

**The role of PPAR γ in insulin resistance/ diabetes:
Insights gained from studies in humans with loss of function mutations**

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Outline of Dissertation

The consequences of loss of function mutations in the nuclear hormone receptor, peroxisome proliferator-activated receptor gamma (PPAR γ), in humans, were studied in order

1. to explore the biological role of PPAR γ in humans and thereby to enhance our understanding of the mechanism of action of thiazolidinediones (PPAR γ agonists) which are now widely used in the treatment of type 2 diabetes

and

2. to further the molecular and physiological understanding of the mechanisms responsible for insulin resistance and diabetes in humans; with particular emphasis on the part played by altered fatty acid metabolism.

The structure of the dissertation is as follows:

Chapter 1: General introduction and review of scientific literature

Chapter 2: Phenotypic studies in carriers of dominant negative PPAR γ mutations incorporating:

- clinical and biochemical details
- precise body composition measurements
- quantification of insulin resistance by hyperinsulinemic-euglycemic clamps
- assessment of adipose tissue morphology and function (both in terms of fatty acid/ triglyceride metabolism and adipocytokine production)

- microarray studies of changes in gene expression induced by the presence of a dominant negative PPAR γ mutation during *ex vivo* pre-adipocyte differentiation
- mononuclear cell studies demonstrating PPAR γ ligand resistance
- description of the response to PPAR γ agonist therapy in two subjects with dominant negative PPAR γ mutations

Chapter 3: Identification and characterisation of a family in which five severely insulin resistant subjects and no unaffected family members were doubly heterozygous for frameshift/premature stop mutations in PPAR γ and PPP1R3A, an unlinked gene encoding the muscle-specific regulatory subunit of protein phosphatase-1. The initial *in vitro* characteristics of both mutants are described, as is the basic phenotypic description of these subjects.

Chapter 4: Results of the studies are briefly summarised and potential studies arising from this work are considered.

Summary

The overall aim of these studies was to explore the physiological mechanisms responsible for insulin resistance, a key component in the pathogenesis of type 2 diabetes. In particular, the studies herein explore the role of the nuclear hormone receptor, PPAR γ , in modulating insulin sensitivity in humans. PPAR γ plays a critical role in adipogenesis and adipocyte biology, and has recently attracted considerable attention on account of it being the receptor for the thiazolidinediones (TZDs), a group of drugs now widely used as insulin sensitising agents in the treatment of type 2 diabetes. The primary approach used was to study humans with loss of function mutations in PPAR γ .

a) Studies in humans with dominant negative mutations in PPAR γ .

The recent identification of three human subjects with dominant negative mutations in PPAR γ highlighted the role of this receptor in insulin resistance and diabetes (Barroso et al., 1999). Further genetic studies in one of the kindreds led to the identification of two prepubertal children with a proline-467-leucine PPAR γ mutation. Fasting insulin levels were strikingly elevated in both children, highlighting the dramatic impact of impaired PPAR γ activity on insulin action. Detailed body composition measurements indicated that all three adult subjects had a stereotyped form of partial lipodystrophy with loss of limb and gluteo-femoral fat. In addition to the reduction in adipose tissue mass, adipose tissue function was impaired. In particular, *in vivo*

measurements of free fatty acid and triglyceride fluxes revealed a striking failure of adipocytes to trap and store free fatty acids appropriately in the postprandial period, a situation likely to induce inappropriate delivery of free fatty acids to skeletal muscle and the liver. In keeping with this notion, hepatic steatosis was present in all affected subjects. Somewhat surprisingly however, intramyocellular triglyceride levels were within the normal range. As well as modulating lipid fluxes, adipocytes secrete a number of proteins with putative endocrine activity. Adiponectin levels were very low in all the subjects - whether this contributed to the insulin resistance or merely reflects reduced adipose tissue mass awaits further studies on the biological roles of this adipokine.

Hyperinsulinemic-euglycemic clamps confirmed the presence of severe peripheral and hepatic insulin resistance. Whilst the lipodystrophy, impaired adipose tissue function and low adiponectin levels are likely to be the principal determinants of this insulin resistance, the possibility of direct effects of the dominant negative PPAR γ receptors in myotubes and/ or hepatocytes cannot be entirely excluded. Tissue specific knockouts will help to elucidate the role of the small amounts of PPAR γ expressed in these tissues. In addition to insulin resistance, all the subjects of this study had all the features of syndrome X, namely diabetes, hypertension, dyslipidemia and steatohepatitis.

Expression profiling in pre-adipocytes harbouring a dominant negative PPAR γ mutation led to the identification of 22 genes whose expression during

differentiation was substantially altered by the presence of the PPAR γ mutant. Of particular note was the identification of PDK4, a key regulator of macronutrient substrate selection, as a PPAR γ responsive gene in human adipocytes.

Ex vivo studies of PPAR γ target gene expression in monocytes from subjects harbouring a dominant negative PPAR γ mutation demonstrated 1) the presence of PPAR γ agonist resistance and 2) the ability of PPAR γ agonists to at least partially overcome this resistance. In the light of these findings and *in vitro* observations indicating the ability of PPAR γ agonists to overcome the dominant negative behaviour of the mutants, a clinical trial of rosiglitazone was undertaken in two adult subjects. Interestingly, whilst a subject harbouring the proline-467-leucine PPAR γ mutation responded dramatically, effectively normalising his insulin sensitivity, the second patient with a valine-290-methionine mutation failed to respond. This discrepant response may, at least in part, reflect molecular differences in the behaviour of the mutant receptors.

b) Digenic inheritance of severe insulin resistance/ type 2 diabetes in a human kindred

In a large UK kindred, where the proband presented at age 15 years with severe insulin resistance (acanthosis nigricans, hyperinsulinemia, polycystic ovarian syndrome), a novel heterozygous frameshift premature stop mutation was identified in the PPAR γ gene. The mutant receptor is truncated within the

second zinc-finger of the DNA binding domain, rendering it unable to bind to DNA with its heterodimeric partner RXR (retinoid X-receptor) and therefore transcriptionally silent. However, unlike the dominant negative PPAR γ mutants, the truncated mutant receptor did not inhibit wild type PPAR γ action in a dominant negative manner. Heterozygous PPAR γ knockout mice retain insulin sensitivity and two of the individuals in this kindred with the PPAR γ mutation did not appear to be insulin resistant, arguing against PPAR γ haploinsufficiency as the sole mediator of the observed phenotype.

Ongoing candidate gene studies led to the identification of a novel frameshift premature stop mutation in a skeletal muscle specific isoform of the glycogen targeting subunit of protein phosphatase-1 (PPP1R3A). This mutation results in a prematurely truncated protein with loss of the sarcoplasmic reticulum binding domain. When transiently expressed in Chinese hamster ovarian (CHO) cells the mutant protein failed to localise appropriately to endoplasmic reticulum. The consequences of this mislocalisation upon glycogen synthesis in skeletal muscle are the subject of ongoing *in vitro* and *in vivo* studies. Whereas individuals with either the PPAR γ (n=2) or the PPP1R3A (n=2) gene defects alone exhibited normal insulin sensitivity, double heterozygosity for both genetic abnormalities (n=5) cosegregated completely with severe insulin resistance.

This represents the first example of human digenic insulin resistance and in contrast to previously described human digenic conditions which have involved direct physical interactions between the two mutant gene products it

would appear that defects separately affecting carbohydrate and lipid metabolism can combine to result in an extreme phenotype of insulin resistance. This provides a model for the type of gene-gene interaction which may lead to common metabolic disorders such as type 2 diabetes. Intriguingly, the PPP1R3A variant was prevalent and found at a higher frequency in type 2 diabetics (20/1029) than in controls (8/1033); odds ratio 2.53, $p=0.03$. In a second independent kindred with severe insulin resistance, the PPP1R3A frameshift premature stop variant appeared to induce severe insulin resistance only when associated with obesity. This observation is consistent with the evidence derived from the initial kindred suggesting the need for a 'second hit' to induce severe insulin resistance in carriers of the PPP1R3A frameshift. A large multi-centre collaborative epidemiological study is in progress to determine whether or not the PPP1R3A mutation is significantly associated with type 2 diabetes in the general population.

