was subsequently excluded form the final data analysis, because of the discovery, during the study, of a diagnosis of diabetes.

South Asians and Europeans were well matched for age (South Asians 26.9 ± 3.9 years, Europeans 24.5 ± 5.5 years, $p = 0.12$) and BMI (South Asians 23.6 ± 2.9 $kg.m^{-2}$, Europeans 22.6 ± 2.7, $p = 0.31$).

3.2.2 Study design

All participants underwent evaluation of physical characteristics, body composition, and cardiorespiratory fitness, and participated in metabolic assessment as detailed in sections 2.3, 2.4, 2.6 and 2.12. All subjects also completed a food questionnaire and physical activity questionnaire.

3.2.3 Evaluation of energy and macronutrient intake

In order to calculate energy and macronutrient intake, all subjects completed a 120-item food frequency questionnaire (appendix A5) (Fehily, Yarnell, & Butland 1987).

3.2.4 Evaluation of habitual physical activity

In order to evaluate habitual physical activity levels, the long version of the validated International Physical Activity Questionnaire (IPAQ) was also completed (appendix A4) (Craig et al. 2003).

3.2.5 Evaluation of cardiorespiratory fitness

Cardiorespiratory fitness was measured by predicted VO_{2max} and lactate threshold, as described in section 2.7.

3.2.6 Metabolic testing

As described in section 2.12, after a 12-hour fast, all subjects attended the metabolic investigation suite at the Institute of Diet, Exercise and Lifestyle (IDEAL). Resting metabolic rate was measured and substrate utilisation calculated (see sections 2.6.2 and section 2.9) and fasting blood samples were taken (section 2.10.1). A percutaneous skeletal muscle biopsy, using the semi-open technique described by

Henriksson (Henriksson 1979) (see section 2.12.1) was preformed and samples were immediately snap frozen in liquid nitrogen. An oral glucose load was then consumed (75g of glucose in 300ml) and blood samples were taken for glucose, insulin, NEFA and TG at 30-minute intervals for 2 hours.

3.2.7 Blood sample analysis

After centrifugation all plasma samples were stored at -80ºC in labelled 2 ml Eppendorf tubes (Alpha Laboratories, Hampshire, UK). Glucose, NEFA, total cholesterol, HDL-cholesterol, TG and CRP were all measured by spectrophometric techniques as described in Section 2.11.3. LDL-cholesterol was calculated by the Friedewald equation (Friedewald, Levy, & Fredrickson 1972). Insulin, adiponectin, resistin, leptin, TNFα, IL-6 were measured by ELISA technique as described in section 2.11.5

3.2.8 Calculation of glucose, insulin and NEFA concentrations and insulin sensitivity during oral glucose tolerance test

The mean concentration of glucose, insulin and NEFA during the oral glucose tolerance test (OGTT) was calculated by dividing the area under variable *vs*. time curve (total AUC) by the duration of the OGTT (mean during OGTT). The rise (or suppression) of glucose, insulin and NEFA was calculated by subtracting the area under the fasting concentration vs. time curve from the total AUC and dividing the obtained value by the duration of the OGTT (rise during OGTT). For variables where values decreased from baseline after glucose ingestion (e.g. NEFA), the rise during OGTT was negative and represented the area above the curve extending up to the baseline value, providing an index of post-glucose suppression. Insulin sensitivity was

calculated using the Insulin Sensitivity Index (ISI) as described by Matsuda and DeFronzo (Matsuda & DeFronzo 1999).

3.2.9 Analysis of muscle samples for quantification of mitochondrial enzyme activity/ gene expression

This analysis was performed as described in section 2.13.2.

3.2.10 Analysis of muscle samples to determine skeletal muscle mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratio.

This analysis was performed as described in section 2.13.3.

3.2.11 Statistical analysis

Data were analysed using Statistica (version 6.0, StatSoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennsylvania). Prior to analysis, all data were tested for normality using the Anderson-Darling normality test and, if necessary, logarithmically transformed. All data between South Asians and Europeans was compared using unpaired t-tests. Univariate linear regressions were performed, using logarithmically transformed data where required. Correlations between ISI and other variables were performed for the South Asians and European groups.

To determine the extent to which variables were related to ISI independently of age, BMI and fat mass, univariate linear regressions were then performed between the residuals for ISI of the regression between ISI and age, ISI and BMI and ISI and total fat mass. This, in effect, provides the correlations between ISI and other variables adjusted for the effect of age, BMI and total fat mass. Significance was accepted at $p \leq 0.05$.

3.3 Results

3.3.1 Demographic and heath screening data

All volunteers lived in Glasgow at the time of the study, with 18 of the European men and 4 of the South Asian men having lived in the UK for their whole lives. Sixteen of the South Asians who were born outside the UK, and mean (± SD) duration of UK residence was 2.5 ± 5 years, although 11 of them had been resident in the UK for less than 1 year. Ten of the South Asians were Indian, 9 were Pakistani and one was Sri Lankan, and all of the Europeans were from northern Europe, 18 being British. One European volunteer was French and 1 was British/Finnish.

There was no ethnic difference in mean DepCat score, which is a measure of socioeconomic deprivation, with a scale ranging from DepCat 1, which is most affluent, to DepCat 7, which is most deprived (Carstairs & Morris 1991). Mean (± SD) DepCat scores were 4.7 ± 1.9 in South Asians, and 5.2 ± 1.6 in Europeans ($p = 0.65$).

All participants were non-smokers, aged 18-40 years, in generally good health, with blood pressure <160/90 mmHg, and no known history of diabetes or cardiovascular disease. The only volunteers taking any medication were one European using steroid and β2 agonist inhalers for asthma and topical steroids for eczema and another European using topical steroids for eczema.

3.3.2 Anthropometric characteristics and body composition data

A summary of anthropometric data is shown in Table 3.1. There was no significant difference in age, body mass or BMI between South Asians and Europeans in

unadjusted analysis, but South Asians were shorter in height with significantly higher total, trunk, arm and leg fat mass and, with correspondingly lower total, trunk, arm and leg lean mass than the Europeans. These differences persisted after adjustment for age and BMI. Further adjustment for fat mass abolished differences in trunk, arm and leg fat between groups, indicating no significant differences in regional fat distribution between South Asians and Europeans. Adjustment for physical activity did not affect these data. There was no significant difference in waist or hip circumference or in waist-hip ratio between the two groups.

Table 3.1 Anthropometric and body composition data.

	South Asians	Europeans	p-value	Age and BMI-adjusted p-value	Age, BMI and fat mass- adjusted p-value
Age (years)	26.9 ± 3.9	24.5 ± 5.5	0.12	-	-
BMI ($kg.m^{-2}$)	23.6 ± 2.9	22.6 ± 2.7	0.31	-	-
Body mass (kg)	71.8 ± 10.1	72.5 ± 8.8	0.82	0.13	0.034
Height (cm)	174.4 ± 7.1	179.0 ± 6.8	0.046	0.11	0.028
Total fat mass (kg)	18.4 ± 5.3	13.6 ± 5.2	**0.007**	**< 0.0005**	-
Trunk fat mass (kg)	10.0 ± 3.3	7.1 ± 3.3	0.009	< 0.0005	0.520
Arm fat mass (kg)	1.4 ± 0.5	1.0 ± 0.5	0.02	0.008	0.606
Leg fat mass (kg)	6.3 ± 1.6	5.0 ± 1.5	0.009	0.004	0.618
Fat-free mass (kg)	53.4 ± 6.6	58.9 ± 5.9	**0.010**	**0.001**	**0.034**
Total lean mass (kg)	50.0 ± 5.8	56.3 ± 5.6	**0.001**	**< 0.0005**	**0.009**
Trunk lean mass (kg)	22.7 ± 2.8	26.4 ± 2.6	**< 0.0005**	**< 0.0005**	**0.003**
Arm lean mass (kg)	6.2 ± 0.8	6.5 ± 0.9	0.25	0.08	0.327
Leg lean mass (kg)	17.5 ± 2.3	19.5 ± 2.1	**0.007**	**0.002**	**0.026**
Waist circumference (cm)	82.3 ± 7.5	78.8 ± 6.8	0.14	0.30	0.778
Hip circumference (cm)	97.3 ± 5.8	96.6 ± 1.1	0.69	0.59	0.463

Values are mean ± SD

3.3.3 Diet data

There was no significant difference in total daily energy intake or in macronutrient intake between the two groups. Self reported alcohol intake was significantly higher in the European group than the South Asian group, and other notable differences

included significantly higher dietary calcium, Vitamin D and Vitamin C intake in the European group. These data are illustrated in Tables 3.2 and 3.3.

Table 3.2 Macronutrient intake data

	South Asians	**European**	**p-value**
Total energy intake ($MJ.day^{-1}$)	8.8 ± 2.0	9.4 ± 2.0	0.41
Fat ($g.day^{-1}$)	71 ± 18	80 ± 26	0.19
Carbohydrate ($g.day^{-1}$)	290 ± 90	260 ± 50	0.20
Protein ($g.day^{-1}$)	87 ± 17	98 ± 25	0.12

Values are mean ± SD

Table 3.3 Micronutrient and alcohol data

	South Asians	**European**	**p-value**
Vitamin C ($mg.day^{-1}$)	82.1 ± 20.8	112.3 ± 52.2	**0.02**
Vitamin D ($\mu g.day^{-1}$)	1.88 ± 1.2	3.61 ± 2.6	**0.01**
Calcium ($mg.day^{-1}$)	1046 ± 177.7	1232 ± 247.1	**0.01**
Vitamin B12 ($\mu g.day^{-1}$)	6.3 ± 1.8	8.04 ± 2.90	**0.03**
Vitamin B6 ($mg.day^{-1}$)	2.1 ± 0.5	2.57 ±0.72	**0.02**
Riboflavin ($mg.day^{-1}$)	2.1 ± 0.4	2.44 ± 0.53	**0.01**
Sodium ($mg.day^{-1}$)	1779.6 ± 499.7	2310.7 ± 598.0	**0.004**
Zinc ($mg.day^{-1}$)	9.9 ± 1.8	12.1 ± 3.2	**0.008**
Alcohol ($g.day^{-1}$	3 ± 7	20 ± 17	**0.0002**

Values are mean ± SD

Interestingly, the South Asians consumed significantly more complex carbohydrates (i.e. dietary starch) than the Europeans, although there was no difference between the groups in consumption of simple carbohydrate and no difference in glycaemic index or glycaemic load between the two groups. Data regarding carbohydrate type and quality are shown in Table 3.4. Dietary intake of the B-vitamins B12, B6 and riboflavin was also significantly lower in the South Asian group, as was intake of

elemental sodium, zinc and chloride. There was no significant difference in consumption of any other mineral

Table 3.4 Carbohydrate data

	South Asians	European	p-value
Complex carbohydrate ($g.day^{-1}$)	181.4 ± 61.2	140.8 ± 30.3	**0.01**
Simple carbohydrate ($g.day^{-1}$)	107.6 ± 36.9	118.0 ± 31.1	0.35
Glycaemic index	49.8 ± 4.3	47.2 ± 5.0	0.08
Glycaemic load	145.0 ± 48.3	122.4 ± 25.6	0.08

Values are mean ± SD

3.3.4 Metabolic data

There was no significant difference in fasting pre and post biopsy glucose and insulin concentrations in either group. There was no significant difference in fasting glucose or insulin concentrations between South Asians and Europeans. Two hours post oral glucose tolerance test (OGTT) glucose concentrations were not significantly different in the two groups, nor was the area under the curve (AUC) of plotted glucose concentrations, over the 2 hour period of the GTT.

However, insulin concentration 2 hours post GTT and insulin AUC were significantly higher in South Asians, compared to Europeans, both in unadjusted analysis and following adjustment for age, BMI and fat mass. Adjustment for trunk fat mass, rather than total fat mass did not alter the findings, but adjustment for percentage body fat, rather than total fat mass resulted in loss of the significant difference in 2-hour ($p = 0.13$) insulin. These data are illustrated in Figure 3.1.

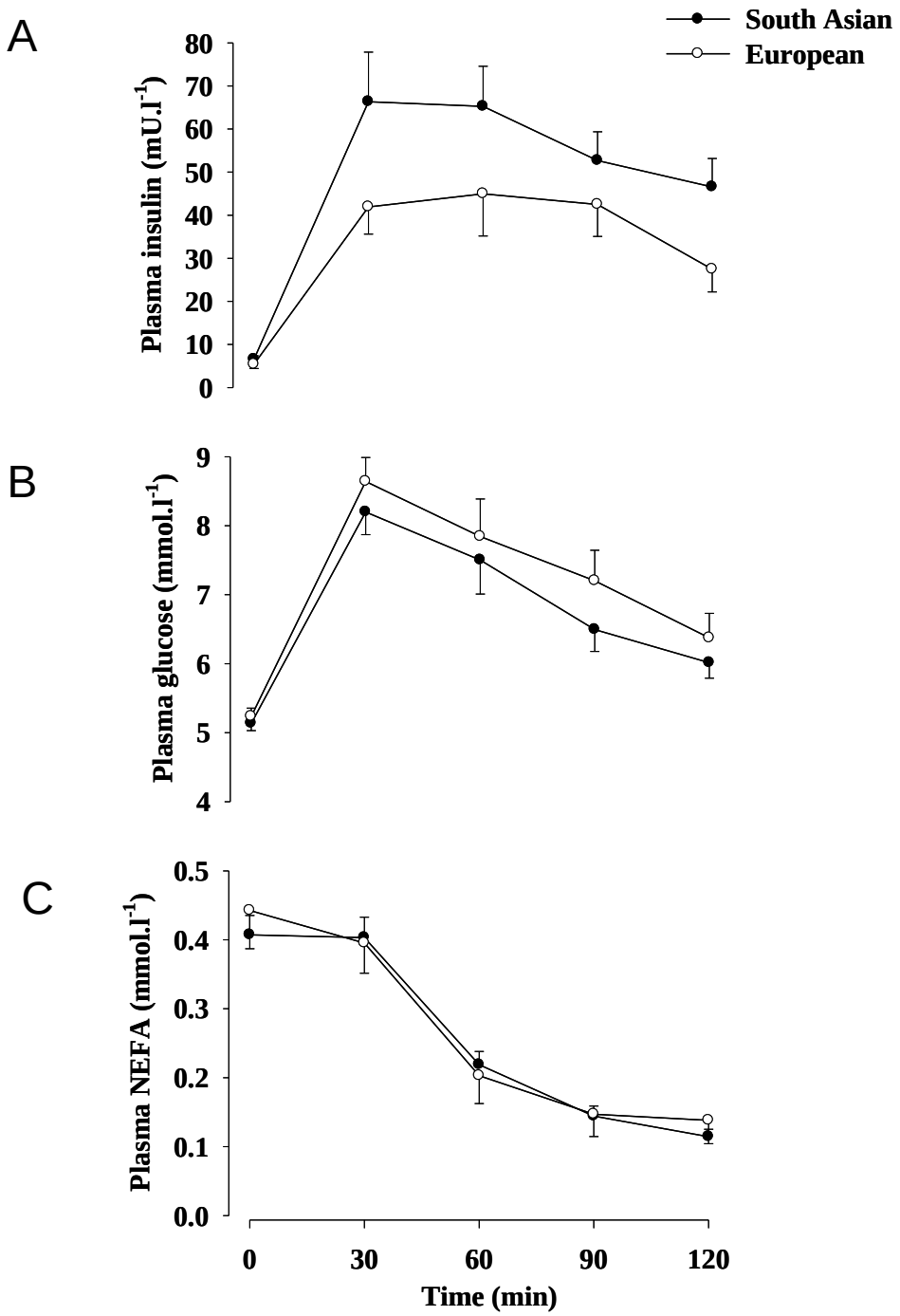

Figure 3.1 Plasma insulin (A), glucose (B) and NEFA (C) concentrations in South Asians and European subjects, fasting, and in responses to a 75g oral glucose tolerance test.

Insulin sensitivity index was significantly lower in South Asians, and fasting HDL concentration was also significantly lower, both differences which were retained after adjustment for age, BMI and fat mass and also after adjustment for percentage body fat, rather than total fat mass. When ISI values were further adjusted for VO_{2max} or for the rate of fat oxidation during sub-maximal exercise at 55% VO_{2max}, the significant difference between South Asians and Europeans was abolished ($p = 0.16$ and 0.12 respectively). There was no observed significant difference between the 2 groups in insulinogenic index, which is a measure of β-cell function, and no significant difference in fasting NEFA concentration, NEFA AUC, fasting total cholesterol, triglyceride, AST or ALT concentrations. Resting metabolic rate was significantly lower in South Asians but this difference was abolished after adjustment for fat mass. There was no difference in rate of fat oxidation (expressed per kg fat-free mass) between the groups. These data are summarised in Table 3.5.

Table 3.5 Metabolic data

	South Asians	Europeans	p-value	Age and BMI adjusted p-value	Age,BMI and fat mass adjusted p-value
Fasting glucose* ($mmol.l^{-1}$)	5.14 ± 0.47	5.24 ± 0.52	0.53	0.50	0.94
Fasting insulin* ($mU.l^{-1}$)	6.56 ± 3.53	5.39 ± 4.20	0.11	0.17	0.023
Glucose AUC ($mmol.l^{-1}.h$)	13.89 ± 2.35	14.74 ± 2.82	0.35	0.46	0.74
Insulin AUC ($mU.l^{-1}.h$)	105.4 ± 53.6	72.9 ± 48.0	**0.036**	**0.045**	0.099
2 hour insulin* ($mU.l^{-1}$)	46.6 ± 29.6	27.5 ± 5.3	**0.017**	**0.031**	**0.043**
Insulin sensitivity index†	5.89 ± 2.93	7.96 ± 3.49	**0.048**	**0.047**	**0.012**
Insulinogenic index Φ*	19.20 ± 14.75	11.10 ± 6.10	0.075	0.57	0.52
Fasting NEFA* ($mmol.l^{-1}$)	0.41 ± 0.13	0.44 ± 0.57	0.85	0.99	0.85
NEFA AUC* ($mmol.l^{-1}.h$)	0.51 ± 0.16	0.52 ± 0.28	0.38	0.39	0.60
Fasting total cholesterol ($mmol.l^{-1}$)	4.46 ± 0.89	4.07 ± 0.85	0.17	0.37	0.34
Fasting HDL cholesterol ($mmol.l^{-1}$)	1.08 ± 0.22	1.37 ± 0.20	< **0.0005**	**0.001**	**0.002**
Fasting TG* ($mmol.l^{-1}$)	1.18 ± 0.65	0.87 ± 0.56	0.054	0.13	0.13
Resting metabolic rate ($kJ.kg\ fat\text{-}free\ mass^{-1}.day^{-1}$)	114.1 ± 9.0	118.6 ± 10.8	0.170	0.378	0.059
Resting fasted fat oxidation ($mg.kg^{-1}$fat-free mass.min^{-1}	1.47 ± 0.44	1.38 ±0.43	0.548	0.510	0.862

Values are mean ± SD. *statistical analysis performed on log transformed data,

†statistical analysis performed on square-root transformed data. Φ insulinogenic index

= insulin (30-0)/glucose (30-0)

3.3.5 Physical activity and exercise test data

Although it is possible that the physical activity questionnaire used may not have been sufficiently sensitive to detect real differences in physical activity, no significant difference in habitual physical activity level between the two groups was demonstrated, nor was therea difference in most individual activities contributing to this, including walking and work-related activity. The only type of physical activity where there was a reported difference between the 2 groups was cycling, with Europeans reporting significantly more cycling than South Asians. Physical activity data are shown in Table 3.6.

Table 3.6 Physical activity data

	South Asians	**European**	**p-value**
Sitting (min.day^{-1})	124.9 ± 48.6	130.0 ± 52.1	0.78
Walking (min.day^{-1})	75.8 ± 92.3	51.4 ± 47.1	0.3
Moderate exercise (min.day^{-1})	17.0 ± 44.8	10.8 ± 19.9	0.57
Vigorous exercise (min.day^{-1})	6.3 ± 12.6	8.5 ± 8.0	0.51
Cycling (min.day^{-1})	0	5.6 ± 10.8	**0.03**
Total physical activity (MET-mins.day^{-1})	254 ± 114	290 ± 229	0.75

Values are mean ± SD

South Asians had lower levels of cardiorespiratory fitness (reflected by VO_{2max}, expressed both per kilogram of total body mass and per kilogram of fat-free mass) than Europeans. Fat oxidation (per kilogram whole body mass and fat-free mass) during incremental submaximal exercise was also significantly lower in South Asians, when expressed as an absolute value and as a percentage of VO_{2max}, but fat oxidation

at rest was not significantly different between the two groups. Carbohydrate oxidation during incremental submaximal exercise was similar in both groups at the same relative exercise intensity (i.e. with intensity expressed as a percentage of VO_{2max} but higher in South Asians at the same absolute exercise intensity). Fitness, physical activity and fat oxidation data are presented in Table 3.7, Figure 3.2 and Figure 3.3.

Table 3.7 Fitness and fat oxidation data

	South Asians	**Europeans**	**p-value**	**Age and BMI adjusted p-value**	**Age,BMI and fat mass - adjusted p-value**
VO_{2max} ($l.min^{-1}$)	2.89 ± 0.48	3.80 ± 0.60	**<0.0005**	**< 0.0005**	**0.001**
VO_{2max} ($ml.kg^{-1}.min^{-1}$)	40.6 ± 6.6	52.4 ± 5.7	**< 0.0005**	**< 0.0005**	**0.001**
VO_{2max} ($ml.kg^{-1}$ fat-free $mass.min^{-1}$)	54.1 ± 6.6	64.3 ± 5.8	**< 0.0005**	**< 0.0005**	**0.001**
Fat oxidation ($mg.kg^{-1}.min^{-1}$) @ 55% VO_{2max}	3.77 ± 2.02	6.55 ± 2.60	**0.001**	**0.010**	**0.012**
Fat oxidation (mg.kg fat-free $mass^{-1}.min^{-1}$) @ 55% VO_{2max}	5.08 ± 2.75	8.01 ± 0.63	**0.005**	**0.003**	**0.011**
Fat oxidation ($mg.kg^{-1}.min^{-1}$) @ 25 $ml.kg^{-1}.min^{-1}$	3.46 ± 2.20	6.00 ± 1.93	**0.001**	**0.002**	**0.020**

Values are mean ± SD

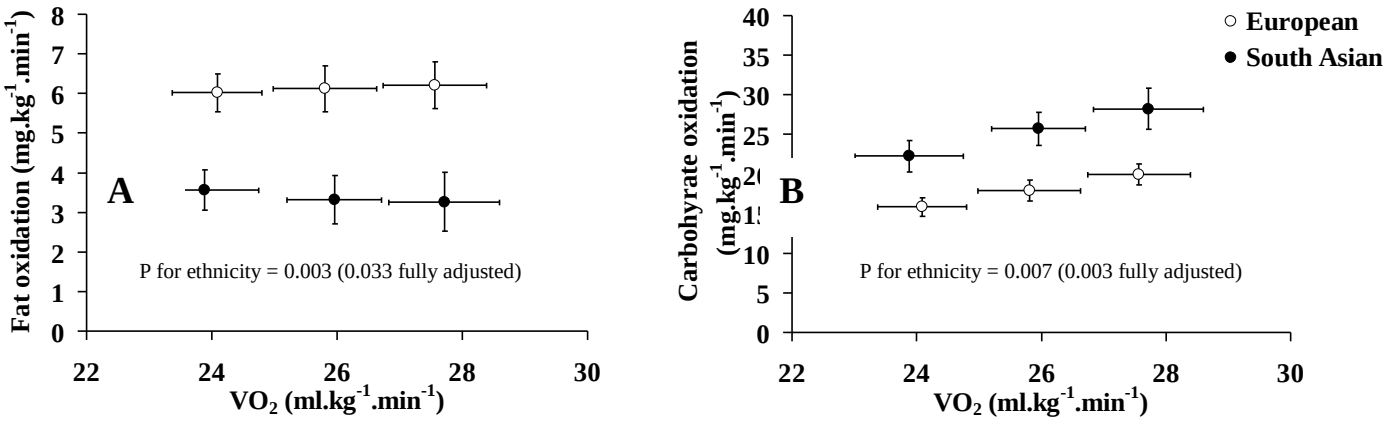

Figure 3.2 Fat oxidation (A) and carbohydrate oxidation (B) during incremental submaximal exercise. Intensity expressed in terms of absolute oxygen uptake (i.e. ml.kg^{-1}.min^{-1}). P-values shown are for the main-effect difference between European and South Asian groups, either unadjusted, or adjusted for age, BMI and fat mass.

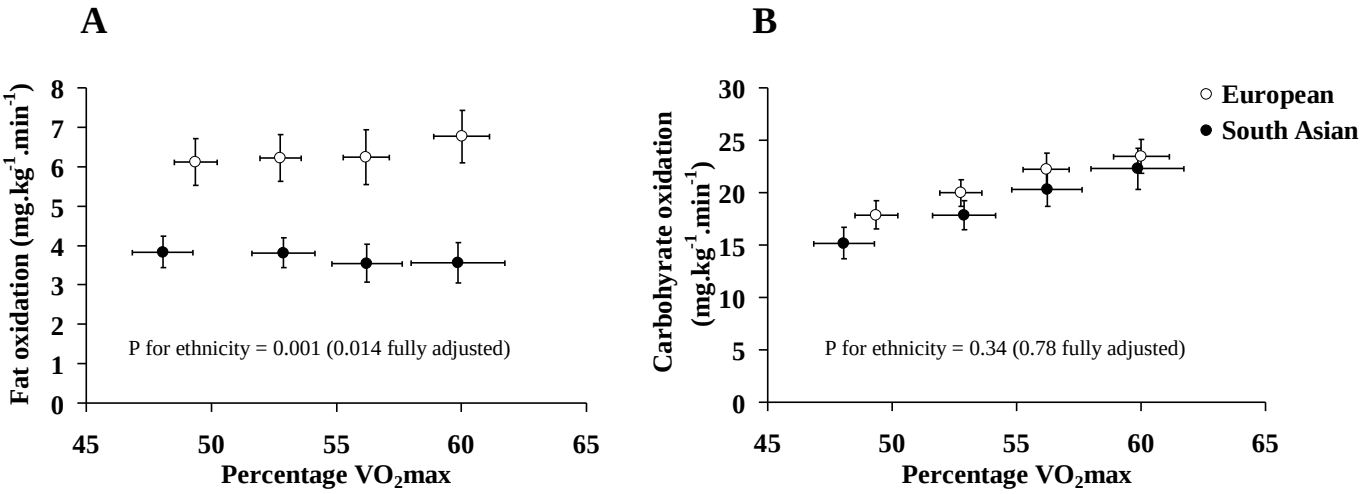

Figure 3.3 Fat oxidation (A) and carbohydrate oxidation (B) during incremental submaximal exercise. Intensity expressed relative to each individual's maximum oxygen uptake (i.e. percentage VO_{2max}). P-values shown are for the main-effect difference between European and South Asian groups, either unadjusted, or adjusted for age, BMI and fat mass.

3.3.6 Skeletal muscle mtDNA to nDNA ratio

There was no difference in skeletal muscle mtDNA to nDNA ratio between South Asians and Europeans (mean (95%CI) South Asians 0.94 (0.71 to 1.21), Europeans 1.13 (0.82 to 1.49), p = 0.39).

3.3.7 Skeletal muscle expression of oxidative and lipid metabolism genes

Expression of oxidative and lipid metabolism genes in skeletal muscle is shown in Table 3.8. Expression of carnitine palmitoyltransferase 1A (CPT1A) was 1.79-fold greater (p = 0.023) and expression of fatty acid synthase (FASN) was 1.96-fold greater (p = 0.036) in South Asians than Europeans.

Table 3.8 Oxidative and lipid metabolism gene expression in skeletal muscle

	South Asians	Europeans	p	Age and BMI adjusted p	Age, BMI and fat mass adjusted
CD36	1.15 (0.94 to 1.41)	1.00 (0.81 to 1.23)	0.343	0.210	0.168
CPT1A	**1.79 (1.32 to 2.43)**	**1.00 (0.69 to 1.46)**	**0.023**	**0.013**	**0.007**
CPT1B	1.22 (0.89 to 1.68)	1.00 (0.71 to 1.40)	0.406	0.244	0.088
CPT2	1.16 (1.00 to 1.34)	1.00 (0.74 to 1.36)	0.388	0.282	0.459
HADHA	1.25 (0.98 to 1.59)	1.00 (0.77 to 1.30)	0.233	0.078	0.049
HADHB	1.23 (0.96 to 1.57)	1.00 (0.75 to 1.33)	0.296	0.356	0.538
ACACA	1.32 (0.86 to 2.04)	1.00 (0.7 to 1.43)	0.342	0.201	0.109
ACACB	1.25 (0.84 to 1.86)	1.00 (0.69 to 1.46)	0.422	0.183	0.141
CS	1.10 (0.84 to 1.44)	1.00 (0.79 to 1.27)	0.606	0.303	0.066
CS (long transcript)	**1.29 (1.04 to 1.6)**	**1.00 (0.8 to 1.26)**	0.122	0.061	0.031
CS (short transcript)	1.09 (0.79 to 1.5)	1.00 (0.73 to 1.37)	0.712	0.323	0.064
COX1	1.07 (0.72 to 1.59)	1.00 (0.69 to 1.44)	0.805	0.515	0.447
FASN	**1.96 (1.19 to 3.25)**	**1.00 (0.72 to 1.39)**	**0.036**	**0.025**	0.111
FADS3	1.65 (1.19 to 2.28)	1.00 (0.64 to 1.56)	0.082	0.037	0.023

Values are mean (95% CI, expressed relative to mean value in European group).

Abbreviations: CD36 Cluster of differentiation 36; CPT carnitine palmitoyltransferase; HADHA hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit; HADHB hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, beta subunit; ACACA acetyl-Coenzyme A carboxylase alpha; ACACB acetyl-Coenzyme A carboxylase beta; CS citrate synthase; COX 1 cytochrome c oxidase 1; FASN fatty acid synthase; FADS 3 fatty acid desaturase 3

3.3.8 Adipose derived hormones, pro-inflammatory cytokines and cell - adhesion molecules

There were no significant differences between South Asians and Europeans in plasma concentrations of the adipose derived hormone adiponectin. Plasma leptin concentration was significantly higher in the South Asians. There were no differences in plasma concentrations of the pro-inflammatory cytokines TNF-α and interleukin-6 (IL-6) or in concentrations of the call-adhesion molecule selectin. These data are illustrated in Table 3.9

Table 3.9 Adipose derived hormones, cytokines and selectin

	South Asians	**Europeans**	**p value**	**Age, BMI and fat mass -adjusted p value**
Adiponectin ($\mu g.ml^{-1}$)	4.10 ± 2.35	4.84 ± 2.41	0.34	0.28
Leptin ($ng.ml^{-1}$)*	4.52 ± 1.76	3.36 ± 1.56	**0.020**	0.31
Selectin ($ng.ml^{-1}$)	37.2 ± 12.89	35.66 ± 14.51	0.73	0.82
IL-6 ($pg.ml^{-1}$)*	1.23 ± 0.77	1.19 ± 0.75	0.535	0.849
TNFα ($pg.ml^{-1}$)#	0.78 ± 0.89	0.57 ± 0.57	0.366	0.248

Values are mean ± SD *statistical analysis performed on log transformed data,

#statistical analysis performed on square-root transformed data

3.3.9 Anthropometric correlates with insulin sensitivity index

The regression slopes for these relationships did not differ significantly between South Asians and Europeans so data are presented for the combined group. As predicted, when the group was examined as a whole, there was a significant correlation between square root ISI and BMI ($r = -0.373$, $p = 0.019$), waist circumference ($r = 0.387$, $p = 0.015$) and trunk fat mass ($r = 20.359$, $p= 0.025$). Square-root ISI correlated significantly with height ($r = 0.329$, $p= 0.041$) and lean mass ($r = 0346$, $p = 0.028$). None of the other measured body composition variables significantly correlated with square root ISI. These data are presented in Figures 3.4 and 3.5. None of the other anthropometric or body composition variables, including total body mass significantly correlated with square root ISI.

The groups were examined separately to determine whether there were any relationships between anthropometric variables and ISI particular to either of the ethnic groups. In the South Asian group, there were significant negative correlations between square root ISI and waist circumference ($r = -0.633$, $p = 0.03$), hip circumference ($r = -0.447$, $p = 0.048$) and waist-hip ratio ($r = -0.503$, $p = 0.024$). In the European group there was a significant negative correlation between square root ISI and BMI ($r = -0.461$, $p = 0.047$), which was not observed in the South Asians group. In addition, ratio of trunk to limb fat mass ($r = -0.474$, $p = 0.04$) and waist-hip ratio ($r = -0.492$, $p = 0.033$) correlated negatively with square root ISI, and a positive correlation existed between square root ISI and height ($r = 0.498$, $p = 0.030$).