Chapter 1

Introduction and review of scientific literature

In this chapter I will:

- briefly outline my reasons for studying diabetes and in particular its pathogenesis
- summarize what is currently known about the pathogenesis of type 2 diabetes and insulin action, as well as introducing the concept of “insulin resistance”
- introduce the “lipocentric view” of type 2 diabetes/ insulin resistance as a prelude to an introduction to PPAR γ , a nuclear hormone receptor whose insulin sensitising and hypoglycemic properties appear to be largely due to primary effects on lipid metabolism
- explain why we believe that studying rare mono-/ oligogenic forms of severe insulin resistance can provide unique insights into metabolism

Why study diabetes?

Non-communicable diseases have become the major health challenge of the 21st century. The worldwide prevalence of type 2 diabetes mellitus is expected to rise from 151 million in the year 2000 to 221 million by 2010, a 46% increase (Zimmet et al., 2001), (Amos et al., 1997). This global increase is predicted to be most dramatic in the Far East (54%) and Africa (50%), and parallels the epidemic of obesity and insulin resistance (Zimmet et al., 2001). Type 2 diabetes accounts for over 90% of diabetes cases and is even beginning to overtake type 1 diabetes as the predominant cause of childhood diabetes. The principal reason for this pandemic is the staggering increase in obesity, the single most important predisposing factor in the pathogenesis of diabetes. The rising prevalence of childhood obesity is therefore particularly ominous (Sinha et al., 2002). Diabetes is not only a common condition, but also causes an enormous amount of human suffering in the form of chronic complications such as blindness, renal failure, amputations, and a dramatically increased risk of coronary artery and cerebrovascular disease. In fact, the risk of myocardial infarction in patients with type 2 diabetes and no history of cardiac disease is roughly equal to the risk in non-diabetic patients with known cardiac disease (Haffner et al., 1998). This risk is exacerbated by the tendency for type 2 diabetes to develop in individuals with insulin resistance and features of the "metabolic syndrome". 'Metabolic syndrome', also known as "syndrome X", encompasses insulin resistance, obesity (particularly visceral obesity), hypertension and diabetic dyslipidemia (raised

triglycerides and low high-density lipoprotein (HDL)-cholesterol), and is associated with a markedly increased risk of macrovascular disease.

While several therapeutic agents exist for type 2 diabetes, the majority were developed without defined molecular targets (e.g. metformin). A better understanding of the pathogenesis of diabetes is beginning to reveal an unprecedented range of novel molecular drug targets (Moller, 2001). For example, recent attempts to elucidate the genes regulated by PPAR γ have revealed a number of potential drug targets, such as 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) (Berger et al., 2001), Cbl-associated protein (CAP) (Berger et al., 2001) and adiponectin (Yang et al., 2002b). Further efforts of this type may well be fruitful.

Pathophysiology of type 2 diabetes

Diabetes is a state in which carbohydrate and lipid metabolism are improperly regulated by insulin. This results in an increase in fasting and postprandial serum glucose that leads to complications if left untreated. Diabetes has been divided into two major categories, types 1 and 2. Whereas type 1 diabetes is a consequence of the destruction of pancreatic β -cells (autoimmune) and hence an absolute deficiency of insulin, type 2 diabetes might be considered to be a state of "relative insulin deficiency" in the face of insulin resistance. Despite enormous scientific endeavour the complexity of type 2 diabetes continues to baffle investigators. Nevertheless several points of agreement do exist:

1) Genetic factors determine the risk of developing type 2 diabetes – this notion originated with the recognition by ancient physicians that type 2 diabetes was familial in nature (reviewed in “The pathophysiology and inheritance of familial type 2 diabetes”; Dissertation submitted for the degree of Doctor of Medicine by Stephen O’Rahilly; The National University of Ireland; 1987). Work done by Pincus and White in the 1930’s added some objectivity to these impressions (Pincus, 1933), (Pincus, 1934a), (Pincus, 1934b) but it was only after the recognition that type 1 (juvenile –onset and insulin requiring) and type 2 (adult-onset and not necessarily insulin requiring) diabetes were the result of distinct pathophysiological processes that further familial and twin studies provided convincing evidence of the genetic basis for type 2 diabetes. Studies of isolated, genetically homogeneous populations such as South Pacific Islanders (Sicree et al., 1987) and the Pima Indians of North America (Knowler et al., 1983) later provided additional corroborative evidence for the genetic basis of diabetes.

2) Insulin resistance, which can be defined as a state of reduced responsiveness to normal circulating levels of insulin, plays a major role in the development of type 2 diabetes. This conclusion is based upon the following observations: i) cross sectional studies demonstrating the consistent presence of insulin resistance in patients with type 2 diabetes (Lillioja et al., 1988), (Haffner et al., 1990), (Reaven et al., 1976) ii) the presence of insulin resistance in the non-diabetic offspring of patients with type 2 diabetes (Warram et al., 1990) and iii) prospective studies demonstrating the

usefulness of insulin resistance as a predictive marker of the future development of type 2 diabetes (Lillioja et al., 1988), (Warram et al., 1990).

3) Early in the disease, β -cells secrete sufficient insulin to compensate for insulin resistance and maintain euglycemia. Ultimately however, relative or absolute insulin deficiency supervenes precipitating hyperglycemia and overt diabetes. β -cell dysfunction is therefore a *sine qua non* of the diabetic state but need not be the primary abnormality (Boden, 2001).

4) Type 2 diabetes is a heterogeneous cluster of conditions rather than a uniform entity. The spectrum includes individuals with maturity-onset diabetes of the young or MODY, manifesting predominantly β -cell dysfunction due to genetic defects in one of several β -cell proteins (Fajans et al., 2001), and people with Donohue's syndrome due to insulin receptor mutations, whose phenotype is dominated by insulin resistance (Krook and O'Rahilly, 1996).

How does insulin act?

(The ensuing discussion of insulin's actions is primarily focused on the metabolic actions of insulin. Insulin does, however, also have a number of important anti-apoptotic properties.)

Under normal circumstances blood glucose is maintained within a very narrow range (4 – 7 mM). A typical Western diet provides approximately 300 g of carbohydrate and 100 g of fat per day. An average meal will therefore contain about 100 g of carbohydrate and 30 - 40 g of fat (Frayn, 2002). Following digestion in the gastrointestinal tract, this influx of energy rich nutrients results

in the prompt release of insulin, the principal coordinator of glucose disposal. As well as promoting glucose uptake in skeletal muscle and fat, insulin suppresses endogenous glucose production by the liver. The majority of insulin stimulated glucose uptake occurs in skeletal muscle (>75%) with most of the rest being taken up by adipocytes and the liver. Insulin promotes anabolic substrate storage in fat, liver and muscle by stimulating lipogenesis, glycogen synthesis and protein synthesis; and inhibiting lipolysis, glycogenolysis and protein breakdown. In addition to its metabolic actions insulin also promotes anabolic activity in the form of cell growth and differentiation.