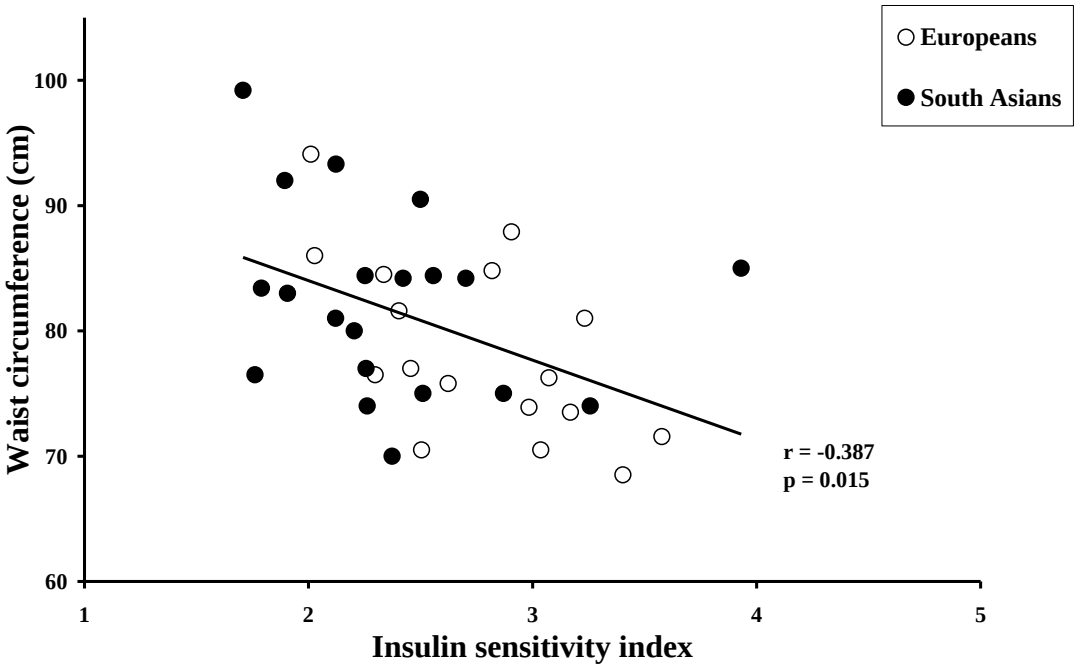

Figure 3.4 Relationship between waist circumference and square-root insulin sensitivity index

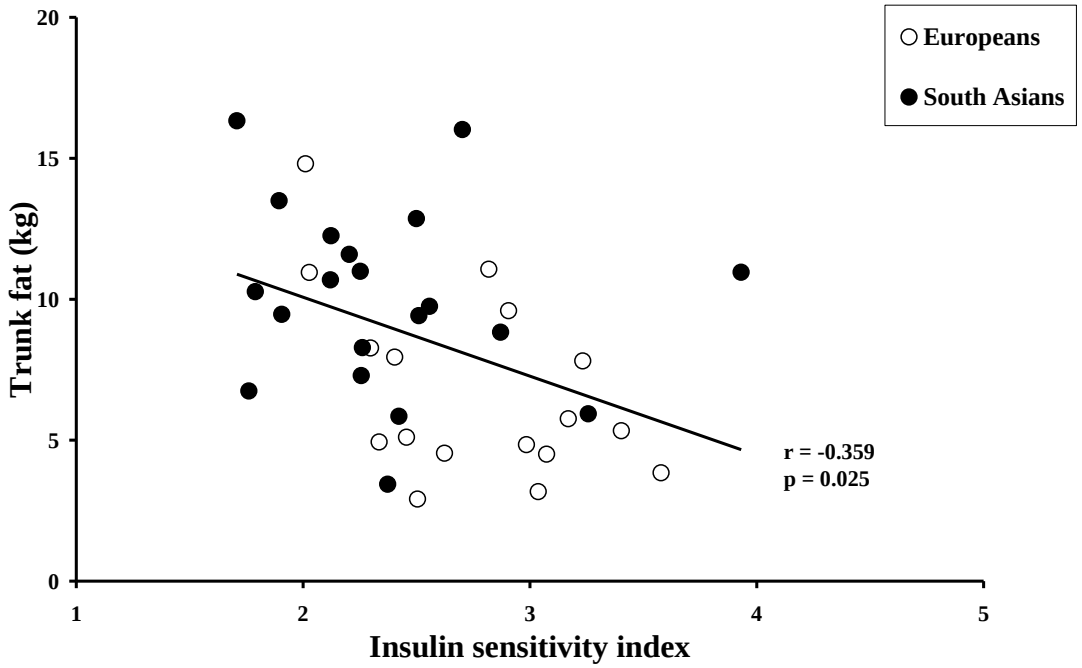

Figure 3.5 Relationship between trunk fat and square-root insulin sensitivity index

3.3.10 Physiological correlates with insulin sensitivity index

In unadjusted univariate correlations, VO_{2max}, expressed in $ml.kg^{-1}.min^{-1}$ (r = 0.399, p = 0.012) or $ml.kg^{-1}$ fat-free $mass.min^{-1}$ (r = 0.352, p = 0.028) correlated significantly with square-root ISI. In addition, fat oxidation during sub-maximal exercise at 55% VO_{2max}, expressed in $ml.kg^{-1}.min^{-1}$ (r = 0.423, p = 0.011) (Figure 3.6) or in $ml.kg^{-1}$ fat-free $mass.min^{-1}$ (r = 0.409, p = 0.015), and fat oxidation at an absolute VO_2 of 25 $ml.kg^{-1}.min^{-1}$ (r = 0.370, p = 0.029) correlated significantly with square-root ISI.

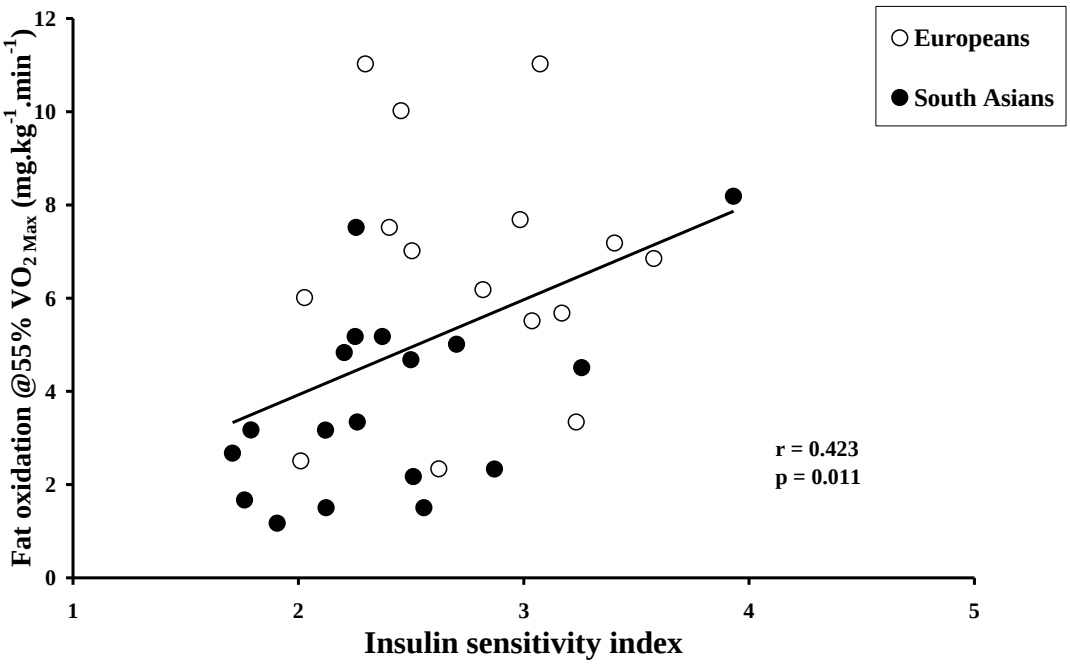

Figure 3.6 Relationship between rate of fat oxidation during submaximal exercise and square-root insulin sensitivity index

Adjustment for age, BMI, fat mass and physical activity slightly attenuated the relationships. However, even after adjustment for these variables significant relationships were observed between square root ISI, and fat oxidation during

submaximal exercise at 55% VO_{2max}, expressed in $mg.kg^{-1}.min^{-1}$ ($r = 0.335$, $p = 0.049$) or in $mg.kg^{-1}$ fat-free $mass.min^{-1}$ ($r = 0.370$, $p = 0.029$). After adjustment, there remained a borderline significant relationship between square root ISI and VO_{2max} expressed in $ml.kg^{-1}$ fat-free $mass.min^{-1}$ ($r = 0.315$, $p = 0.051$). Thus, independent of age, body composition and physical activity level, VO_{2max} or fat oxidation during submaximal exercise explained 10-13% of the variance (i.e. r^2) in square-root ISI.

Neither fat oxidation rate at rest nor resting metabolic rate (however expressed) correlated significantly with square-root ISI. In addition, fat oxidation rate at rest did not correlate significantly with fat oxidation rates during exercise (however expressed).

3.4 Discussion

The aims of the study were firstly to determine whether there were any differences in insulin sensitivity or in other metabolic variables associated with insulin resistance between South Asians and Europeans which were independent of differences in body composition. An additional aim was to investigate possible lifestyle differences, such as dietary intake and habitual exercise and to examine differences in cardiorespiratory fitness and skeletal muscle fat oxidation. Further, the study aimed to investigate relationships between fat oxidation and insulin sensitivity, to establish whether or not this could explain differences in insulin sensitivity between the two ethnic groups.

Data from the study demonstrate no significant difference in total energy or macronutrient intake between South Asians or Europeans, although South Asians

reported higher complex carbohydrate intake. There were also significant differences in intake of Vitamin C and Vitamin D, and in intake of a number of micronutrients, with the South Asians reporting significantly lower intake of all of these dietary components. These findings are of interest, as hypovitaminosis D is increasingly recognized in UK South Asians, especially women (Ford et al. 2006; Roy et al. 2007) and serum vitamin D concentrations have been found to be associated positively with insulin sensitivity and measures of β cell function in non-diabetic individuals (Chiu et al. 2004) and negatively with diabetes risk (in heterogeneous US populations, not including subjects of South Asian origin) (Scragg et al. 2004). Plasma vitamin C is a good surrogate measurement of fruit and vegetable intake and has also, in at least one study been shown to inversely correlate with diabetes risk (Harding et al. 2008).

The observation that there was no significant difference in habitual physical activity between South Asians and Europeans in this study is not in keeping with previous published studies, which suggest that UK South Asians have lower physical activity levels than the background population. However most of the published studies to date investigating ethnic differences in physical activity in adults relied on self-report questionnaires, which are of limited validity and reliability compared to objective measures of physical activity (Shephard 2003). Data obtained from questionnaires may also be subject to differences in cultural interpretation of the questions (Fischbacher et al. 2004). Only one recent study has provided objective evidence, using accelerometers, that South Asian children aged 9-10 years old have lower levels of physical activity than white European children of the same age (Owen et al. 2009). This finding requires confirmation in adults, with a view to examining the relationship between physical activity and insulin resistance, but it does seem to be the case that

South Asians may adopt particularly sedentary lifestyles when they migrate to Westernised countries, which could potentiate their innate predisposition to insulin resistance.

Despite no significant differences in diet or physical activity, the South Asian subjects displayed the characteristic phenotype of increased adiposity and particularly abdominal fat distribution (significantly higher total body fat mass, with increased truncal obesity than the European subjects) and a characteristic lipid profile with similar total cholesterol concentrations to Europeans but significantly lower HDL cholesterol concentrations.

As expected, and in keeping with this characteristic phenotype, the South Asian subjects were significantly more insulin resistant than the European subjects. Interestingly the reduction in insulin sensitivity was not fully explained by differences in adiposity – the insulin sensitivity index remained significantly different even after adjustment for age, BMI, physical activity and fat-mass. There was no significant difference in insulinogenic index, which is a marker of β cell function, between the South Asians and Europeans, suggesting that β cell function is preserved, with compensatory hyperinsulinaemia overcoming the effects of insulin resistance and post-prandial hyperglycaemia.

A large adipose tissue mass is known to be associated with increased lipolysis and resultant higher circulating NEFA concentrations (Boden 1997; Delarue & Magnan 2007), and NEFA concentrations are sometimes, but not always elevated in insulin resistant states (Corpeleijn et al. 2009), so the present study results finding South

Asian subjects to have similar NEFA concentrations (and post-glucose NEFA AUCs) to European subjects, despite significantly larger fat mass are somewhat surprising, although could be explained by the fact that circulating NEFA levels generally tend to be very low, difficult to measure, and are a poor marker of fatty acid flux/ mobilisation.

The South Asians also had strikingly reduced cardiorespiratory fitness as measured by VO_{2max} than the Europeans with values, which were, on average 25% lower, whether this was expressed per kilogram of total body mass or per kilogram of fat-free mass (with a highly significant p value of 0.001).

The finding of no ethnic differences in plasma concentrations of the adipose derived hormone adiponectin contrasts with prior studies in South Asians (Chandalia et al. 2007), and suggests that this may not be an important factor in insulin resistance in South Asians. Plasma leptin concentrations were higher in the South Asians, simply reflecting increased fat mass, so as expected, the difference in plasma leptin concentration did not persist after adjustment for fat mass. The concentrations of IL-6, TNF-α and the cell-adhesion molecule selectin were also comparable between the two groups, suggesting that increased production and /or activity of pro-inflammatory cytokines is not causal of or associated with insulin resistance in South Asians, again conflicting evidence to that from previous studies (Abate et al. 2004; Nair, Bigelow et al. 2008).

In contrast to the observations made at the whole body level, South Asians did not exhibit lower expression of oxidative and lipid metabolism genes in skeletal muscle

biopsies than Europeans, and indeed expression of CPT1A and FASN were higher in South Asians. In addition, other than a negative correlation between expression of CPT1A and ISI in the South Asians – the opposite direction of the expected association – expression of none of these genes was related to whole-body insulin sensitivity. Furthermore, the mtDNA to nDNA ratio, which provides an index of mitochondrial biogenesis (Williams et al. 1986), did not differ between the two groups. Our data therefore indicate that reduced skeletal muscle expression of oxidative and lipid metabolism genes does not explain the increased insulin resistance observed in South Asians. The author is unaware of previous studies examining differences in whole body fat oxidation in any ethnic population, either at rest or in the context of exercise. Although the new observation of reduced whole-body fat oxidation during exercise in South Asians was made, it is not possible, from the present data, to state conclusively that an innate defect in skeletal muscle is the cause of this phenomenon.

This study has a number of limitations. Firstly, insulin sensitivity was assessed from glucose and insulin responses to an oral glucose tolerance test, rather than using the gold-standard euglycaemic hyperinsulinaemic clamp. However, the insulin sensitivity index used in this study correlates well with clamp-derived measures of insulin sensitivity (Matsuda & DeFronzo 1999), and has been widely accepted in the literature, with over 1000 citations in the last decade. In addition it was only the expression of genes involved in oxidation and lipid metabolism in skeletal muscle which was measured, and while none of these was lower in the South Asians than the Europeans, indicating that transcription of these genes is not defective in South Asian muscle, further study is needed to determine whether differences in protein levels or

activity of these enzymes exist between South Asian and European skeletal muscle. Finally, although the men were extensively phenotyped, IMTG and skeletal muscle concentrations of lipid intermediates were not directly measured in this study and further investigation is needed to ascertain the extent to which these differ between South Asians and Europeans, and how they relate to capacity for fat oxidation during exercise and insulin sensitivity.

The novel findings of this study were that South Asians oxidized less fat than Europeans during exercise, and that VO_{2max} and fat oxidation during submaximal exercise correlated significantly with whole-body insulin sensitivity, independently of age, body composition and physical activity level, explaining 10-13% of the variance in insulin sensitivity. This suggests that reduced oxidation and utilization of fatty acids by skeletal muscle is an important association with the insulin resistant phenotype typically seen in South Asians.

The significantly higher expression of CTP1A and FASN observed in the South Asians suggest that reduced fat oxidation is not a result of reduced skeletal muscle expression of oxidative and lipid metabolism genes.

Chapter 4

Skeletal muscle insulin signalling in South Asian and European Men

4.1 Introduction

The studies performed in Chapter 3 found the South Asian subjects to be more insulin resistant than their matched Europeans counterparts. They displayed the characteristic phenotype seen in South Asians of increased adiposity, particularly increased truncal fat, indicative of central obesity. However, differences in insulin sensitivity between South Asian and European subjects persisted after adjustment for age, BMI and fat mass. This finding, together with the observation of reduced whole body fat oxidation during exercise, and the fact that skeletal muscle during exercise is the major contributor to fat oxidation, suggests a novel mechanism for insulin resistance i.e. that altered skeletal muscle function could be implicated.

Altered skeletal muscle function causing reduced oxidation of fatty acids could result in the accumulation of lipid intermediates with deleterious effects, and this could conceivably affect insulin signalling within skeletal muscle. If altered insulin signalling was to be demonstrated in South Asians this could arise either as a direct result of the effect of IMCL accumulation or it could be an innate alteration in insulin signalling. Regardless of the cause, if the alteration impaired insulin signalling, glucose transport into skeletal muscle cells would be reduced, therefore skeletal muscle would be insulin resistant. Because of the significant influence of skeletal muscle on whole body metabolism, insulin resistance within skeletal muscle results in a series of metabolic perturbations which over years leads to whole-body insulin resistance (Petersen & Shulman 2002).

As mentioned in Chapter 1, aberrations in insulin signalling at various stages in the insulin signalling pathway have been observed in patients with type 2 diabetes and other insulin resistant states. There is no clear consensus about specific defects as many studies have produced conflicting results. For example, studies examining IRS-1 phosphorylation in the skeletal muscle of patients with type 2 diabetes, have found it to both reduced (Bjornholm et al. 1997; Stentz & Kitabchi 2007) and unchanged (Krook et al. 2000), suggesting that IRS-1 may not be a key protein in insulin resistance.

Reductions in expression of the p85α, the regulatory subunit of PI3K has been observed in obesity in one study (Colomiere et al.2009) but most studies have fond increased p85 expression in insulin resistance (Bandyopadhyay et al. 2005; Barbour et al. 2005; Friedman et al.1999; Hammarstedt et al. 2003).

Downstream of PI3K, protein kinase B (PKB), or Akt, is an important protein in the insulin signaling pathway. Of its several isoforms PKBβ is thought to have the most significant role in insulin signalling, based on studies examining the PKB β knockout mouse, which is insulin resistant and develops a type 2 diabetes-like syndrome (Cho et al. 2001; Cleasby et al. 2007). Activation of protein kinase B/Akt, requires it to be phosphorylated at 2 sites; threonine 308 and serine 473. Impaired insulin-stimulated PKB phosphorylation/ activity has been observed in skeletal muscle from subjects with type 2 diabetes (Karlsson et al. 2005; Krook et al. 1998), low birth weight (Jensen et al. 2008) and PCOS (Hojlund et al. 2008), yet other studies have reported no significant difference in insulin-stimulated PKB activity or phosphorylation in

skeletal muscle of subjects with type 2 diabetes (Bandyopadhyay et al. 2005; Kim et al. 1999; Meyer et al. 2002).

Animal studies exploring the effect of induced insulin resistance by continuous glucose infusion have demonstrated that chronic hyperglycaemia and resultant increased circulating NEFA concentrations activate skeletal muscle protein kinase C isoforms via the accumulation of diacyglycerol and long chain acyl Co-A (LCACoA) which are potent PKC activators (Griffin et al. 1999; Laybutt et al. 1999). Human studies using lipid infusion to induce insulin resistance by causing an acute increase in circulating NEFA have reproduced these findings, observing increased DAG mass and corresponding increased PKC activity (Itani et al. 2002). In addition, PKC activity has been found to be increased in the skeletal muscle of obese insulin resistant subjects, compared with that in non-obese controls (Itani et al. 2000). There have not yet been any studies examining PKC activity in type 2 diabetes in human skeletal muscle but in this context PKC is likely to be linked to impaired insulin signalling and reduced glucose transport. More recently, a number of atypical protein C isoforms (zeta, lambda and iota), have been discovered which seem to have paradoxically reduced activation in the skeletal muscle of insulin-resistant animal models (Standaert et al. 2002) but which also have not yet been investigated in human insulin resistance.

In obese subjects, with and without type 2 diabetes, GLUT 4 expression in skeletal muscle is reduced (Dohm et al. 1991), whereas in type 2 diabetes GLUT 4 expression is similar to that in non-diabetic controls (Handberg et al. 1990; Pedersen et al. 1990). This suggests that although quantity of GLUT 4 is similar, function or trafficking may be impaired in diabetic subjects. There is some evidence for abnormal GLUT 4

function/ distribution in type 2 diabetes, with one study finding abnormal GLUT 4 transport and location in human subjects with insulin resistance and type 2 diabetes (Garvey et al. 1998) and a further study in a mouse model of type 2 diabetes showing significantly reduced insulin-stimulated GLUT 4 translocation to the plasma membrane in diabetic mice compared with controls (Miura et al. 2001). Interestingly GLUT 4 translocation and subsequently glucose transport has been found to be increased by exercise (Wallberg-Henriksson et al. 1988), which may partially explain the improvements in insulin sensitivity seen in exercise training.

There have not yet been any published studies examining protein expression of components of the insulin signalling pathway specifically in South Asian populations, nor in any other ethnic groups. The aim of the study described in this chapter was, therefore to characterise the expression of proteins in the insulin signalling pathway in South Asians and Europeans to identify at which points in the pathway differences exist. This would not only facilitate understanding of the mechanism underpinning insulin resistance in South Asians, but may also assist the identification of potential targets for future pharmacological therapies.

4.2 Methods

4.2.1 Volunteers

Volunteers were those described in section 3.2.1. They were recruited, screened and enrolled in the study as detailed in section 2.2. Specific inclusion criteria are also defined in section 2.2, where further information detailing recruitment response and excluded volunteers can also be found.

The final study groups therefore consisted of the well-matched 20 South Asians and 19 Europeans fully described in Chapter 3.

4.2.2 Study design

All participants underwent muscle biopsy using the technique described in section 2.12.1. Samples were analysed, as outlined in section 2.13.1 to determine differential skeletal muscle insulin signalling in the two groups, under both basal and insulin-stimulated conditions.

4.2.3 Muscle biopsy

After a 12 hour fast, percutaneous skeletal muscle biopsy was performed, as described fully in section 2.12.1. Biopsy samples were allowed to recover for 30 minutes in 10 ml of Krebs-Ringer-Hepes buffer supplemented with 25mmoll^{-1} D-glucose and 0.1% (w/v) BSA at 37^{o}C, which had been pre-gassed with 100% (v/v) O_2 for 5 minutes. They were then incubated in the presence or absence of human soluble insulin (Actrapid, NovoNordisk, Bagsvaerd, Denmark) at a concentration of 10 nmol/l for 10 minutes, after which both samples were placed into screw cap microcentrifuge tubes, snap frozen in liquid N_2 and then stored at -80^{o}C.

4.2.4 Analysis of muscle samples

Muscle biopsy samples were analysed as fully described in section 2.13. Briefly, samples were homogenised and centrifuged to separate cytosolic and particulate fractions. Following protein estimation and transfer to nitrocellulose, samples were subjected to Western blotting and probed with antibodies against insulin receptor (insulin receptor (β subunit), insulin receptor substrate (IRS) proteins –1 and –2,

phosphatidylinositol 3'-kinase (p85 and p110 subunits), PTEN, PKB (α and β), GLUT4 and PKC isoforms. Resultant bands were quantified by densitometry and compared with an internal standard to assess expression levels. Phosphorylation of PKB at Ser473 was assessed in the muscle homogenates described above by Western blotting using phosphorylation site-specific antibodies.

4.2.5 Statistical analysis

Data were analysed using Statistica (version 6.0, StatSoft Inc., Tulsa, Oklahoma) and Minitab (version 13.1, Minitab Inc., State College, Pennsylvania). Prior to analysis, all data were tested for normality using the Ryan-Joiner normality test and, if necessary, logarithmically transformed. All data between South Asians and Europeans was compared using unpaired t-tests. Univariate linear regressions were performed, using logarithmically transformed data where required. Correlations between ISI and other variables were performed for the South Asians and European groups. Significance was accepted at $p \leq 0.05$.

4.3 Results

4.3.1 Basal insulin signaling protein expression

Due to technical problems, it was not possible to obtain data on skeletal muscle insulin signalling protein expression for all participants. There was also an outlier in the data for protein expression of the p110β subunit of PI3K, in that, on statistical analysis this value was over 9 standard deviations from the mean so it was not possible to normalise the data. This data point was therefore removed from the analysis. PKC θ was not measured because of difficulties acquiring the appropriate

antibody. Insulin signalling protein expression data and the number of observations for each variable are shown in Table 4.1 and Figure 4.1.

In muscle samples which were not insulin-stimulated, protein expression of IRS-1 was significantly lower in South Asians than European in unadjusted analysis. However these data were only available for a small subset of the total cohort (n = 8 South Asians and 7 Europeans) and this difference was abolished on adjustment for age and BMI; and age, BMI and fat mass. Protein expression of the p85 subunit of PI3K was ~50% lower in South Asians than Europeans, and although this finding was also based on a small number of observations (n = 8 South Asians and 8 Europeans), this difference remained significant after adjustment for age, BMI and fat mass.

Unstimulated PKB protein expression was also ~50% lower in South Asians (n = 14) than Europeans (n = 12). This difference remained after adjustment for age and BMI, and became borderline significant after further adjustment for fat mass (p = 0.054). Phosphorylation of PKB at Ser473 was over 60% lower in South Asians (n = 14) than Europeans (n = 11) and this remained significant after adjustment. Neither protein expression of the p110β subunit of PI3K, nor protein expression of PKC β1 in either the cytosolic or microsomal fractions differed significantly between South Asians and Europeans.

Table 4.1 Skeletal muscle insulin signalling protein expression under basal conditions.

	South Asians	Europeans	p-value	Age and BMI-adjusted p-value	Age, BMI and fat mass-adjusted p-value
IRS-1 (n = 8 South Asians, 7 Europeans)	0.35 (0.13 to 0.56)	1.00 (0.50 to 1.50)	**0.028**	0.699	0.699
PI3K, p85 subunit (n = 8 South Asians, 8 Europeans)	0.51 (0.28 to 0.75)	1.00 (0.84 to 1.16)	**0.005**	0.079	**0.049**
PI3K, p110β subunit (n = 5 South Asians, 14 Europeans)	1.05 (0.82 to 1.28)	1.00 (0.78 to 1.22)	0.800	0.281	0.159
PKB* (n = 16 South Asians, 12 Europeans)	0.51 (0.36 to 0.71)	1.00 (0.57 to 1.75)	**0.041**	**0.003**	0.054
PKB Ser473 phosphorylation* (n = 14 South Asians, 11 Europeans)	0.38 (0.27 to 0.52)	1.00 (0.74 to 1.38)	**< 0.0005**	**< 0.0005**	**0.019**
Cytosolic PKC β1 (n = 8 South Asians, 9 Europeans)	0.94 (0.61 to 1.27)	1.00 (0.64 to 1.37)	0.813	0.739	0.759
Microsomal PKC β1 (n = 8 South Asians, 9 Europeans) [#]	0.85 (0.55 to 1.20)	1.00 (0.25 to 2.30)	0.824	0.907	0.734

Values are mean (95% CI), expressed relative to mean value in European group.

*statistical analysis performed on log transformed data, [#]statistical analysis performed on square-root transformed data.

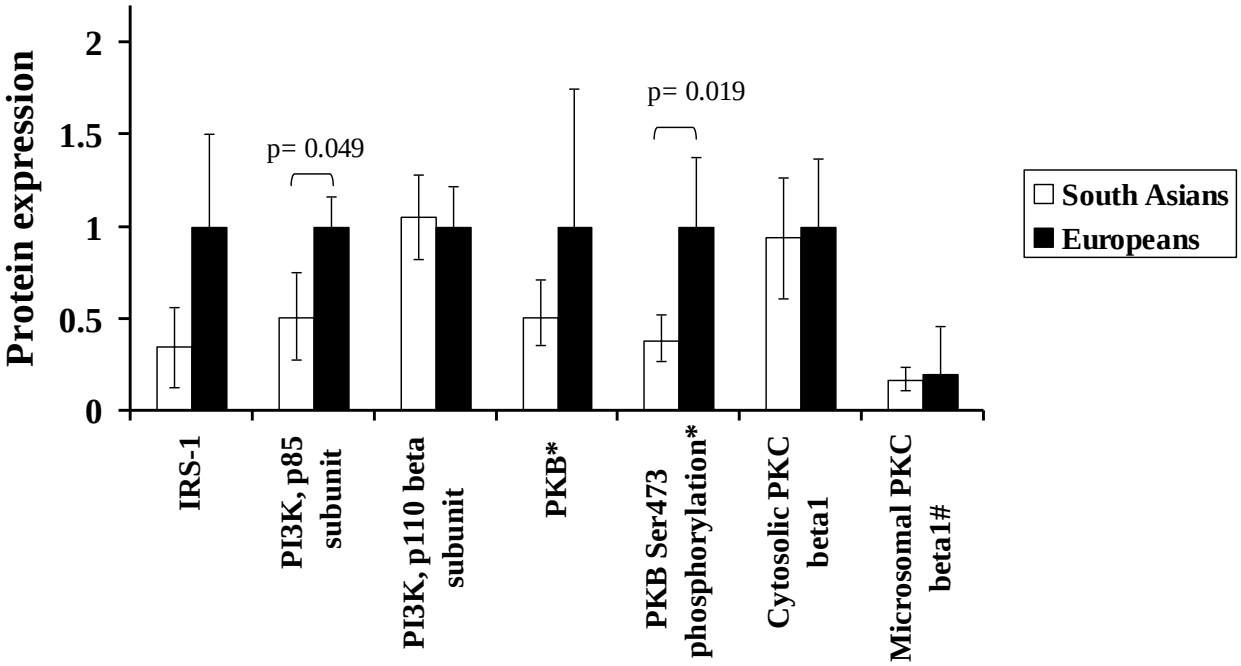

Figure 4.1 Skeletal muscle insulin signalling protein expression in South Asian and European men under basal conditions.

Values are mean (95% CI), expressed relative to mean value in European group. *statistical analysis performed on log transformed data, #statistical analysis performed on square-root transformed data. Significant p values (after adjustment for age, BMI and fat mass) are shown on the graph. All other differences are not significant after adjustment

4.3.2 Insulin signaling protein expression with insulin stimulation

In unadjusted analysis, insulin stimulated IRS-1 protein expression was significantly lower in South Asians, but this difference was lost after full adjustment for age, BMI and fat mass. Notably, protein expression of the p85 subunit of PI3K was also again significantly lower, as it was under basal conditions, and this difference persisted after full adjustment. There was no difference in the expression of any of the other insulin

signaling proteins in response to insulin stimulation, not in the ratio of total PKB to Ser473 phosphorylated PKB. These data are presented in Table 4.2. Figure 4.2 shows a representative blot, which illustrates the method used to quantify insulin signalling protein expression in South Asians and Europeans with and without insulin stimulation.

Table 4.2 Insulin stimulated skeletal muscle insulin signalling protein expression

	South Asians	Europeans	p-value	Age and BMI-adjusted p-value	Age, BMI and fat mass-adjusted p-value
IRS-1 (n = 8 South Asians, 7 Europeans)	0.43 (0.17 to 0.69)	1.00 (0.74 to 1.26)	**0.009**	0.29	0.15
PI3K, p85 subunit (n = 8 South Asians, 8 Europeans)	0.61 (0.42 to 0.81)	1.00 (0.78 to 1.22)	**0.02**	0.81	**0.03**
PI3K, p110β subunit (n = 5 South Asians, 14 Europeans)	1.15 (0.78 to 1.54)	1.00 (0.82 to 1.18)	0.41	0.21	0.25
PKB* (n = 16 South Asians, 12 Europeans)	0.55 (0.43 to 0.68)	1.00 (0.52 to 1.48)	**0.067**	0.61	0.58
PKB Ser473 phosphorylation (n = 14 South Asians, 11 Europeans)	0.91 (0.70 to 1.12)	1.00 (0.67 to 1.33)	0.63	0.37	0.49
Cytosolic PKC β1 (n = 8 South Asians, 9 Europeans)	1.14 (0.85 to 1.44)	1.00 (0.66 to 1.34)	0.55	0.93	0.83
Microsomal PKC β1 (n = 8 South Asians, 9 Europeans)	1.25 (0.41 to 1.85)	1.00 (0.33 to 1.67)	0.80	0.29	0.15
Ratio PKB/ Ser473 PKB	1.25 (0.76 to1.76)	1.00 (0.62 to 1.38)	0.43	0.99	0.57

Values are mean (95% CI), expressed relative to mean value in European group.

*statistical analysis performed on log transformed data.

As illustrated in Figure 4.3, basal log PKB phosphorylation at Ser473 for the whole study group correlated significantly with square-root ISI ($r = 0.407$, $p = 0.044$), even after adjustment for age ($r = 0.404$, $p = 0.045$), although there was no significant correlation after adjustment of square-root ISI for age and BMI or age and fat mass.

4.3.3 Relationship between insulin signalling protein expression and insulin sensitivity

When the data were analysed separately according to ethnicity, there was no significant correlation between phosphorylation of PKB at Ser473 (or any other insulin signalling protein) and square root insulin sensitivity in South Asians, but a borderline significant correlation between these variables in Europeans, when insulin sensitivity was adjusted for age and BMI ($r = 0.554$, $p = 0.077$) or age and fat mass ($r = 0.546$, $p = 0.082$) (Table 4.3).

Figure 4.2 Example western blot

Soluble cytosolic (C) and microsomal (M) fractions were prepared from muscles incubated *ex vivo* in the presence or absence of insulin. Fractions were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. Representative blots are shown from 2 South Asian subjects and a European subject.