Once released into the circulation insulin binds to its cognate receptor, a tetrameric protein composed of two extracellular alpha and two membrane-spanning beta subunits (see (Virkamaki et al., 1999) and (Saltiel, 2001) for a comprehensive review of insulin signalling pathways). The insulin receptor is a tyrosine kinase and undergoes autophosphorylation of the intracellular portion of the β -subunits in response to ligand binding. The activated insulin receptor then phosphorylates tyrosine residues on a number of proteins, collectively referred to as insulin receptor substrate proteins (or IRSs) (White, 2002). The principal IRSs involved in insulin's metabolic actions function as docking proteins for several additional downstream proteins and initiate a signalling cascade which ultimately has a range of metabolic and mitogenic effects. IRS-1 and 2 are the best characterised insulin receptor substrates. Knockout models suggest that IRS-1 is primarily involved in mediating insulin's metabolic effects in muscle and fat, as well as insulin's mitogenic

effects. In contrast, IRS-2 appears to be more important in insulin signalling within β -cells and the liver, where insulin's major effect is to inhibit gluconeogenesis. Tyrosine phosphorylated IRS-1 and IRS-2 in turn provide docking sites for SH2 domain containing proteins, including phosphatidylinositol 3 kinase (PI3-kinase), a key player in mediating insulin-stimulated glucose transport. PI3-kinase is a lipid kinase, consisting of a regulatory subunit which binds to IRSs and a catalytic subunit responsible for phosphorylation of phosphatidylinositols found in cellular membranes (Virkamaki et al., 1999). The subsequent increase in phosphatidylinositol 3,4,5 trisphosphate (PIP3) leads to activation of a protein kinase cascade. Pyruvate dehydrogenase kinase 1 (PDK1) phosphorylates and activates two classes of serine/ threonine kinases, Akt (also known as protein kinase B (PKB)) and the atypical protein kinase C (PKC) isoforms ζ and λ (Saltiel, 2001).

In addition to the PI3-kinase pathway, recent data supports a role for a PI3-kinase independent pathway in insulin stimulated glucose uptake. This pathway is thought to involve tyrosine phosphorylation of the Cbl proto-oncogene (Ribon and Saltiel, 1997). Cbl phosphorylation is dependent upon the presence of an adaptor protein CAP (Cbl-associated protein), and induces prompt translocation of the Cbl/ CAP complex to lipid rafts in the plasma membrane, where additional signalling proteins are recruited and activate the G protein TC10 (Saltiel and Kahn, 2001). This pathway ("molecular switch") may ultimately provide a second signal (the other signal being that derived from the PI3-kinase cascade) to GLUT4 (glucose

transporter 4) containing vesicles. GLUT4 translocation to the cell surface is the final step in insulin stimulated glucose transport in skeletal muscle and fat. In the basal state GLUT4 continuously cycles between the cell membrane and intracellular compartments. Although the precise mechanism remains unknown, insulin receptor activation markedly increases GLUT4 vesicle exocytosis and marginally reduces GLUT4 internalization (Saltiel, 2001).

Where does insulin act?

Whilst insulin receptors are expressed in almost every tissue in the body the principal insulin responsive tissues include skeletal muscle, liver, fat and the brain. Insulin receptor knockout mice (IRKO) develop diabetes and ketoacidosis soon after suckling begins (Joshi et al., 1996). White and brown adipocytes are present in these mice but have reduced fat content. In contrast, hepatocytes accumulate fat leading to hepatic steatosis. IRKO pups are also growth retarded and have skeletal muscle atrophy. They die within one week of birth. Tissue-specific insulin receptor knockout models have provided both substantial insight into - and several surprises in our understanding of the contribution made by each of the key insulin sensitive tissues to the actions of insulin. For example, muscle-specific insulin receptor knockout mice (MIRKOs) display normal "whole-body insulin sensitivity" and a tendency to increase total fat mass as glucose is shunted from skeletal muscle to adipose tissue (Bruning et al., 1998), (Kim et al., 2000). Fat-specific insulin receptor knockout mice (FIRKO) are protected against the development of obesity and obesity-related glucose intolerance (Bluher et al.,

2002). The latter was particularly surprising as fat-specific GLUT4 knockout mice are insulin resistant and a significant percentage go on to develop diabetes (Abel et al., 2001). This apparent discrepancy supports the notion that insulin has important actions in adipose tissue beyond those of glucose transport. For example, despite having reduced body fat FIRKO mice have elevated plasma leptin levels (Bluher et al., 2002). Knocking out the insulin receptor in the liver of mice (LIRKO) leads to severe insulin resistance within 2 months of birth, but does not produce overt diabetes (Michael et al., 2000). The insulin receptor is also widely expressed in neural tissue where it is believed to have an appetite suppressant effect. Indeed neuron-specific knockout mice manifest hyperphagia, weight gain and insulin resistance (NIRKO) (Bruning et al., 2000). Somewhat counter-intuitively mice with selective loss of the insulin receptor in β -cells do develop diabetes, providing novel insights into the role of paracrine insulin signalling within β -cells (Kulkarni et al., 1999).

Despite the fact that MIRKO mice do not develop diabetes, elegant NMR (nuclear magnetic resonance) studies in humans suggest firstly, that skeletal muscle accounts for the vast majority of insulin stimulated glucose uptake and secondly that over 80% of glucose is subsequently stored as glycogen in the resting state (Shulman et al., 1990). The same workers went on to address the question of the rate-limiting step in insulin mediated glucose disposal. Using ^{31}P NMR they were able to measure intracellular glucose 6-phosphate (G6P) during hyperinsulinemic-euglycemic clamps (Rothman et al., 1992). G6P is the first intracellular intermediate formed in the glucose metabolic

pathway. Whereas normal individuals demonstrated a 0.1 mM rise in intracellular G6P, type 2 diabetics had a significantly blunted response, indicating that the rate-limiting step was either at the level of glucose transport and/ or glucose 6-phosphorylation (Rothman et al., 1992). Subsequent measurements of intracellular ^{13}C labelled glucose indicated that glucose transport itself was the rate-limiting step (Cline et al., 1999).

Is insulin primarily a carbohydrate or lipid regulatory hormone?

In addition to its “gluco-regulatory” activity insulin regulates lipid metabolism. In fact the last decade has seen a shift from the traditional “glucocentric” view of diabetes to an increasingly acknowledged “lipocentric” viewpoint, first proposed by Dennis McGarry in 1992 (McGarry, 1992). This hypothesis holds that abnormal fatty acid metabolism may result in inappropriate accumulation of lipids in muscle, liver and β -cells (McGarry, 2002). It is further proposed that ectopic fat accumulation is involved in the development of insulin resistance in muscle and liver as well as impairing β -cell function (so called “lipotoxicity” (Unger and Orci, 2000)). Proponents of this theory cite the following evidence:

- Lipid accumulation within myocytes and hepatocytes is strongly associated with insulin resistance. This association is true of both diabetics and individuals with impaired glucose tolerance (Boden, 2001). In fact nuclear magnetic resonance (NMR) measurements of intra-myocellular lipids (IMCL) correlate more closely with insulin

resistance than any other commonly measured indices including BMI, waist-hip ratios, or total body fat (McGarry, 2002). Non- alcoholic steatohepatitis is also increasingly recognised as a component of the insulin resistance or metabolic syndrome (Marchesini et al., 2001), (Pagano et al., 2002).

- Infusion of free fatty acids (FFAs) during hyperinsulinemic-euglycemic clamps in both humans and rodents reduces glucose disposal. Furthermore, the fall in insulin sensitivity during such clamp procedures only occurs several hours after elevations in FFA concentrations, in keeping with the idea that FFA accumulation in skeletal muscle and liver is responsible for this phenomenon (Boden, 2001). Again in this paradigm, the presence of insulin resistance coincides with increasing intramuscular triglyceride levels as measured by NMR spectroscopy (Boden, 2001).
- Lipid accumulation in myocytes is apparent in non-diabetic relatives of patients with type 2 diabetes, a cohort at high risk of developing diabetes (Jacob et al., 1999).
- Dietary restriction in obese insulin resistant rodents leads to a significant reduction in IMCL levels and improvement in insulin sensitivity (Man et al., 2000).

In addition to muscle and liver, lipid accumulation in pancreatic islets appears to cause β -cell dysfunction. Unger and co-workers have documented lipid accumulation in islets and β -cell dysfunction in Zucker diabetic fatty (fa/fa) rats, a rodent strain with non-functional leptin receptors (Kakuma et al., 2000). This observation prompted them to propose an anti-steatotic insulin sensitising role for leptin (Unger and Orci, 2000).