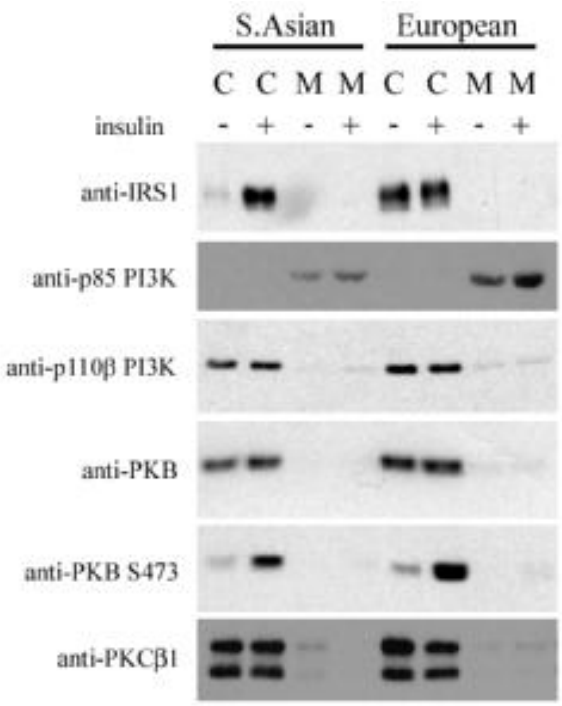

Table 4.3 Relationships between insulin signalling proteins under basal conditions and ISI (unadjusted), ISI adjusted for age, age+BMI and age+fat mass.

	Unadjusted r	Age-adjusted r	Age + BMI adjusted r	Age + fat mass adjusted r
IRS-1	-0.047	-0.054	-0.160	-0.170
PI3K p85	0.138	0.132	0.026	0.046
PI3K p110β	-0.142	-0.143	-0.403	-0.422
PKB*	0.083	0.084	0.169	0.046
PKB Ser473 phosphorylation*	**0.407**	**0.404**	0.379	0.277
Cytoplasmic PKC β1	0.276	0.276	0.275	0.268
Microsomal PKC β1#	0.452	0.452	0.405	0.433

Statistical analysis performed on log transformed data, #statistical analysis performed on square-root transformed data. Statistically significant correlations are shown in bold

PKB phosphorylation at Ser473 for the whole study group also correlated significantly with various indices of adiposity (see Table 4.4). It correlated negatively with total fat mass, arm, leg and trunk fat mass and also with triceps and subscapular skinfold thickness. It correlated positively with total fat-free mass and total, leg and trunk lean mass. There was no significant correlation between biceps or suprailiac skinfold thickness and PKB phosphorylation at Ser473.

PKB Ser473 phosphorylation was, however, very strongly correlated with indices of cardiorespiratory fitness (see Table 4.5), including VO_{2max} and fat oxidation during sub-maximal exercise at 55% of VO_{2max} (whether expressed in $ml.kg^{-1}.min^{-1}$ or in

$ml.kg^{-1}$ fat-free $mass.min^{-1}$), and fat oxidation at an absolute VO_2 of 25 $mg.kg^{-1}.min^{-1}$. There was no significant correlation between PKB Ser473 phosphorylation and resting VO_2 or resting fat oxidation.

None of the other measured insulin signalling proteins correlated significantly with square-root insulin sensitivity index, or any indices of body composition. However, protein expression of IRS-1 correlated significantly with VO_{2max}, expressed in $ml.kg^{-1}.min^{-1}$ ($r = 0.677$, $p = 0.006$) or $ml.kg^{-1}$ fat-free $mass.min^{-1}$ ($r = 0.649$, $p = 0.009$).

Table 4.4 Correlations between log PKB Ser473 and indices of adiposity

Variable	r value	p value
Total fat mass	**-0.440**	**0.028**
Arm fat mass	**-0.433**	**0.031**
Leg fat mass	**-0.401**	**0.047**
Trunk fat mass	**-0.434**	**0.030**
Total lean mass	**0.538**	**0.006**
Arm lean mass	0.282	0.172
Leg lean mass	**0.546**	**0.005**
Trunk lean mass	**0.539**	**0.005**
Biceps skinfold	-0.354	0.083
Triceps skinfold	**-0.399**	**0.048**
Subscapular skinfold	**-0.439**	**0.028**
Suprailiac skinfold	-0.349	0.087

Statistically significant correlations are shown in bold

Table 4.5 Correlation between log PKB Ser473and indices of cardiorespiratory fitness

Variable	r value	p value
VO_{2max} ($ml.kg^{-1}.min^{-1}$)	**0.634**	**0.001**
VO_{2max} ($ml.kg^{-1}$fat-free mass.min^{-1})	**0.539**	**0.005**
Fat oxidation at VO_2 25 $ml.kg^{-1}.min^{-1}$	**0.588**	**0.025**
Fat oxidation at 55% VO_{2max} ($mg.kg^{-1}.min^{-1}$)	**0.475**	**0.004**
Fat oxidation at 55% VO_{2max} ($mg.kg^{-1}$fat-free mass.min^{-1})	**0.449**	**0.036**

Statistically significant correlations are shown in bold

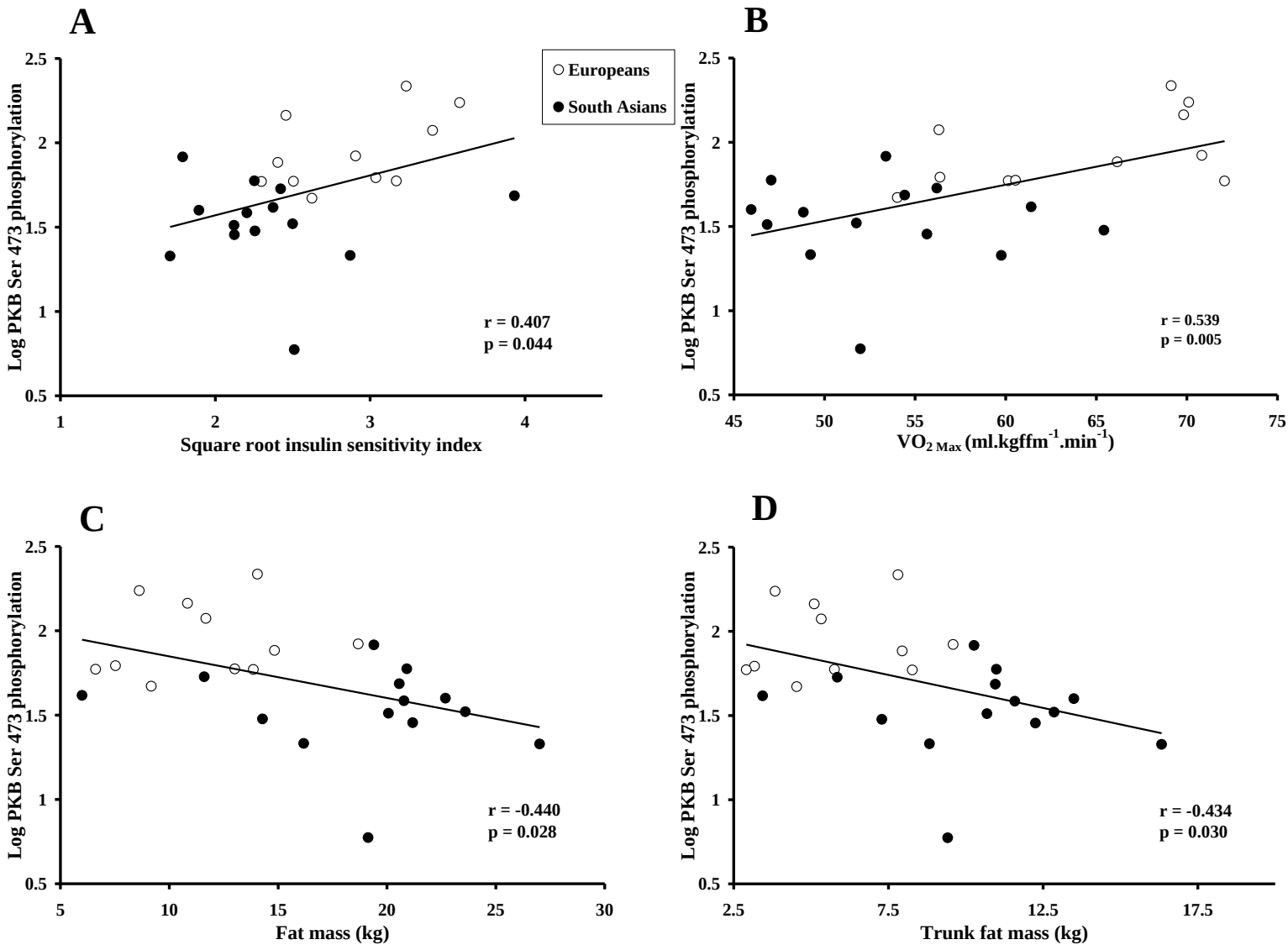

Figure 4.3 Scattergrams illustrating relationships between PKB Ser473 phosphoryation and insulin sensitivity index (A), VO_{2max} (B), total fat mass (C) and trunk fat mass (D).

4.4. Discussion

The aim of the study described in this chapter was to determine whether there was any difference in protein expression of some of the insulin signalling enzymes in the skeletal muscle of South Asians compared to Europeans, which might explain their increased insulin resistance. Protein expression of some of the major components of the insulin signalling pathway in skeletal muscle was found to be lower in South Asians compared to Europeans, even after adjustment for age, BMI and total fat mass.

In the fasted state, the protein expression of both Ser473 phosphorylated PKB and the p83 subunit of PI3K was significantly and markedly lower (by at least 50%) in South Asians than in Europeans. This was despite the fact that measurements were made using muscle biopsy samples which had been exposed only to basal endogenous fasting insulin levels, differences in which are unlikely to be confounders, as the South Asian subjects had a tendency for higher fasting insulin concentrations, which would be expected to, if anything, increase PI3K p80 and Ser473 phosphorylation.

Following insulin stimulation, the only significant difference which remained after full adjustment for age, BMI and fat was that South Asians had significantly lower expression of the p83 subunit of PI3K. There was no correlation between basal or insulin-stimulated p83 expression and insulin sensitivity.

The PKB findings are noteworthy, as basal Ser473 phosphorylation of PKB was over 60% lower in South Asians and it correlated significantly with whole-body insulin sensitivity, which was not the case with any of the other insulin signalling proteins. In addition, PKB Ser473 phosphorylation correlated strongly and significantly with

whole-body fat oxidation rates during exercise, suggesting that differences in capacity for fat oxidation between Europeans and South Asians may influence insulin sensitivity via effects at this point in the insulin signalling pathway.

This finding is important because PKB has, for almost 20 years, been known to be an important enzyme in the insulin signaling cascade (Brazil & Hemmings 2001). Phosphorylation at Ser473 is essential for its activation, and alterations in this enzyme's activity have previously been observed in insulin-resistant states. It has been found to be reduced in type 2 diabetes (Krook et al. 1998) and in insulin resistant low-birth weight men (under insulin stimulated conditions (Jensen et al. 2008)). In addition, the thiazolidinedione class of drugs, which are known to improve insulin sensitivity have been shown, *in vitro*, to increase PKB Ser473 phosphorylation in human umbilical vein endothelial cells (Wu et al. 2009).

PKB Ser473 phosphorylation, in both adipose tissue, and muscle, leads to a chain of events which stimulate glucose transport via increased GLUT 4 expression and recruitment to the cell membrane. Ultimately glucose uptake into the myocyte is increased, so increased PKB Ser473 phosphorylation is associated with increased insulin sensitivity (Cho et al. 2001; Hill et al. 1999; Kohn et al. 1996; Ueki et al. 1998; Wang et al. 1999). Our study findings are consistent with these previous study findings, with the significant positive correlation between PKB phosphorylation at Ser473 and insulin sensitivity indicating that findings at skeletal muscle level influence whole body insulin sensitivity i.e. increasing PKB Ser473 phosphorylation increases whole-body insulin sensitivity.

Also of note, the p85 subunit of PI3K was the only enzyme which had consistently reduced expression in South Asians, both in basal and insulin stimulated conditions. This finding was particularly intriguing as it is unexpected in the context of most of the previously published literature examining p85 expression, which mostly reports increased, rather than reduced p85 expression in insulin resistance. This issue will be explored further in the general discussion.

In addition to the above differences, there was also a trend towards lower protein expression of IRS-1, another key insulin signaling protein, and lower expression of PKB in South Asians, which although significantly lower in unadjusted analysis, became non-significantly different on adjustment for adiposity. Because the numbers were small, it may simply be that the study was under-powered to detect differences in IRS-1, or it may be that increased adiposity is causally linked to reduced IRS-1 expression. It would be interesting to investigate whether differences in IRS-1 protein expression would become significant despite adjustment for adiposity in a larger number of subjects, as it could be that South Asians have variations in insulin signaling at various points in the pathway, rather than just at one point. This would, potentially provide more opportunities to intervene from a therapeutic point of view.

There were no differences between South Asian and European muscle in protein expression of cytosolic or microsomal PKC β1, suggesting that PKC β1 does not appear to be implicated as a mechanism for the increased skeletal muscle insulin resistance observed in South Asians.

The main limitation of this study was that expression of insulin signalling proteins was only assessed in the basal state – i.e. signalling protein expression in response to fasting insulin concentrations – and in response to maximal insulin stimulation. While the direction of the differences in expression in the basal data between South Asians and Europeans precludes potential confounding by differences in fasting insulin concentrations (South Asians had reduced signalling despite a tendency for higher insulin concentrations), this study does not provide data on insulin signalling across the range of physiological insulin concentrations and further study is needed to ascertain whether insulin signalling differs between South Asians and Europeans at insulin concentrations between basal and maximal.

Nonetheless, these findings suggest key differences in insulin signalling in the skeletal muscle of South Asians and Europeans. One possible explanation for this is the effect of the increased IMCL observed in South Asians, on expression of key insulin signaling proteins. At least one study (Forouhi et al. 1999) however, has demonstrated no significant relationship between IMCL (triglyceride) and whole-body insulin sensitivity. This may be because the quantity of IMCL itself is not responsible for insulin resistance, but rather the rate of its turnover (Corcoran et al. 2007) or the proportion IMTG which is made up of the lipid intermediates DAG and ceramides, which have been associated with insulin resistance (Petersen & Shulman 2006) and which have the specific effects on insulin signalling, mainly via PKC, which are mentioned in detail above (Griffin et al. 1999; Laybut et al. 1999)

Another possibility is that the difference in insulin signalling observed between the South Asians and Europeans are a result of an innate skeletal muscle defect. It is not possible from this study to determine if either of the above mechanisms, or a combination of both, is responsible, as one of the limitations of the study was that IMCL and lipid intermediates were not quantified. If increased lipid intermediates in South Asians were the only explanation for impaired insulin signalling in South Asian muscle, a difference in PKC might be expected, given the known effects of DAG as a potent activator of PKC (Griffin et al. 1999; Laybutt et al. 1999; Wolf 2008). However there was no observed difference in PKC, rather PKC expression in South Asian and European muscle was found to be similar. This suggests either that an alternative mechanism is at least partly responsible for impaired insulin signalling in South Asian skeletal muscle or that DAG has a lesser effect on the β1 isoform of PKC, the activity of which was measured in this study, compared with the striking effect it is thought to have on the θ isoform of PKC (Wolf 2008).

The novel findings of this study were significantly lower skeletal muscle protein expression of PI3K and PKB Ser473 phosphorylation in South Asians and a correlation between Ser473 phosphorylation and both fat oxidation during submaximal exercise and VO_{2max}. These findings suggest that the reduced capacity for fat oxidation oberved in South Asians may affect insulin sensitivity via effects on these particular enzymes.

Chapter 5

General Discussion

5.1 Experimental Chapter Summaries

The aims of Chapter 3 were to describe the lifestyle and phenotypic differences between South Asians and Europeans, with a focus particularly, on differences in adiposity, cardiorespiratory fitness and insulin sensitivity and to examine relationships between these variables. The results confirm that South Asians display increased adiposity and lower lean mass, markedly reduced cardiorespiratory fitness and increased insulin resistance, compared with Europeans, and that this may be, in part, as a result of South Asians' reduced ability to oxidize fat during exercise, which was a novel finding of the study. VO_{2max} and fat oxidation during submaximal exercise were correlated significantly with whole-body insulin sensitivity suggesting that these are important associations. In addition South Asians exhibited higher expression of the CPT1A and FASN genes, suggesting that impaired mitochondrial oxidative capacity is unlikely to be the mechanism underpinning their reduced skeletal muscle oxidative capacity.

Chapter 4 examined differences in insulin signalling between South Asians and Europeans, and found reduced protein expression of PI3K p85 subunit in both basal and insulin-stimulated states, and markedly reduced (60% lower) phosphorylation of PKB at Ser473 in South Asians. These differences persisted after adjustment for age, BMI and fat mass, suggesting a real difference in insulin signalling between South Asian and European skeletal muscle. In addition PKB Ser473 phosphorylation correlated positively with insulin sensitivity and with whole body fat oxidation during

exercise, suggesting that differences in capacity for fat oxidation between South Asians and Europeans may alter insulin signaling via effects at this specific point in the insulin signaling pathway.