Although triglyceride accumulation in non-adipose tissue is what is usually measured, it is widely believed that rather than inducing insulin resistance themselves, triglycerides are a surrogate marker for another fatty acid derived entity. Candidates for the molecules directly responsible for inducing insulin resistance include long chain fatty acyl-coenzyme-A (acyl-CoA) molecules, diacylglycerol (DAG) and ceramides. Yu et al (Yu et al., 2002) attempted to resolve this question by infusing a lipid emulsion into healthy rats and then measuring intracellular levels of long chain acyl-CoA, ceramides, DAG and triglycerides. During this infusion there was no change in intracellular ceramide or triglyceride levels, whereas intracellular long chain acyl-CoA increased 6-fold and DAG increased transiently after three hours. These changes were associated with protein kinase-C theta (PKC- θ) activation, serine phosphorylation of IRS-1, reduced insulin stimulated tyrosine phosphorylation of IRS-1 and a fall in IRS-1 associated PI3-kinase activity leading in turn to reduced glucose uptake.

Yuan et al (Yuan et al., 2001) hypothesized that the serine kinase activity of IKK- β might be involved in mediating fatty acid induced insulin resistance.

They then demonstrated that both pharmacological inhibition of IKK- β activity by high dose salicylates and heterozygous deletion of the IKK- β gene ameliorated insulin resistance in obese rodents during high fat feeding or lipid infusion. This salicylate effect has also been observed in humans, although high dose salicylates remain a research tool only due to significant side-effects (Hundal et al., 2002). In short, it would appear that inappropriate free fatty acid delivery to muscle leads to accumulation of esterified long chain fatty acids within myocytes. This accumulation triggers PKC and IKK- β mediated serine phosphorylation of IRS-1, inhibiting insulin induced tyrosine phosphorylation of IRS-1 and IRS-1 associated PI3-kinase activity, ultimately leading to a fall in insulin stimulated GLUT-4 translocation to the cell membrane and reduced glucose uptake.

In summary, the lipocentric view of diabetes suggests that an abnormal accumulation of fat in muscle and other tissues plays an important role in the development of insulin resistance and possibly also in the demise of the β -cell. Somewhat counter-intuitively, studies of lipodystrophic rodents and humans have provided several important pieces of information in the development of this hypothesis, the basic idea being that both lipodystrophy and obesity represent states of inadequate storage capacity for surplus energy (Friedman, 2002). In obesity the adipose depot is "overloaded" due to excess energy intake and reduced energy utilization, whereas in lipodystrophy a depleted adipose tissue depot cannot cope with normal energy intake (the problem is exacerbated by the tendency of people with lipodystrophy to "over-eat" as a consequence of low plasma leptin levels).

Lipodystrophic rodents and humans accumulate lipids in liver and muscle. One particularly compelling piece of evidence in support of the lipotoxicity theory of diabetes was recently provided by Reitman and co-workers, who successfully transplanted adipose tissue into lipodystrophic mice (Gavrilova et al., 2000). Despite only partially restoring adipose tissue mass they dramatically improved insulin sensitivity in the recipient mice as well as reducing intramuscular and intrahepatic triglycerides. Although adipose tissue transplantation has been considered in humans, to date this option remains theoretical. As an alternative to surgical transplantation fat mass can be increased pharmacologically, by PPAR γ agonists, in lipodystrophic humans (Arioglu et al., 2000) and rodents (Olefsky, 2000). Interestingly the increase in fat mass induced by PPAR γ agonist therapy is associated with improved insulin action and a redistribution of lipid stores from liver and skeletal muscle to adipose tissue depots (Oakes et al., 2001), (Mayerson et al., 2002).

Introduction to PPAR γ , a nuclear hormone receptor

Whereas membrane-bound receptors are generally responsible for propagating signals initiated by hydrophilic protein molecules, the nuclear hormone receptor superfamily is responsible for signalling by lipophilic molecules, including steroids and derivatives thereof, and fatty acids and their derivatives. Changes in concentration of lipid moieties are "sensed" by these receptors which respond by modifying gene transcription in the cell. An obvious example of this type of homeostatic signalling is the role of steroid response element binding proteins (SREBPs) in the regulation of cellular cholesterol and fatty acid levels (see (Horton et al., 2002) for a comprehensive review). Whilst SREBP1a and c are particularly important in maintaining fatty acid levels in the cell (Horton et al., 2002), the PPAR subfamily of nuclear receptors appear to be involved in modulating cellular fatty acid signalling.

PPAR γ is one of three PPARs (Escher et al., 2001): 1) PPAR α is principally expressed in liver and skeletal muscle in humans where it is thought to orchestrate fatty acid oxidation. Fibrates are PPAR α agonists and are widely used to treat dyslipidemia. 2) PPAR δ (also referred to as PPAR β in some texts) is ubiquitously expressed with putative roles in lipid metabolism, carcinogenesis and placentation. The lack of a PPAR δ -specific agonist has hampered understanding of the physiological role of this protein to date, although recent work suggests that this situation is about to change (Oliver et

al., 2001). 3) Finally, PPAR γ is most heavily expressed in mature adipocytes. Three isoforms exist; the γ 1 isoform is identical to the γ 3 isoform and is ubiquitously expressed, albeit at very low levels in tissues other than fat. The γ 2 isoform has an additional 30 amino acids at the N-terminal and is almost exclusively expressed in adipocytes. Isoform specific inhibition of PPAR γ function in cell culture suggests that the γ 2 isoform is essential for adipogenesis whereas the γ 1 isoform is not an absolute requirement (Ren et al., 2002). The creation of isoform specific knockout rodents is eagerly anticipated and should improve our understanding of the roles of the different isoforms.

PPAR γ has recently become the focus of massive biomedical interest largely for two reasons (recent reviews on PPAR γ include (Spiegelman, 1998), (Willson et al., 2000), (Rosen and Spiegelman, 2001)). Firstly, it plays a central role in adipogenesis as reflected by its high levels of expression in fat tissue and by its capacity to drive differentiation along the adipocyte lineage when over-expressed in uncommitted fibroblasts. Secondly, it appears to be the molecular target of a new class of drugs (thiazolidinediones) which improve insulin sensitivity and glucose tolerance in type 2 diabetes.

Determinants of PPAR activity

1) Ligand binding (Willson et al., 2000): Although it is widely believed that ligand binding is required for receptor activation, the nature of PPAR γ 's endogenous ligand/s remains uncertain. Thiazolidinediones and a number of

non-TZD pharmacological agonists certainly bind to and activate PPAR γ . Similarly a variety of fatty acids and derivatives thereof bind to PPAR γ with relatively low affinity but the concentrations of these molecules in the nuclei of target cells are likely to be too low for them to be *bone fide* ligands. Some have suggested that PPAR γ may be a promiscuous receptor for several fatty acids, acting as a non-specific fatty acid sensor. Certain eicosanoids bind PPAR γ with higher affinity. In particular, 15-deoxy-12,14-prostaglandin J2 (15dPGJ2) can bind to PPAR γ with a K_d in the low micromolar range. However, there is ongoing uncertainty as to whether 15dPGJ2 actually exists *in vivo*.

2) Co-activators/ co-repressors (McKenna and O'Malley, 2002): Co-regulatory molecules are cellular factors recruited by transcription factors that complement their function as mediators of endocrine signals. They may either enhance (co-activators) or inhibit (co-repressors) transcriptional responses. Co-activator recruitment is dependent upon prior ligand binding, a process thought to induce a shift in the position of several critical regions in the ligand-binding domain of the nuclear receptor. The co-activator molecules, of which there are a large number, then facilitate access of the basal transcription machinery to the promoter to initiate transcription.

Co-repressors are thought to bind to nuclear receptors in the absence of ligand. One of the well-characterized results of co-repressor binding is the recruitment of histone deacetylases to the promoter regions of target genes. These molecules deacetylate histones thereby facilitating a histone

maintained “off-state” of promoter regions of target genes. One of the actions of co-activators is to reverse this process by histone acetylation. An important potential consequence of co-repressor recruitment is that of dominant negative activity of mutant receptors with an enhanced tendency to recruit co-repressors. This model has been thoroughly explored in thyroid hormone resistance syndromes (Collingwood et al., 1998). In contrast, wild type PPARs exhibit negligible co-repressor recruitment in the basal state.