5.2 Ethnic differences in whole body and skeletal muscle fat oxidation

Two key findings of Chapter 3 were reduced whole-body fat oxidation by around 40% in South Asians during submaximal exercise, and a correlation between fat oxidation during exercise and insulin sensitivity. There was no difference in whole-body fat oxidation at rest, and no relationship between resting fat oxidation and insulin sensitivity. This disparity could potentially result from the fact that whole-body fat oxidation during exercise largely reflects fat oxidation in skeletal muscle, whereas at rest skeletal muscle only contributes about 20-30% of resting energy expenditure (Zurlo et al. 1990), thus whole-body fat oxidation at rest is to a large degree determined by other tissues. In the studies described in Chapter 3, fat oxidation increased, 3-6 fold during exercise compared to at rest and this increase would almost exclusively be attributable to changes in skeletal muscle. Thus, if skeletal muscle, rather than whole-body fat oxidation is the key regulator of insulin sensitivity, the relationship will be more clearly revealed in whole-body measurements made during exercise rather than at rest.

It is also possible that a deficiency in the capacity of muscle to oxidise fat becomes more evident when energy demand is high, and is thus revealed during exercise. These findings highlight the value of exercise testing in providing new insights into muscle metabolism in a relatively non-invasive manner.

Cardiorespiratory fitness is closely associated with skeletal muscle lipid oxidative capacity (Helge et al. 2006; Sahlin et al. 2007; Venables et al. 2005) and it is likely that fitness influences insulin sensitivity, at least partly, via effects on muscle lipid metabolism (Goodpaster & Brown 2005; Goodpaster et al. 2003). In the Chapter 3 studies, adjusting ISI values for either VO_{2max} or rate of fat oxidation during exercise abolished the difference in insulin sensitivity between the South Asian and European groups, and strong correlations were evident between VO_{2max} and fat oxidation during exercise ($r = 0.58$ to 0.67, depending on units of measurement, all $p < 0.0005$). This supports the suggestion that low oxidative capacity/ capacity for fatty acid utilisation is a central feature of the South Asian insulin resistance phenotype, and highlights the fact that the lower cardiorespiratory fitness and reduced capacity to oxidize fat in this group are likely to largely reflect the same underlying mechanism.

Unexpectedly, and in contrast to the observations made at the whole body level the reduction in fat oxidation seen in South Asians was not reflected by a reduction in skeletal muscle expression of mitochondrial oxidative and lipid metabolism genes in skeletal muscle biopsy samples, but rather expression of some of these genes was higher in South Asians. There was no relationship between whole-body insulin sensitivity and expression of any of the mitochondrial oxidative or lipid metabolism genes.

In Europeans, capacity for mitochondrial fat oxidation in *ex vivo* muscle biopsy samples has been shown to correlate strongly with whole-body fat oxidation during sub-maximal exercise (but not at rest) (Sahlin et al. 2007); this is consistent with observations in the study where positive correlations were observed between skeletal

muscle expression of oxidative and lipid metabolism genes and fat oxidation during submaximal exercise in the European group. However, strikingly, this pattern was reversed in the South Asian group where negative correlations were observed between skeletal muscle gene expression and fat oxidation during exercise, and significant interactions in the relationships between skeletal muscle gene expression and fat oxidation during exercise were evident between the European and South Asian groups. These relationships are illustrated in Figure 5.1.

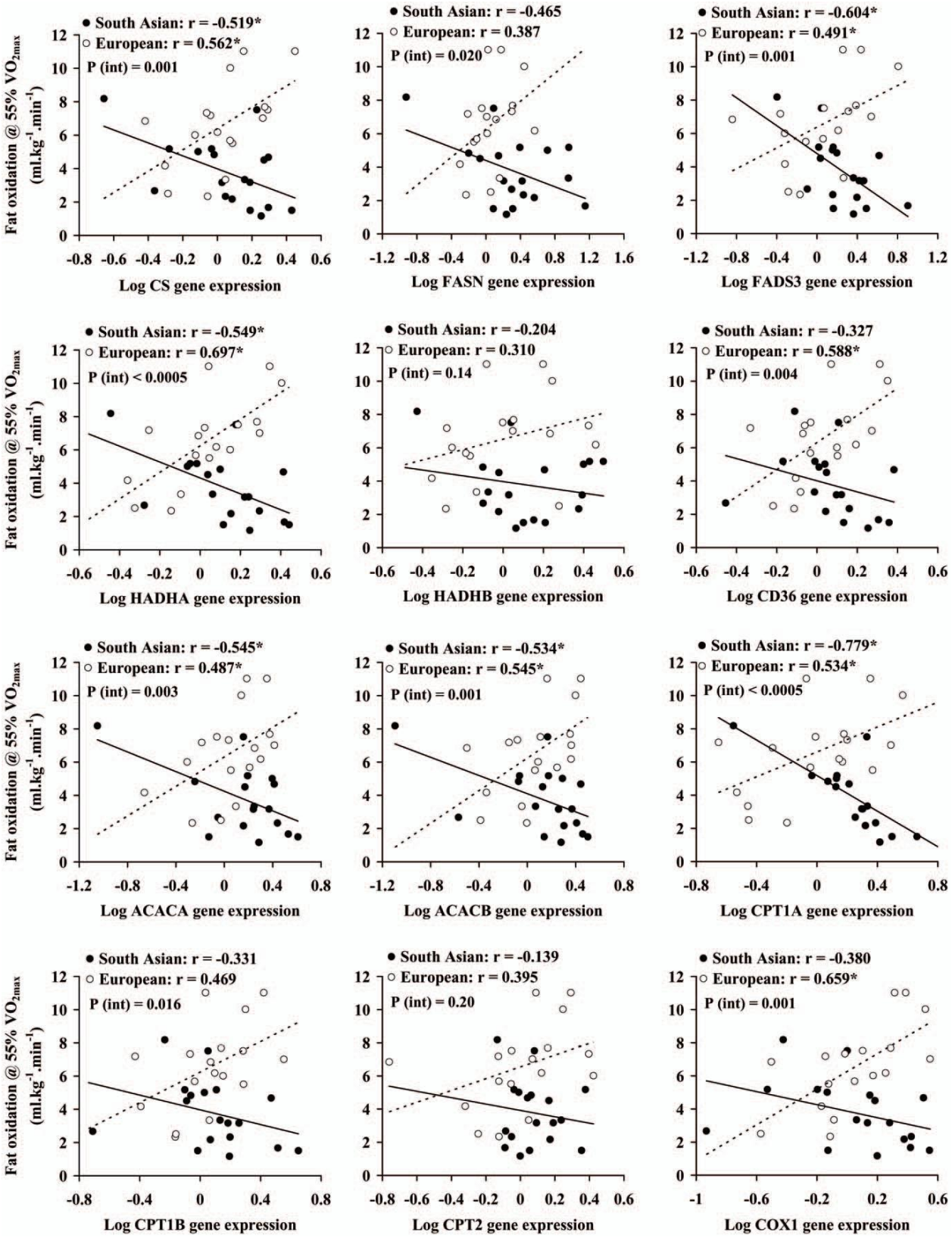

Figure 5.1 Relationships between exercise fat oxidation and skeletal muscle expression of oxidative and lipid metabolism genes. Significant correlations ($p<0.05$) are denoted by an asterisk. P (int) signifies p-value for interaction in relationship between gene expression and fat oxidation between European and South Asians groups.

Thus, the South Asians with the highest expression of oxidative and lipid metabolism genes oxidised the least fat during exercise, indicating a dissociation between mitochondrial function and the reduced whole body oxidative capacity with increased insulin resistance observed in South Asians, such that reduced mitochondrial function does not seem to be the mechanism underlying increased insulin resistance in this population. This finding is consistent with the study by Nair and colleagues (Nair et al. 2008), which reported increased skeletal muscle capacity for oxidative phosphorylation and a higher mitochondrial DNA copy number in South Asians.

An alternative hypothesis is that the reduced capacity of South Asians to oxidise fat during exercise represents a defect in substrate delivery rather than impaired mitochondrial function. Conceivably, the observed increase in oxidative and lipid metabolism gene expression within South Asians' skeletal muscle may represent a compensatory "up-regulation" to attempt to attenuate the effect of, for example, reduced fuel delivery to skeletal muscle or to the mitochondria itself.

It is not possible to quantify skeletal muscle oxidative capacity, capacity for fat utilisation and insulin sensitivity by examining only expression of these oxidative and lipid metabolism genes, without investigating enzyme activity, or indeed by examining mitochondrial function alone, as mitochondrial size, density and distribution may also be important. Indeed a recent study investigating mitochondrial location found that intermyofibrillar mitochondrial content was lower in insulin resistant and diabetic subjects than in lean, insulin-sensitive subjects, and that intermyofibrillar mitochondrial content was correlated significantly with clamp-

derived measures of insulin sensitivity and indices of fat oxidation (Chomentowski et al 2011).

In addition, macrovascular and microvascular circulation (i.e. capillary density and recruitment) will also have an effect on oxidative capacity. As mentioned in Chapter 1, South Asian men are known to have impaired endothelial function in forearm resistance vessels (Murpy et al. 2007)and reduced nitric oxide bioavailability both at rest and during exercise (Cubbon et al. 2010), both of which could be important factors in determining skeletal muscle oxidative capacity, as both will influence substrate delivery to skeletal muscle. Substrate delivery data is not available from this study, nor are there relevant published data in the literature, so clearly further research is required to determine whether there are indeed ethnic differences in rates of substrate delivery to skeletal muscle, and to confirm the finding of apparently increased "mitochondrial efficiency" in South Asians, as both this study and the only other study examining ethnic differences in mitochondrial function involved small numbers of subjects.

It is also important to recognise that increased fatty acid mobilisation could produce the same effect as reduced fat oxidation, as it is the mismatch between mobilisation and oxidation of fatty acid, rather than a low ability of muscle to oxidise fat *per se*, which can result in insulin resistance. Recent studies have demonstrated improvements in insulin sensitivity with weight loss which results in a reduction in fatty acid mobilisation (Schenk et al. 2009), in the absence of changes in oxidative capacity or fatty acid oxidation rates (Berggren et al. 2008; Schenk et al. 2009; Toledo et al. 2008) and have also shown that increasing fatty acid mobilisation by

lipid infusion can abolish improvements in insulin sensitivity elicited by exercise training, despite fatty acid oxidation rates remaining elevated (Schenk et al. 2009). Thus, capacity for fatty acid oxidation clearly only provides one part of the story, and further investigation is also needed to determine whether differences in fatty acid mobilisation exist between South Asians and Europeans that may contribute to the differences in insulin sensitivity.

5.3 Ethnic differences in insulin signalling

There have been no previous data comparing insulin signalling molecule expression between South Asians and Europeans or indeed data examining insulin signalling differences in any ethnic groups.

The principal findings from Chapter 4 were of reduced basal and insulin-stimulated protein expression of PI3K p85 subunit and markedly reduced PKB phosphorylation at Ser473 in South Asians, but in unadjusted analysis, South Asians also demonstrated lower protein expression of IRS-1 and of basal PKB.

IRS-1 is the principal IRS involved in muscle insulin-stimulated glucose transport (Sesti et al. 2001), but evidence regarding the role of altered IRS-1 expression in insulin resistant states is lacking, due to conflicting results from several studies examining this, as detailed in previous chapters. This suggests that IRS-1 itself may not have a major role in insulin resistance, but rather that changes in components of the insulin signalling pathway downstream of IRS-1 may play a more important role.

The data described in Chapter 4 do imply reduced IRS-1 signalling in South Asians, which may manifest as impaired insulin-stimulated glucose transport and a degree of skeletal muscle insulin resistance. IRS-1 expression did not correlate significantly with whole-body insulin sensitivity, so it may be that the observed reduction in IRS-1 expression simply reflects greater adiposity in the South Asian group, rather than a fundamental difference in South Asians' skeletal muscle function. The fact that the ethnic difference in IRS-1 expression was abolished after adjustment for adiposity supports this interpretation.

The finding of significantly lower basal and insulin stimulated expression of the p85 subunit of PI3K in South Asians persisted after adjustment for age, BMI and fat mass, implying a constitutional difference in this aspect of insulin signalling between South Asian and European skeletal muscle. The implications of altered PI3K p85 subunit expression on insulin sensitivity are also unclear in the literature, with the majority of published studies reporting increased p85 expression as an association with increased insulin resistance in both human and animal skeletal muscle (Bandyopadhyay et al. 2005; Barbour et al. 2005; Friedman et al. 1999; Hammarstedt et al. 2005). In addition, down-regulation of p85 has been reported to reverse the inhibition of insulin-stimulated glucose transport in 3T3-L1 adipocytes caused by constitutively active PKB (Adochio et al. 2009). However, reduced p85 expression has also been reported to be associated with obesity in a single study in placenta (Colomiere et al. 2009).

The reduction observed in South Asians in the current study is, therefore, in disagreement with many studies of insulin-resistant cohorts, yet this may indicate that there is a requirement for optimal levels of p85 expression, such that increased and

decreased p85 expression both have detrimental consequences in terms of insulin sensitivity.

PKB exists as three isoforms, a (Akt1), b (Akt2) and c (Akt3), and, as mentioned in Chapter 4, studies in knockout mice have indicated that PKBβ is the principal regulator of glucose homeostasis (Cho et al. 2001; Cleasby et al. 2007). As with the other insulin-signalling proteins, studies examining PKB phosphorylation/activity in insulin-resistant states have reported differing results.

In chapter 4, expression and phosphorylation of PKB at Ser473 was assessed using antibodies that do not distinguish between the different isoforms. Interestingly total PKB protein expression was 50% lower in South Asians than Europeans, although after adjustment for age, BMI and fat mass, this difference was reduced to borderline statistical significance ($p = 0.054$). In addition, basal Ser473 phosphorylation of PKB was over 60% lower in South Asians and differences persisted after adjustment for age, BMI and fat mass. It also correlated significantly with whole body insulin sensitivity. The increased basal Ser473 phosphorylation observed in Europeans is unlikely to be a result of effects from endogenous insulin associated with the muscle biopsies after washing, as South Asians exhibited higher fasting insulin concentrations than Europeans. In addition, basal PKB Ser473 phosphorylation correlated strongly and significantly with whole-body fat oxidation rates during exercise, suggesting that differences in capacity for fat oxidation between Europeans and South Asians may influence insulin sensitivity via effects at this point in the insulin signaling pathway.

There were no differences between South Asian and European muscle in protein expression of cytosolic or microsomal PKC β1, which negatively regulates insulin signaling (Standaert et al. 1999), suggesting that PKC β1 does not underlie the increased skeletal muscle insulin resistance observed in South Asians.

5.4 Interactions between adipose tissue and skeletal muscle

Results from Chapter 3 and from numerous previous studies demonstrate that adipose tissue has a strong association with whole body insulin resistance, and the relationship between fat mass and insulin resistance in South Asians suggests increased susceptibility to the adverse effects of adiposity.

Because the DEXA imaging technique used in this study was insufficiently detailed to distinguish distinct adipose tissue compartments, these were not examined in this study, so it is unclear whether the South Asians in the present study had increased visceral fat, deep subcutaneous fat or superficial subcutaneous fat, although the issue of which of these compartments is most harmful with respect to NEFA mobilisation, adipose derived hormones and insulin resistance has yet to to be fully elucidated (Banerji et al. 1999; Chandalia et al. 2007; Gautier et al. 1999; Sniderman et al. 2007). Computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US) are able to distinguish visceral and subcutaneous adipose tissue by identification of the fascia superficialis, which enables quantification of deep and superficial subcutaneous compartments (Smith et al. 2001), and further studies using these imaging techniques to compare adipose tissue location in different ethnic groups would be useful.

As mentioned in Chapter 1, it is likely that adipose tissue function rather than quantity or location the more important determinant of insulin sensitivity, supported by the finding of reductions in both circulating insulin and leptin concentrations, independent of fat mass in response to weight loss (Doucet et al. 2000), along with the intriguing discovery of increased adipocyte size in South Asians, associated with insulin resistance by Chandalia *et al* (Chandalia et al. 2007) and Anand *et al* (Anand et al. 2011).

The hypothesis of dysfunctional adipose tissue and early ectopic fat deposition in South Asians' skeletal muscle is broadly in keeping with the study finding of differences in insulin signalling between the two ethnic groups described above, as ectopic skeletal muscle fat would result in an increased quantity of the harmful lipid intermediates which are known to disrupt insulin signalling. Previously published studies, however, have suggested that this increase in lipid intermediates would be likely to manifest itself as an effect on PCK activity, with DAG being a potent activator of this enzyme (Griffin et al. 1999; Laybutt et al. 1999; Wolf 2008). This might imply that increased ectopic fat and lipid intermediates impaired insulin signalling in South Asian muscle in a novel way, for example, by reducing PKB Ser473 phosphorylation, an effect which is not seen in other insulin resistant groups, or it could simply be that DAG has a lesser effect on the β1 isoform of PKC, the activity of which was measured in this study, compared its known effect on PKC θ (Wolf 2008), which it was unfortunately not possible to measure.

Dysfunctional adipose tissue and ectopic skeletal muscle fat accumulation could also explain increased expression of the oxidative and lipid metabolism genes. CPT-1 activity has been previously repored to be reduced in insulin resistant states) (Kim et al. 2000; Simoneau et al.1999) and the unexpected finding of significantly increased CPT-1A expression in South Asians, indicating increased delivery of fatty acids to the mitochondria for oxidation, probably indicates a problem further upstream. This could implicate increased fatty acid mobilisation from dysfunctional adipose tissue depots as a mechanism, and the increased mitochondrial oxidative enzyme gene expression might represent an attempt by skeletal muscle to compensate for increased fatty acid mobilisation/ delivery. If this theory is correct, the increase in intracellular transport and fat oxidation does not seem to be sufficient to compensate for the increase fatty acid mobilisation, resulting in a net gain of fat into the mucle, and resultant effects on insulin signalling.

5.5 Implications for future studies

Given that findings of the present study and those of Nair *et al* demonstrate reduced whole body fat oxidation in the face of increased oxidative and lipid metabolism enzyme activity, the next logical step would be to do a further comparison study to attempt to identify some of the missing pieces of the puzzle. This might involve examining indices of endothelial function, fatty acid mobilisation and IMCL (including location of lipid within myocyes) together, and confirming the findings of reduced oxidative enzyme gene expression and enzyme activity in South Asians. More detailed investigation of mitochondrial size and location would also be interesting.

A further worthwhile future study would be to examine the effects of an exercise intervention on insulin sensitivity and skeletal muscle oxidative function in South Asians compared to Europeans, incorporating robust measurement of physical activity with accelerometers, both at baseline and during the intervention.

Conceivably larger numbers of volunteers could be recruited if a non-invasive measure of muscle metabolism, such as magnetic resonance spectroscopy was used as an alternative to muscle biopsy, and detailed imaging with CT or MRI could help further investigate the various hypotheses relating to ethnic differences in adipose tissue compartments and adipose tissue function. Measurement of differential rates of adipose tissue mobilisation from the VAT, superficial and deep subcutaneous adipose tissue depots in South Asians and Europeans may also provide some insight, and genetic studies examining adipose tissue expression of some of the genes implicated in insulin resistance could also assist our understanding of the role of adipose tissue dysfunction in insulin resstance in South Asians.

5.6 Final Conclusions

In conclusion, this thesis describes studies which have attempted to increase understanding of the mechanisms which underlie the characteristic insulin resistant phenotype seen in South Asians. Novel findings from the studies suggest that a key feature is reduced whole-body oxidative capacity and capacity for fatty acid utilisation, but this does not seem to be a consequence of reduced skeletal muscle expression of oxidative and lipid metabolism genes. These studies provide a small part of the picture and further investigation is required to elucidate the mechanism underpinning reduced capacity for fat oxidation in South Asians and to explain how this relates to their reduced insulin sensitivity.

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Appendices

Appendix A1 Volunteer information sheet

Appendix A2 Consent form

Appendix A3 Health screen questionnaire

Appendix A4 International Physical Activity Questionnaire (IPAQ) long version

Appendix A5 Food frequency questionnaire

Appendix B1 Method of analysis for insulin

Appendix B2 Method of analysis for glucose

Appendix B3 Method of analysis for NEFA

Appendix B4 Method of analysis for TG

Appendix B5 Method of analysis for lactic acid

Appendix B6 Method for analysis of leptin

Appendix A1

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VOLUNTEER INFORMATION SHEET

Insulin resistance in South Asians: role of skeletal muscle and adipose tissue function

Does the function of muscle and fat tissue affect risk of diabetes?

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

It is well known that South Asians living in the UK have a much greater risk of adult-onset diabetes than their European counterparts. This is likely to be due to differences in their metabolism making insulin less effective at controlling their blood sugar levels. This is known as "insulin resistance" but it is unclear what makes South Asians' body tissues more resistant to the effects of insulin. This study will investigate for the first time whether differences in South Asians' muscles ability to burn fat or differences in the way that fat tissue functions can explain the significant differences in their metabolism, compared to Europeans and provide new insights into their increased risk for diabetes. This may lead to better therapies to prevent and treat diabetes.

Why have I been chosen?

You have been chosen because you are a healthy adult aged between 18 and 40, with both parents of either European or South Asian (i.e. Indian, Pakistani, Bangladeshi or Sri Lankan) origin. The study will compare the two different groups to establish whether there are differences in insulin resistance and muscles' ability to burn fat and the function of fat tissue between Europeans and South Asians.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

Who is organising and funding the research?

- Diabetes UK, the largest UK organisation working for people with diabetes. It aims to help people live with diabetes through campaigning and funding research.

- Wyeth pharmaceuticals are a major drug company in the United States with a UK base in Surrey, England.

What is my part in the study?
If you agree to take part in the study and meet entry requirements then your participation will consist of 4 or 5 visits over a period of two to four weeks. Most visits will last around two hours.

What do I have to do?
Firstly you will be asked to attend for a screening visit where we will discuss with you and complete confidential questionnaires regarding your health, family history, physical activity and diet. We will also measure your blood pressure and take a small blood sample (20 ml or 4 teaspoons) to check the fat and sugar levels in your blood. We will also check for any abnormalities in the function of your liver, kidneys and thyroid gland to ensure that it is perfectly safe for you to participate in this study. There will be an opportunity for you to ask questions.

During the remaining visits we will ask you to undertake a number of tests to determine your level of health and fitness.

- On the second visit a screening exercise (walking) test will be performed. This test will involve walking on a treadmill with self-adhesive electrodes attached to your chest to monitor how hard your heart is working. This test will last for a maximum of 12 minutes and will confirm whether it is safe for you to take part in this study. This visit can be combined with the initial screening visit.

- The third and fourth visits we will perform tests to look at the amount of oxygen you are breathing in and the amount of carbon dioxide your body is producing at rest. This is a way of measuring your body's resting metabolism. There will then be two additional tests where you will walk on a treadmill at different speeds and gradients and we will monitor your heart rate and collect the air you breathe out to determine

your fitness level. We will also take tiny 'finger prick' blood samples during one of the tests. One test will involve a maximal effort and will last for about 10 to 20 minutes. The other test will not involve a maximal effort and will last for about 40 minutes.

- On the final visit we will repeat the resting metabolic rate test. Then we will perform muscle and fat tissue biopsies. The muscle biopsy involves taking a very small sample of muscle (less than half a gram) from the side of your thigh. We will numb the area with anaesthetic beforehand and take the sample using a special device designed to take very small pieces of muscle through a small (3 to 5 mm) cut in the skin. The biopsy site will then be closed with sterile strips which stick to the skin and covered with a dressing. No aftercare is needed and you can the remove the strips and dressing yourself after 2 or 3 days. You may feel slight discomfort during the biopsy and experience some bruising at the site afterwards but this should subside within 2-3 days. For the fat tissue biopsy we will take a small fat sample from your tummy by 'liposuction'. This involves numbing the skin with a local anaesthetic then sucking up 1 to 2 grams of fat (about the size of a pen top) with a needle and syringe. This is no more painful than having a blood sample taken.

- In addition, during the final visit you will have a glucose tolerance test. We will ask you to drink a sugary drink and take blood samples before the drink and for two hours after the drink to assess how your body deals will this sugar. This will help us to determine how well the insulin in your body is working. This will be no more painful than a simple blood test as samples will be obtained from a tiny plastic tube called a 'cannula' placed in a forearm vein. We will take approximately 120 ml (about an eggcup full) of blood over the course of this test.

- We will also measure your weight and height and measure around your waist and hips. We will perform a scan of your body to calculate how much fat you are carrying. These tests will take about 15 minutes in total and can be carried out on the same day as other tests. You will also be asked to complete questionnaires about your diet and levels of physical activity.

For all of the visits to the University, you will need to be fasted (i.e. not having had anything to eat or drink, except water, for at least 12 hours). It may, therefore be more convenient for you arrange visits in the morning.

What are the possible disadvantages and risks of taking part?

- One of the exercise tests will be at a maximal level and the possibility exists that, very occasionally, certain changes may occur during or shortly after the test. They include abnormal blood pressure, fainting or a change in the normal rhythm of the heartbeat.
- Blood sampling via the cannula may cause minor bruising, an inflammation of the vein or haematoma (a small accumulation of blood under the skin). Good practice, however, minimises this risk. Some people may feel faint when they give blood.
- Taking the muscle and fat biopsies carries a small risk of minor bruising or an infection. Good sterile practice reduces this risk.
- The scan to determine your level of body fat uses a very small dose of radiation. Each scan is equivalent to about on twentieth of a chest X-ray or the amount of natural background radiation that we are exposed to in 4 hours.
- There is a small possibility that taking part in this study will reveal a health problem that you already have such as high cholesterol or high blood pressure. If such a problem is revealed, we will inform your GP to ensure that you receive appropriate treatment. If necessary, a clinical consultant physician (Professor Naveed Sattar) will supervise any follow-up care.

What are the possible benefits of taking part?

The information gained during the study will allow us to give you detailed feedback about your fitness level, body fat, dietary intake, blood pressure, cholesterol, blood sugar and level of “insulin resistance”. In addition, the knowledge gained from your participation may benefit people who have diabetes in the future by increasing our understanding of why South Asians are at increased risk of developing diabetes. It will also help guide future research investigating how to reduce this increased risk through lifestyle changes and may also help direct the development of drugs to prevent and treat diabetes in groups at increased risk of the disease.

What if something goes wrong?

The chance of something going wrong is extremely small. All of the procedures involved in this study are low risk and our screening tests are designed to ensure that you will only participate if it is safe for you to do so. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

Will my taking part in this study be kept confidential and who will have access to my personal information?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the University will be anonymised (i.e. will have your name and address removed so that you cannot be recognised from it and you will only be identified by a code number). Anonymised information collected about you may be shared in future with other parties including researchers working on behalf of Wyeth Pharmaceuticals in the United States, the UK and in other countries. None of your personal information will be shared with anybody or any organisation outside the University of Glasgow without your specific consent.

What will happen to my samples after the study has finished?
The blood and fat tissue samples that you provide for this study may be useful for future research into the prevention and treatment of diabetes and heart disease; this may involve analysis of certain genes associated with these diseases. These samples may be shared in future with other parties including researchers working on behalf of Wyeth Pharmaceuticals in the United States, UK and in other countries. Any use of your samples for future research will require further approval from a Research Ethics Committee and samples will be analysed in such a way that the results will not be directly traceable to you. If you do not wish your samples to be used for future research, please indicate this on the consent form.

Who has reviewed the study?
This study has been reviewed and approved by the North Glasgow NHS Trust Research Ethics Committee.

Contact for Further Information
You may ask any questions you like now or at any time about your rights as a participant in a research study or about the research study itself. Dr Lesley Hall will be available to discuss these issues with you if you phone her on 0141 330 6588 or or e-mail l.hall@bio.gla.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep for your records.

Appendix A2

UNIVERSITY *of* GLASGOW

Volunteer Identification Number for this trial:

CONSENT FORM

Title of Project: Insulin resistance in South Asians: role of skeletal muscle and adipose tissue function.

Name of Researcher:_____

Please initial box

1. I confirm that I have read and understand the information sheet dated 14th June 2007 (version 3) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to take part in the above study. ☐

4. I agree for my samples to be used for future research into the prevention and treatment of diabetes and heart disease. This may involve analysis of genes associated with these diseases. Yes ☐ No ☐

_____	_____	_____
Name of Volunteer	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent (if different from researcher)	**Date**	**Signature**

Researcher	**Date**	**Signature**

Appendix A3

HEALTH SCREEN FOR STUDY VOLUNTEERS

Name: …….

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

At present, do you have any health problem for which you are:

(a) on medication, prescribed or otherwise	yes [ ]	no [ ]
(b) attending your general practitioner	yes [ ]	no [ ]
(c) on a hospital waiting list	yes [ ]	no [ ]

In the past two years, have you had any illness which required you to:

(a) consult your GP	yes [ ]	no [ ]
(b) attend a hospital outpatient department	yes [ ]	no [ ]
(c) be admitted to hospital	yes [ ]	no [ ]

Have you ever had any of the following:

(a) Convulsions/epilepsy	yes [ ]	no [ ]
(b) Asthma	yes [ ]	no [ ]
(c) Eczema	yes [ ]	no [ ]
(d) Diabetes	yes [ ]	no [ ]
(e) A blood disorder	yes [ ]	no [ ]
(f) Head injury	yes [ ]	no [ ]
(g) Digestive problems	yes [ ]	no [ ]
(h) Hearing problems	yes [ ]	no [ ]
(i) Problems with bones or joints	yes [ ]	no [ ]
(j) Disturbance of balance/co-ordination	yes [ ]	no [ ]
(k) Numbness in hands or feet	yes [ ]	no [ ]
(l) Disturbance of vision	yes [ ]	no [ ]
(m) Thyroid problems	yes [ ]	no [ ]
(n) Kidney or liver problems	yes [ ]	no [ ]
(o) Chest pain or heart problems	yes [ ]	no [ ]
(p) Any other health problems	yes [ ]	no [ ]

4. Have any of your family (parents, grandparents, brothers, sisters, children, aunts, uncles, cousins) ever had any of the following: (if yes please give details including age of first diagnosis)

(a) Any heart problems	yes [ ]	no [ ]
(b) Diabetes	yes [ ]	no [ ]
(c) Stroke	yes [ ]	no [ ]
(d) Any other family illnesses	yes [ ]	no [ ]

7. Do you currently smoke — yes [ ] no [ ]
   Have you ever smoked — yes [ ] no [ ]

If so, for how long did you smoke and when did you stop? ……

8. How many units of alcohol do you typically drink in a week? …….
9. What is your mother's country of origin?.....
10. What is your father's country of origin?.....

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.) (Use a separate sheet if necessary)

……

……

……

……

……

……

……

Name and address of GP

……

……

……

Blood pressure measured at screening……..mm Hg

Appendix A4

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ
The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ
Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation
Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ
International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information
More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Appendix A5

FOR OFFICE USE ONLY

FOOD INTAKE QUESTIONNAIRE

Surname..... ....

First Name(s) ....

Address ....

....

Phone No. .. ..

Date of Birth .... Date of Survey ....

Subject ID ☐☐☐☐ 1-4 ☐☐☐☐ 5-8

Questionnaire No ☐ 9

Group Code ☐ 10

Survey No ☐☐ 11-12

Male / Female ☐ 13

☐☐ 14-15

☐☐ 16-17

☐☐☐☐ 18-19/162-163

☐☐ 20-21

☐☐ 22-23

☐☐☐☐ 24-25/164-165

The following questions are about the foods you USUALLY eat.
Please indicate the number of days per week that you eat each item on average. Ring the answer as in these examples:

If you eat the food every day, ring 7 (7) 6 5 4 3 2 1 F R
If you eat the food three days/week, ring 3 7 6 5 4 (3) 2 1 F R
If you eat the food once a fortnight, ring F 7 6 5 4 3 2 1 (F) R
If you rarely or NEVER eat the food, ring R 7 6 5 4 3 2 1 F (R)

PLEASE ANSWER EVERY QUESTION

BREAD

How often do you eat the following breads and how many slices do you have per day?

	No. days/week	*No. slices or rolls per day*	*Size of slices or rolls*	
White or high fibre white	7 6 5 4 3 2 1 F R	.....	Thick/medium/thin Large/small	☐☐☐ 26-28
Brown or wheatgerm	7 6 5 4 3 2 1 F R	.....	Thick/medium/thin Large/small	☐☐☐ 29-31
Wholemeal/chapatis	7 6 5 4 3 2 1 F R	.....	Thick/medium/thin Large/small Chapatis	☐☐☐ 32-34
Bread rolls/crumpets	7 6 5 4 3 2 1 F R	.....	White or crumpets /brown/wholemeal	☐☐☐ 35-37
Crispbread, Ryvita or cream crackers	7 6 5 4 3 2 1 F R	.....		☐☐ 38-39

How often do you eat jam, marmalade or honey on bread? 7 6 5 4 3 2 1 F R ☐ 40

FOR OFFICE USE ONLY

BREAKFAST CEREALS

How often do you eat the following cereals?