3) As alluded to above, each PPAR isoform has a specific tissue distribution, which is likely to modify both the nature and concentrations of endogenous ligands as well as the co-activator/ co-repressor milieu to which the receptor is exposed.

What is the physiological role of PPAR_γ?

Adipogenesis and adipocyte biology

Previously considered to function largely as a passive energy storage depot, adipose tissue is now known to be a key player in the regulation of energy balance (Spiegelman and Flier, 2001). It is capable of cell recruitment (adipogenesis) and hypertrophy in “times of excess”, and secretes a number of proteins with paracrine and endocrine effects. Leptin is one such adipocyte-derived hormone. Plasma levels are increased in obesity and provide feedback signals to central appetite regulatory centres in the hypothalamus. Leptin receptors are also present in peripheral tissues and

although less well established than its role in appetite regulation, leptin may have an anti-steatotic role in peripheral tissues (Unger, 2002). Several cytokine-like molecules such as tumour necrosis factor alpha ($\text{TNF}\alpha$), interleukin-6 (IL-6), resistin and adiponectin (also known as Acrp30) are also secreted by adipose tissue, and may be involved in metabolic cross-talk between fat and other tissues (Steppan and Lazar, 2002).

PPAR γ has emerged as the master-regulator of adipogenesis, the process of transformation of fibroblast like pre-adipocytes into triglyceride laden mature adipocytes. Evidence for this role includes the following:

- Ectopic over-expression of PPAR γ in fibroblasts, myoblasts and embryonic stem cells results in adipocyte transdifferentiation (Spiegelman, 1998)
- PPAR γ agonists potentiate adipogenesis *in vitro* and increase fat mass *in vivo* (Spiegelman, 1998)
- Stem cells lacking PPAR γ cannot differentiate into adipocytes (Rosen et al., 1999)
- PPAR γ antagonists (Wright et al., 2000) and a dominant negative construct inhibit adipogenesis (Gurnell et al., 2000)
- Although mice lacking PPAR γ die *in utero*, a single live born pup (the result of tetraploid rescue) lacked all adipose tissue (Barak et al., 1999)

The CCAAT/ enhancer binding proteins, C/EBP α , β and δ , and sterol regulatory element binding proteins, SREBP-1a and 1c, are also important in adipocyte differentiation (reviewed in (Rosen et al., 2000)). A transcriptional

cascade is thought to exist in which C/EBP β and δ simultaneously induce the expression of both C/EBP α and PPAR γ . They in turn appear to induce one another's expression as well as increasing SREBP-1 levels. This constellation of transcription factors goes on to promote the fully differentiated adipocyte phenotype. C/EBP α knockout fibroblasts express low levels of PPAR γ and do not differentiate into mature adipocytes. Over-expressing PPAR γ in these cells does lead to adipocyte differentiation, raising the possibility of redundancy in the roles of PPAR γ and C/EBP α . However, recent studies demonstrating that PPAR γ deficient cells cannot differentiate even if C/EBP α is over-expressed, indicate that the role of C/EBP α is ancillary to that of PPAR γ (Rosen et al., 2002).

In addition to the regulation of adipogenesis, PPAR γ is thought to substantially modify the metabolic activities of mature adipocytes. In particular, it appears to modulate lipid metabolism and adipocytokine production. This aspect of PPAR γ biology is alluded to in the next section of this chapter and is considered in detail in chapter 2.

Insulin resistance

First identified as anti-diabetic agents in pharmacological screening of fibrate analogues, thiazolidinediones were later shown to act via PPAR γ . Evidence to support a role for PPAR γ in thiazolidinedione action includes (Spiegelman, 1998):

- TZDs bind and activate PPAR γ in the same concentration range that has antidiabetic activity
- The rank order of their receptor-binding affinities mirrors their *in vivo* anti-diabetic activity
- Non-TZD PPAR γ agonists have anti-diabetic effects
- RXR ligands have anti-diabetic activity
- No alternative receptors for TZDs have been identified.

Whilst the evidence from numerous animal and human studies clearly indicates that PPAR γ agonists improve insulin sensitivity the mechanism of this action awaits elucidation (Olefsky, 2000). They certainly promote weight gain, tending to increase subcutaneous adipose depots via an increase in the number of small, insulin sensitive adipocytes. They also appear to reduce triglyceride accumulation in both hepatocytes and myotubes, potentially ameliorating fatty acid induced insulin resistance (Mayerson et al., 2002). Furthermore they may favourably influence adipokine production by adipocytes (Combs et al., 2002). Saltiel and others have also identified CAP (Saltiel and Kahn, 2001) which appears to be involved in a non-PI3-kinase dependant glucose uptake pathway as a possible PPAR γ target. Another angle to the debate involves whether all the beneficial effects associated with TZDs involve direct effects on fatty acid metabolism in adipose tissue with secondary effects on skeletal muscle and liver or whether PPAR γ agonists have direct effects in skeletal muscle. A study in one lipodystrophic mouse model indicated that rosiglitazone improved insulin sensitivity even in the absence of body fat (Burant et al., 1997), but a subsequent rosiglitazone trial

in a more severely lipodystrophic mouse strain suggested the opposite i.e. there was no improvement in insulin sensitivity (Chao et al., 2000). Tissue specific PPAR γ knockout models should help to answer this question.

Cardiovascular

Outside adipocytes, PPAR γ is most highly expressed in monocytes and macrophages, where it appears to modulate lipid transport. Original observations of an increase in the scavenger receptor CD36 mRNA levels in thiazolidinedione treated macrophages raised concern about enhanced foam cell formation and potential worsening of atherosclerosis (Lazar, 2001). However, subsequent work suggests that PPAR γ agonists promote both lipid uptake and efflux to HDL via the ATP-binding cassette receptor –A1 (ABCA1), in macrophages (Lazar, 2001). *In vivo* use of PPAR γ agonists in rodent models predisposed to atherosclerotic disease also suggests that PPAR γ agonists retard atheroma formation (Li et al., 2000). This remains an area of intensive study as diabetics, the population taking PPAR γ agonists, are particularly prone to macrovascular disease. In addition to its effects on lipid trafficking in macrophages, TZDs have been shown to improve all the other components of the metabolic syndrome (Martens et al., 2002): 1) Pioglitazone reduces plasma triglycerides and increases HDL (this may not be a class effect as rosiglitazone has not been associated with this response). 2) They reduce plasminogen activator inhibitor-1 (PAI-1) levels and hyperuricemia. 3) They lower blood pressure consistently in insulin resistant rodents. This observation has been more difficult to consistently reproduce in

humans. However, the fact that tyrosine based agonists also produce this effect in rodents is highly suggestive of a direct role for PPAR γ in this effect.

Whether these effects are secondary to the improvements in insulin sensitivity or direct effects of PPAR γ agonists awaits clarification.

PPAR γ and cancer

The ability of PPAR γ to inhibit fibroblast proliferation during adipocyte differentiation initially suggested that this receptor might be capable of inhibiting malignant cell proliferation. A small clinical trial of troglitazone in liposarcoma provided early impetus for this line of research (Tontonoz et al., 1997). Subsequent work involving PPAR γ agonists in several additional cancers appears to support this role. Furthermore, somatic loss of function mutations in PPAR γ have been identified in a number of colonic (Sarraf et al., 1999) and thyroid tumours (Kroll et al., 2000). Paradoxically however, PPAR γ agonist administration to min mice (a model of APC deficiency), lead to an increase in colon tumour number (Saez et al., 1998). This complication did not occur in wild type mice, nor has it been reported in humans treated with TZDs. Nevertheless, these observations are of some concern and warrant further investigation.

Why study rare human conditions?

In an attempt to explore the pathophysiology of insulin resistance in humans our group has focused on extreme forms of this condition, believing that this subgroup of the diabetic population is likely to contain a number of individuals in whom single gene defects are responsible for the observed phenotype. This approach is analogous to the generation of transgenic and knockout models in rodents; techniques which have facilitated substantial progress in the understanding of diabetes (Kadowaki, 2000), (Moller, 1994). Although monogenic disorders are, on the whole, rare in humans, they frequently yield significant breakthroughs in physiological understanding. Examples in the endocrine arena include (1) the recent identification of an estrogen deficient man with severe osteoporosis (Smith et al., 1994); a finding which highlighted the requirement for estrogen signalling in maintaining bone mass in males, and (2) the recent description of morbid obesity in leptin deficient humans (Montague et al., 1997). These subjects lack any detectable plasma leptin and manifest morbid obesity in early childhood. Treatment of these children with recombinant human leptin established the notion that leptin's effects in humans are mediated through the regulation of food intake and appetite (Farooqi et al., 1999).

We have undertaken candidate gene studies in a cohort of approximately 130 severely insulin resistant subjects (SIR cohort). Subjects have been recruited by Professor O'Rahilly, Cambridge, UK for a number of years. Recruitment was largely based upon patient referral by attending physicians.

As the heterogeneous manifestations of severe insulin resistance preclude the use of tight definitions, the following criteria were used as a guide to appropriate referral:

- 1) the presence of a fasting plasma insulin > 150 pmol/L, or in the case of diabetics, a requirement of > 200 units of insulin daily
- 2) the presence of acanthosis nigricans
- 3) although body mass index (BMI) is not specified, obese subjects are generally excluded in an attempt to enrich the cohort for monogenic forms of severe insulin resistance

What is a “severe insulin resistance syndrome”?

(This section has been published as part of a chapter on “Hereditary and acquired syndromes of severe insulin resistance” in “Textbook of Diabetes”, 3rd edition, Edited by Pickup and Williams, Blackwell Science, 2003)

Syndromes of severe insulin resistance constitute an extreme phenotype likely to be due to mono-/ oligogenic abnormalities. Severe insulin resistance usually manifests itself in one of two ways. Firstly, in the form of established type 2 diabetes requiring massive (arbitrary cut-off being > 200 U daily) insulin doses to maintain euglycemia, and secondly, as one or other of a group of conditions which tend to present with acanthosis nigricans, features of polycystic ovarian syndrome, growth retardation and /or acral enlargement.

Acanthosis nigricans is present in the majority of patients with severe insulin resistance, whatever the underlying cause. It is a dark velvety hyperpigmented skin lesion, sometimes accompanied by multiple skin tags, occurring most strikingly in flexural locations such as the axillae, the back of the neck and in the groin. It is also frequently seen over pressure points. In the most extreme cases it can be generalised but the palms and soles are usually spared. Histologically, it is a hyperkeratotic epidermal papillomatosis with some evidence for increased melanocyte number. Its absence in states of insulin deficiency and its presence in many different hyperinsulinemic conditions suggest that it is likely to result from the hyperinsulinemia. While it has been suggested that it might result from insulin's actions on IGF1 receptors in skin, acanthosis nigricans is not common in acromegaly, a condition where IGF1 itself is raised.

Features of hyperandrogenism are very common in post-pubertal girls with severe insulin resistance and are often the presenting feature of the condition. Amenorrhea/ oligomenorrhea, hirsutism, acne, and ultrasonographically demonstrable polycystic ovaries are all common clinical manifestations. Plasma testosterone levels can reach the order of 10 nmol/l in some cases, often leading to a fruitless search for an adrenal or ovarian tumour if the association with severe insulin resistance is not recognised. In some of the more severe congenital severe insulin resistance syndromes, clitoromegaly and precocious puberty can occur. The mechanisms responsible for the close association between severe insulin resistance and polycystic ovary syndrome remain poorly understood.

People presenting with these features may, somewhat paradoxically, present with hypoglycemic episodes (O'Rahilly et al., 1994). This phenomenon occurs because the normal accuracy of the physiological systems that control circulating insulin and glucose levels cannot be maintained at extreme levels of insulin resistance, resulting in a period of "overcompensation". The markedly delayed insulin clearance that occurs in some of the disorders may also contribute to reactive hypoglycemic episodes. Insulin clearance predominantly takes place in the liver and is impaired in severe insulin resistance. In due course the majority of subjects with severe insulin resistance syndromes do progress to overt diabetes.

In a patient who is suspected of having a severe insulin resistance syndrome but does not have insulin-treated diabetes, a useful and simple way of establishing the condition is to undertake an oral glucose tolerance test with measurements of plasma insulin. While no formal criteria for severe insulin resistance are widely accepted, a fasting insulin level $> 150\text{pmol/l}$ and/or a post glucose load insulin level of $> 1500\text{pmol/l}$ indicates a marked degree of insulin resistance. However it should be noted that such levels are not infrequently seen in patients with morbid obesity, a condition not usually classified as a severe insulin resistance syndrome. Additional differential diagnoses which should be born in mind include: 1) Apparent insulin resistance due to surreptitious manipulation of insulin therapy. 2) Insulin resistance due to the development of antibodies against injected insulin. This possibility warrants particular consideration in the setting of other auto-

immune disease. 3) Apparent insulin resistance due to the production of a genetically abnormal insulin molecule (insulinopathies).

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Chapter 2

Clinical and physiological consequences of dominant negative mutations in PPAR_γ

Introduction

Gene targeting in rodents has substantially improved our understanding of the physiological functions of several nuclear hormone receptors (Horton et al., 2002), (Chawla et al., 2001). Although PPAR_γ knockout mice die *in utero*, several groups managed to at least partially circumvent this problem (Lowell, 1999). One group managed to generate a single live born knockout (tetraploid rescue) which was a runt with a body mass 70% of that of its wild type littermates (Barak et al., 1999). It was further notable for the complete absence of any white adipose tissue and a fatty liver. Others noted that embryonic stem cells derived from PPAR_γ knockouts were unable to differentiate into adipocytes *in vitro* (Rosen et al., 2002). Both results highlighted the essential role played by PPAR_γ in mammalian adipogenesis. Another intriguing observation pertaining to the biological role of PPAR_γ, was made in PPAR_γ haplo-insufficient mice, which are viable. Kubota et al (Kubota et al., 1999) initially reported that PPAR_γ +/- mice demonstrated improved insulin sensitivity compared with their wild type counterparts when given a high fat diet. They attributed this observation to a reduced tendency to gain weight, smaller adipocytes and relatively higher leptin levels. This surprising result was later supported by Miles et al (Miles et al., 2000) who

noted that PPAR γ +/- mice were also protected against age-induced insulin resistance.

Prior to the studies described in this thesis two single N-terminal amino acid substitutions within human PPAR γ had been described. A proline-12-alanine mutation at the extreme N-terminal of PPAR γ 2 is relatively common among Caucasians (allele frequency of 15%) (Altshuler et al., 2000). *In vitro* characterization of this mutant suggests that it reduces transcriptional activity and the initial genetic studies reported an association with a reduction in BMI and lower risk of type 2 diabetes (Deeb et al., 1998). Although subsequent studies failed to consistently reproduce this observation, a recent meta-analysis did suggest that the Pro12Ala polymorphism is associated with a reduced incidence of type 2 diabetes (Altshuler et al., 2000). A much rarer, PPAR γ "gain-of-function" proline-115-arginine mutation was identified in four morbidly obese people (Ristow et al., 1998). Although, it was suggested that these subjects had surprisingly little impairment in insulin sensitivity, they were diabetic and formal measurements of insulin sensitivity were not undertaken.

More recently, three subjects with extreme hyperinsulinemia and hypertension were found to harbour mutations (proline-467-leucine (P467L) and valine-290-methionine (V290M)) within the ligand-binding domain of the nuclear hormone receptor PPAR γ (Barroso et al., 1999). *In vitro* characterization of these mutations suggested (Barroso et al., 1999):

- that the ability of the mutant proteins to bind pharmacological PPAR γ agonists was significantly impaired
- that the transcriptional activity of the mutants was significantly reduced
- that when co-expressed with equal amounts of the wild type receptor, the mutants inhibited wild type transcriptional activity i.e. they exhibited dominant negative behaviour.