1. Cornflakes	7 6 5 4 3 2 1 F R	
2. Sugar Puffs, Special K, Ricicles, Rice Krispies, Coco Pops, Frosties or Crunchy Nut Cornflakes	7 6 5 4 3 2 1 F R	
3. Muesli, Fruit n' Fibre or Cheerios	7 6 5 4 3 2 1 F R	41-42
4. Weetabix, Wheat Flakes or Shredded Wheat	7 6 5 4 3 2 1 F R	
5. Bran Flakes or Sultana Bran	7 6 5 4 3 2 1 F R	43-44
6. Porridge or Ready Brek	7 6 5 4 3 2 1 F R	
7. All Bran	7 6 5 4 3 2 1 F R	
Other Cereal	7 6 5 4 3 2 1 F R	
Please specify brand/type	.....	
How many teaspoons of sugar/honey do you add?	.....	45
How often do you have wheat bran?	7 6 5 4 3 2 1 F R	46

MEATS

How often do you have the following meats?
Include all forms of each meat, eg use in stews, casseroles, lasagne, curry etc.

Beef (including beefburgers)	7 6 5 4 3 2 1 F R	47
Lamb	7 6 5 4 3 2 1 F R	48
Pork	7 6 5 4 3 2 1 F R	49
Bacon	7 6 5 4 3 2 1 F R	50
Ham	7 6 5 4 3 2 1 F R	51
Chicken or other poultry	7 6 5 4 3 2 1 F R	52
Canned meat (e.g., corned beef), paté or meat spread	7 6 5 4 3 2 1 F R	53
Sausages	7 6 5 4 3 2 1 F R	54
What type of sausages do you have?	1 Pork 2 Beef 3 Pork and Beef 4 Turkey 5 Low Fat	55
Meat pie/pastie/sausage roll/samosa - shop bought	7 6 5 4 3 2 1 F R	56
Meat pie/pastie/sausage roll/samosa - home made	7 6 5 4 3 2 1 F R	57
Liver/kidney/heart	7 6 5 4 3 2 1 F R	58
Do you usually eat the fat on meat?	Yes / No	59

FOR OFFICE USE ONLY

FISH

How often do you eat the following fish?

White fish (cod/haddock/plaice/fish fingers/fish cakes)	7 6 5 4 3 2 1 F R	60
Kipper/herring/mackerel/trout (including canned)	7 6 5 4 3 2 1 F R	61
Pilchards/sardines/salmon (including canned)	7 6 5 4 3 2 1 F R	62
Tuna (including canned)	7 6 5 4 3 2 1 F R	63

VEGETABLES & SAVOURY DISHES

How often do you have the following vegetables or dishes?

Potatoes - boiled or mashed	7 6 5 4 3 2 1 F R	64
Potatoes - jacket	7 6 5 4 3 2 1 F R	65
Chips - shop bought, 'oven/microwave chips' or hash browns	7 6 5 4 3 2 1 F R	66
Chips - homecooked	7 6 5 4 3 2 1 F R	67
Potatoes - roast	7 6 5 4 3 2 1 F R	68
Peas	7 6 5 4 3 2 1 F R	69
Other green vegetables, salads or tomatoes	7 6 5 4 3 2 1 F R	70
Carrots	7 6 5 4 3 2 1 F R	71
Parsnips, swedes, turnips or sweetcorn	7 6 5 4 3 2 1 F R	72
Baked beans	7 6 5 4 3 2 1 F R	73
Butter beans, broad beans or red kidney beans	7 6 5 4 3 2 1 F R	74
Lentils, chick peas or dahl	7 6 5 4 3 2 1 F R	75
Onions (cooked/raw/pickled)	7 6 5 4 3 2 1 F R	76
Spaghetti, other pasta or noodles	7 6 5 4 3 2 1 F R	77
Rice (NOT pudding rice)	7 6 5 4 3 2 1 F R	78
Quiche	7 6 5 4 3 2 1 F R	79
Pizza	7 6 5 4 3 2 1 F R	80
Vegetable pie/pasty/samosa	7 6 5 4 3 2 1 F R	81

BISCUITS, CAKES & PUDDINGS

How often do you eat the following items?

Digestive biscuits/plain biscuits	7 6 5 4 3 2 1 F R	82
Other sweet biscuits	7 6 5 4 3 2 1 F R	83
Chocolate, e.g., Galaxy, Mars Bar, Twix, KitKat	7 6 5 4 3 2 1 F R	84

FOR OFFICE USE ONLY

Sweets, e.g., fruit gums, pastilles, mints	7 6 5 4 3 2 1 F R	85
Crisps/savoury snacks, e.g., Quavers, tortilla chips	7 6 5 4 3 2 1 F R	86
Nuts	7 6 5 4 3 2 1 F R	87
Ice cream, iced dessert, fool, mousse or trifle	7 6 5 4 3 2 1 F R	88
Low fat yogurt	7 6 5 4 3 2 1 F R	89
Low calorie yogurt e.g., Shape	7 6 5 4 3 2 1 F R	90
Other yogurt/fromage frais, e.g., thick & creamy	7 6 5 4 3 2 1 F R	91
Fruitcake/sponge cake/sponge pudding - shop bought	7 6 5 4 3 2 1 F R	92
Fruitcake/sponge cake/sponge pudding - homemade	7 6 5 4 3 2 1 F R	93
Fruit tart/jam tart/doughnut/Danish pastry - shopbought	7 6 5 4 3 2 1 F R	94
Fruit tart/jam tart - home made	7 6 5 4 3 2 1 F R	95
Milk pudding e.g., rice/tapioca/macaroni	7 6 5 4 3 2 1 F R	96

What type of milk do you use for milk pudding?

1 Ordinary/whole
2 Semi-skimmed
3 Skimmed
4 Canned milk pudding - ordinary
5 Canned milk pudding - low fat

97

FRUIT

How often do you have fruit canned in syrup?	7 6 5 4 3 2 1 F R	98
How often do you have fruit canned in juice?	7 6 5 4 3 2 1 F R	99
How many apples do you have per week?	.....	100
How many pears do you have per week?	.....	101
How many oranges/tangerines/satsumas/clementines/ grapefruit do you have per week?	.....	102
How many bananas do you have per week?	.....	103

EGGS & MILK PRODUCTS

How many eggs do you usually eat per week? 104-105

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Roughly how much milk do you drink in a day in tea/coffee/milky drinks/cereals?

1 None
2 Half a pint or less
3 Between half and one pint
4 One pint or more

☐ 106

What type of milk do you have?

1 Whole
2 Semi-skimmed
3 Skimmed
4 More than one type

☐ 107

How much cream do you use per week?

(1 tablespoon=20g; small carton=150g; large carton=300g) .....g ☐☐☐ 108-110

How much cheese (excluding cottage cheese) do you usually eat per week? .....g ☐☐☐ 111-113

(Suggestion: divide amount bought for household by number of people in house)

How often do you eat cottage cheese? 7 6 5 4 3 2 1 F R ☐ 114

FATS

What do you usually spread on bread?

1 Butter
2 Polyunsaturated margarine/spread
3 Other soft marg/spread (tub) (not olive spread)
4 Hard margarine (block)
5 Low fat spread - polyunsaturated
6 Low fat spread - other
7 Lard, dripping, solid vegetable oil
8 Very low fat spread (25% fat)
9 Olive oil spread
0 Bread eaten dry

☐☐ 115-116

Brand name & description on packet/tub .....

How much butter/margarine/spread do you usually eat per week?.....g ☐☐☐ 117-119

(One block or small tub = 250g. Spread on one slice of bread: Thinly=5g; Medium=8g; Thickly=13g.)

How often do you have food that is fried?
(e.g., fish/onions/mushrooms/tomatoes/eggs) 7 6 5 4 3 2 1 F R ☐ 120

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What types and BRANDS of fat do you use in cooking?

Frying	.....	solid/liquid	121
Chips	.....	solid/liquid	122
Roast Potatoes	.....	solid/liquid/eaten out	123
Home made cake	.....		124
Home made pastry	.....		125

DRINKS

How many cups of tea do you have per day? 126-127

How many teaspoons of sugar/honey per cup? 128

How many cups of coffee do you have per day? 129-130

How many teaspoons of sugar/honey per cup? 131

How often do you have fruit juice/squash/fizzy drinks (NOT low calorie)? 7 6 5 4 3 2 1 F R 132

Which of these do you usually have?

1 Natural Juice
2 Squash
3 Fizzy Drink
4 More than one

133

How often do you have drinks containing alcohol? 7 6 5 4 3 2 1 F R 134

When you drink, how many do you have? .....

Please specify how many drinks of each type per occasion:

Beer/lager/stout/cider	Number of pints	.....	135-136
Wine	Number of glasses	.....	137
Sherry/port/vermouth	Number of glasses	.....	138
Spirits/liqueurs	No. of single measures	.....	139

FOR OFFICE USE ONLY

HEIGHT, WEIGHT & ACTIVITY

What is your height? ..... ft ..... ins **OR** .....cm

140-143 ☐☐☐.☐ ☐☐☐.☐ 144-147

What is your weight? ..... st ..... lbs **OR** .....kg

How physically active is your occupation?

1 Not very active
2 Moderately active
3 Very active
4 Not working

☐ 148

How physically active is your leisure time?

1 Not very Active
2 Moderately active
3 Very active

☐ 149

Questions for women only..

Are you pregnant? Yes / No ☐ 150

Are you breast feeding? Yes / No ☐ 151

ADDITIONAL QUESTIONS

How often do you have..

Dishes made with TVP (soya mince) or Quorn? 7 6 5 4 3 2 1 F R ☐ 152

Vegetarian sausages / Vegetarian burgers? 7 6 5 4 3 2 1 F R ☐ 153

Are there any other foods that you eat regularly, but which are not recorded in the questionnaire? Yes / No ☐ 154

If Yes, please state each food and how often you usually eat it

Food	Frequency
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

☐☐☐ 155-157

☐☐☐ 158-160

Diet Code ☐ 161

Appendix B1 – Method of analysis for insulin

Reagents

1 x 96 well coated plate

Calibrators: 3, 10, 30, 100 and 200mU.l^{-1}

Calibrator 0

Enzyme conjugate

Enzyme conjugate buffer

Wash buffer

Substrate TMB

Stop solution

Reagent preparation

Wash buffer: the concentrated wash buffer was diluted with distilled water by the addition of 35 ml of buffer to 700 ml of distilled water.

Enzyme conjugate: the enzyme conjugate was diluted with enzyme conjugate buffer by the addition of 1.0 ml of the enzyme conjugate to 10 ml of buffer.

Procedure

1. 25 µl aliquots of calibrators and plasma samples were added to the appropriate wells.
2. 100 µl of enzyme conjugate was added to each well.
3. The plate was incubated for 1 hour at room temperature on a plate shaker.
4. The plate was washed using an automated plate washer, with 350 µl of wash buffer added to and then aspirated from each well a total of six times.
5. 200 µl TMB was added to each well before being incunated in the dark at room temperature for 15 minutes
6. 50 µl stop solution was added to each well and the plate lightly shaken for 5 seconds
7. The plate was read at an optical density of 450 nm.

Appendix B2 - Method of analysis for glucose

Principle of method

$$\text{Glucose + ATP} \xrightarrow{\textbf{HK}} \text{glucose-6-phosphate + ADP}$$

$$\text{Glucose-6-phosphatase + } NADP^{+} \xrightarrow{\textbf{G-6-PDH}} \text{gluconate-6-phosphatase + NADPH + } H^{+}$$

Reagents

R1: Buffer / coenzymes

R2: Dilutent

2. Reagent

Reagent preparation

Reagent was added to R2

Procedure

Plasma glucose concentrations were determined by the author and colleagues at the faculty of biomedical and life sciences at University of Glasgow. The procedure for this analysis is property of the faculty.

Appendix B3 - Method of analysis for NEFA

Principle of method

$$\text{RCOOH (NEFA)} + \text{ATP} + \text{CoA-SH} \xrightarrow{\textbf{ACS}} \text{Acyl-Co-A} + \text{AMP} + \text{PPi}$$

$$\text{Acyl-CoA} + O_2 \xrightarrow{\textbf{ACOD}} \text{2,3-trans-Enoyl-CoA} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminophenazone} + \text{MEHA} \xrightarrow{\textbf{POD}} \text{Quinonelmine-colour} + 4\ H_2O$$

Reagents

R1: Solvent A

R1a: Colour reagent A

R2: Solvent B

R2a: Colour reagent B

CAL: NEFA C standard

Reagent preparation

Colour reagent solution A: The contents of R1a were dissolved with 10 ml of R1 and mixed well

Colour reagent solution B: The contents of R2a were dissolved with 20 ml of R2 and mixed well

Procedure

Plasma NEFA concentrations were determined by the author and colleagues at the faculty of biomedical and life sciences at University of Glasgow. The procedure for this analysis is property of the faculty.

Appendix B4 - Method of analysis for triglyceride

Principle of method

A lipoprotein lipase derived from micro-organism is used to rapidly and completely hydrolyse TG to glycerol. Glycerol is then oxidised to dihydroxyacetone phosphate (DHAP) and hydrogen peroxide. Subsequently, hydrogen peroxide reacts with 4-aminophenazone and 4-chlorophenal under the catalytic action of peroxidise to form a red dyestuff.

$$TG + 3\,H_2O \xrightarrow{\textbf{LPL}} glycerol + 3\,RCOOH$$

$$Glycerol + ATP \xrightarrow[\textbf{Mg}^{2+}]{\textbf{GK}} glycerol\text{-}3\text{-}phosphate + ADP$$

$$Glycerol\text{-}3\text{-}phosphate + O2 \xrightarrow{\textbf{GPO}} DHAP + H_2O_2$$

$$H_2O_2 + 4\text{-}aminophenazone + 4\text{-}cholrophenol \xrightarrow{\textbf{Peroxidase}} 4\text{-}(p\text{-}benzoquinone\text{-}monoimino)\text{-}phenazone + 2\,H_2O + HCl$$

Reagents

R1 Buffer / 4-chlorophenol / enzymes

Calibrator S1: 0.9% NaCl

Calibrator S2

Quality control sera

Procedure

Plasma TG concentrations were determined by the author and colleagues at the faculty of biomedical and life sciences at University of Glasgow. The procedure for this analysis is property of the faculty.

Appendix B5 - Method of analysis for lactic acid

Principle of method

Lactic acid concentration was measured according to the method of Maughan[306], which depends on the use of fluorimetry to determine the concentration of the reduced co-enzyme NADH. The analysis depends on the interconversion of lactate linked to a change in the oxidation state of NAD^+, as demonstrated by the reactions:

$$\text{L-lactate} + NAD^+ \xleftrightarrow{\textbf{LDH}} \text{pyruvate} + NADH + H^+$$

$$\text{D-3-OH butyrate} + NAD^+ \xleftrightarrow{\textbf{HBDH}} \text{acetoacetate} + NADH + H^+$$

$$\text{L-alanine} + NAD^+ + H^2O \xleftrightarrow{\textbf{ADH}} \text{pyruvate} + NADH + NH_4^+$$

Reagents

1 x 96 well coated plate

Hydrazine (1.1 $moll^{-1}$, pH 9.0)

NAD (5 $mmoll^{-1}$)

LDH (2.75 Units/ ml)

Procedure

1. Capillary blood was collected, dispensed into perchloric acid, centrifuged, separated and stored as described in chapter 2.
2. All samples were defrosted at room temperature.
3. 20 µl of calibrators with know lactate concentrations (1,2,3 and 5 $mmoll^{-1}$) and a quality control sample (8 $mmoll^{-1}$) were each added to 200 µl of 0.4 $moll^{-1}$ perchloric acid.
4. 20 µl aliquots of calibrators and samples were added to the appropriate wells.
5. 200 µl of reagent was added to each well
6. The plate was read at an optical density of 325 nm, λex 420 by a microplate fluorometer (Fluoroskan Ascent, Thermo Fisher scientific, Waltham, Massachussets, USA).

Appendix B6 - Method of analysis for leptin

Principle of method

A fixed concentration of a labelled tracer is incubated with a constant dilution of antiserum. In this situation, the concentration of antigen binding sites is limited. The addition of unlabelled antigen introduces competition between the labelled tracer and unlabelled antigen for the limited number of abntibody binding sites. Therefore, as the concentration of unlabelled antigen increases, the amount of tracer attached to the antibody will decrease. This process is measured by separating the bound antibody from the free tracer and counting either if both of the fractions.

Reagents and procedure

1. Radioiodinated leptin was prepared using a solid phase lactoperoxidase procedure
2. Leptin was purified by Sephadex G-25 gel filtration followed by G-50 using 0.1 $mol.l^{-1}$ phosphate, 7.7 mmol sodium $azide.l^{-1}$, BSA and 0.5 $ml.l^{-1}$ Triton X-100 elution buffer
3. 0.1 ml of test plasma was incubated with the leptin standard, 0.1ml donkey serum, 0.1 ml assay buffer, sheep antileptin antiserum and 125I-labelled leptin at $4^{o}C$ for 16 hours.
4. Sepharose-donkey antisheep globulin was added after incubation and the samples were then re-incubated for one hour at room temperature.
5. The free and bound fractions were separated by centrifugation using 3 ml washes with 0.15 $mmol.l^{-1}$ sodium chloride containing Tween 20.
6. The bound fraction was counted for 60 seconds on a multichannel counter.