Detailed clinical and pathophysiological studies in these subjects are described in this chapter. They have resulted in:

- the definition of a novel insulin resistance syndrome characterised by a stereotyped pattern of partial lipodystrophy and multiple features of the metabolic syndrome
- the demonstration of severe peripheral and hepatic insulin resistance in carriers of both mutations
- the demonstration of significantly altered triglyceride and non-esterified fatty acid fluxes across a preserved adipose tissue depot as well as reduced serum levels of adipocyte complement-related protein of 30 kilodaltons (Acrp30)
- the first direct evidence of PPAR γ agonist resistance in mononuclear cells derived from the patients
- the identification of two additional pre-pubertal carriers of the P467L mutation, both of whom manifest striking fasting hyperinsulinemia.

Finally, the response of two subjects to thiazolidinedione therapy is described.

Research subjects

All studies were approved by the local research ethics committees and fully informed consent was provided by each subject, and controls, for all procedures undertaken.

Subject 1 (P467L heterozygote) – Case History:

(see figure 1 for P467L family tree)

The patient presented with oligomenorrhoea and hirsutism at the age of 19 years. Subsequent difficulties with conception were successfully managed with ovulatory induction therapy, but her first pregnancy (age 25 years) was complicated by gestational diabetes and pre-eclampsia. Diabetes persisted after the pregnancy and although dietary modification and oral anti-hyperglycemic therapy were tried, she required insulin by the age of thirty. A second pregnancy (age 32 years) was again complicated by severe pre-eclampsia with fetal loss. Hypertension persisted after this pregnancy, necessitating the institution of anti-hypertensive therapy. Despite increasing insulin doses her diabetic control remained suboptimal and she developed microvascular complications. At the time of referral her therapy included 280U insulin daily and four anti-hypertensive agents.

On examination she was hirsute and had acanthosis nigricans in the axillae and at the base of the neck. Her body mass index was normal (24.4kg/m^2), but her waist: hip ratio was elevated at 1.1 ($N < .86$). This was primarily due to a reduction in gluteo-femoral fat whereas truncal fat was preserved (figure 2).

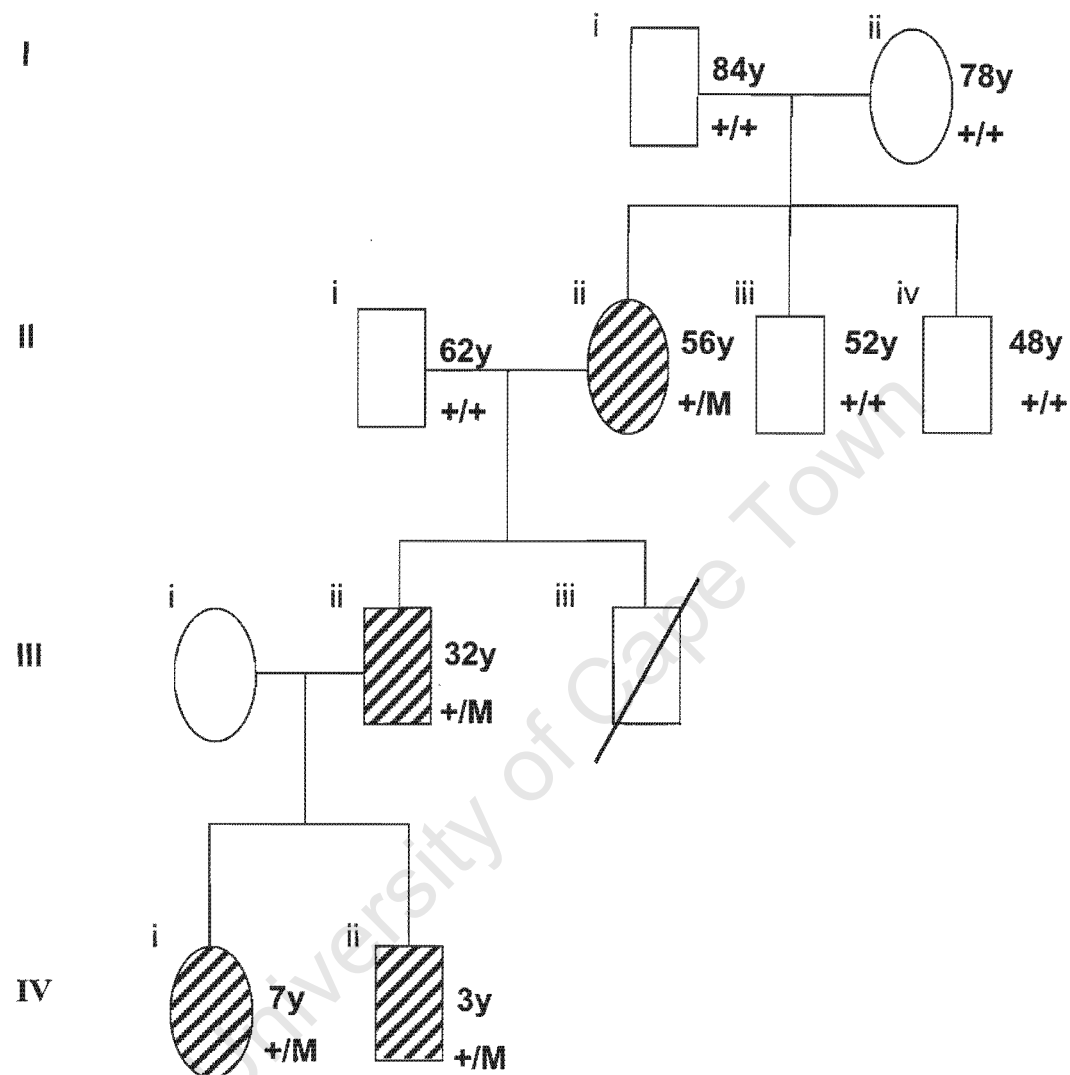


Figure 1

Family pedigree of subjects with heterozygous P467L PPAR γ mutation (hatched symbols), indicating concordance between the presence of the mutation (M, mutant; +, wild type) and insulin resistance in three successive generations. Diagonal line indicates a deceased individual.

She had prominent peripheral veins and well-defined peripheral musculature (figure 2). Her blood pressure remained high at 180/80. She had an ejection systolic murmur, in keeping with aortic stenosis. She had bilateral diabetic retinopathy, proteinuria and peripheral neuropathy, but no clinical evidence of peripheral macrovascular disease. She had no clinically detectable features of chronic liver disease.

Biochemical findings (see table 1) included hyperglycemia, hyperinsulinemia, hypertriglyceridemia with a reduced HDL cholesterol fraction and hyperuricemia. She also had raised liver enzymes and an abdominal ultrasound picture consistent with fatty liver (non-alcoholic steatohepatitis – she customarily consumed less than 40 mg ethanol weekly and, hepatitis B and C serology and auto-immune markers were negative). Splenomegaly, gastric varices and reduced portal vein blood flow were highly suggestive of portal hypertension. In addition a 2 * 2 cm echogenic nodule was noted in the right lobe of the liver. In summary the ultrasound findings were in keeping with hepatic cirrhosis secondary to chronic steatohepatitis, complicated by portal hypertension and possibly hepatocellular carcinoma. Further investigations revealed thrombocytopenia, but normal hepatic synthetic function. Liver histology confirmed steatohepatitis, cirrhosis and hepatocellular carcinoma.

Cardiac echocardiography revealed left ventricular hypertrophy, moderate aortic stenosis and good left ventricular function. Subsequent angiography confirmed moderate aortic stenosis and revealed surprisingly little coronary atheroma. The findings were summarised as follows by a cardiologist:

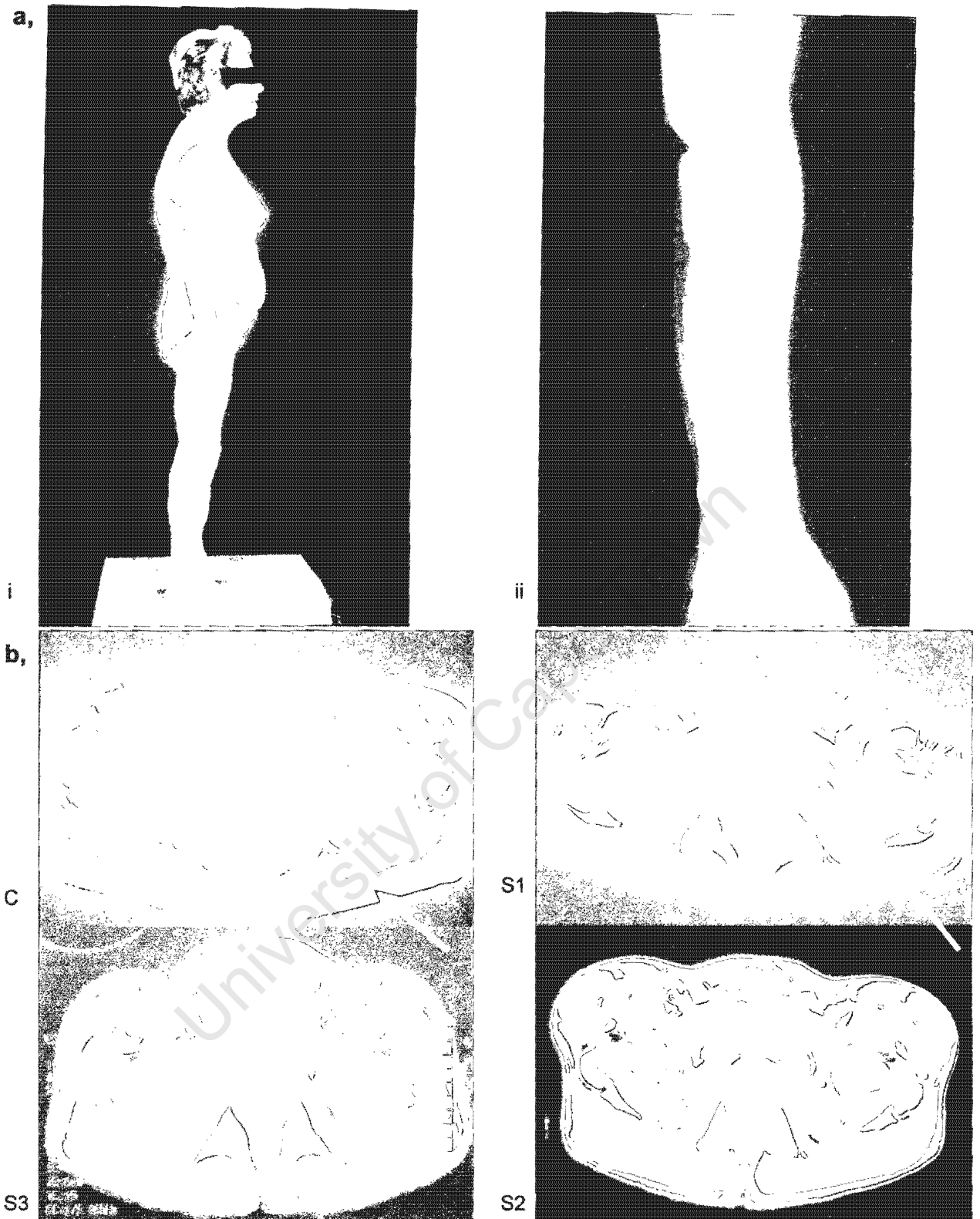


Figure 2

a_{i-ii}) Photographs of a 56 year-old-female P467L carrier (Subject 1, S1). Note the prominent forearm veins and musculature, as well as the preservation of abdominal fat with loss of limb and gluteal fat depots. b) T1-weighted MRI images at the level of the gluteal fat pad indicate the striking loss of gluteal subcutaneous fat (red arrows) in subject 1 (S1). Corresponding images from subjects 2 (S2) and 3 (S3) demonstrate the consistency of these features. Control (C) images were taken from a lean female individual.

Table 1: Metabolic characteristics of S1-3 and prepubertal P467L carriers.

	56-yr-old ♀	32-yr-old ♂	21-yr-old ♀	7-yr-old ♀	3-yr-old ♂	Reference values
	P467L*	P467L	V290M	P467L	P467L	
Glucose (mmol/L)	6.0	10	9.6	4.9	5.6	3.5-6.3
Insulin (pmol/L)	296	85	411	124†	85†	<80 (adults)
Triglycerides (mmol/L)	2.1	6.6	4.5	0.7	0.6	Desirable < 2.0
Total cholesterol (mmol/L)	3.4	4.7	3.4	4.1	4.8	Desirable < 5.2
HDL-cholesterol (mmol/L)	0.9	0.72	0.6	0.84	1.05	Desirable > 1.0
NEFAs (μmol/L)	721	503	558	-	-	280-920
Uric acid (mmol/L)	0.29	0.35	0.37	-	-	0.15-0.35
ALT (U/L)	70	85	39	29	33	0-50
GGT (U/L)	454	68	147	18	20	0-50

All measurements were made on fasting blood samples. * Subject 1 is on hormone replacement therapy. † Reference values for fasting insulin in children:

Healthy 9-yr-olds (n=50, mean±SE):42.8±5.7; healthy 3-yr-olds (n=14, mean±SE):18.7±1.6 (personal communication with DA Dunger and K Ong - Avon

Longitudinal Study of Pregnancy and Childhood (ALSPAC)). HDL-cholesterol, high density lipoprotein cholesterol; NEFAs, non-esterified fatty acids; ALT,

alanine aminotransferase; GGT, gamma glutamyl transferase.

"..I performed coronary angiography via the right radial artery using five French catheters. This showed that she has plaque disease throughout her coronary system, most marked in the RCA (*right coronary artery*) which is small and non dominant but there is no significant coronary disease otherwise. "

In summary this case demonstrated early onset type-2 diabetes in a lean woman, with features of the HAIRAN (hyperandrogenism, insulin resistance and acanthosis nigricans) syndrome. Hypertension, dyslipidemia and hyperuricemia completed the cluster of abnormalities known as syndrome X. Non-alcoholic steatohepatitis (NASH) was the most likely cause of her cirrhosis. After consultation with hepatologists she proceeded to liver transplantation in an attempt to completely remove her hepatocellular carcinoma. Unfortunately this procedure was complicated by acute hepatic artery thrombosis several months later, with sudden decompensation and death.

Subject 2 (P467L heterozygote): Case history

This patient, a 32-year-old man, was found to be diabetic and hypertensive at age 28 years during a routine health assessment. At the time of referral he was treated with oral hypoglycemics and two anti-hypertensive agents. On examination he was lean (BMI 24.9 kg/m²) with a waist: hip ratio of 0.91 and no acanthosis nigricans. Clinical examination was normal except for high blood pressure (BP 160/93). His children, aged 3 and 7 years (subjects IVi

and IVii in figure 1), were both found to be heterozygous for the P467L mutation. Clinically they were both lean and healthy (Z-scores: 3-year old boy 0.1 SD; 7-year old girl -0.6 SD).

Biochemical findings in subject 2 are listed in table 1. In summary, he too manifested fasting hyperinsulinemia, dyslipidemia, steatohepatitis and hyperuricemia. His children were also hyperinsulinemic with fasting insulin levels 3-4 times those of age-matched controls (figure 3). Their biochemistry was otherwise normal (table 1).

Subject 3 (V290M heterozygote): Case history

Subject 3, a 21-year-old woman, presented with primary amenorrhoea, hirsutism, acanthosis nigricans and hypertension at age 15 years. She developed diabetes at age 17 years. At the time of referral she was treated with oral hypoglycemics, atenolol and an oral contraceptive to induce regular menstrual bleeds. On examination she had a BMI of 28.1kg/m² and an elevated waist:hip ratio of 0.99. As in subject 1 truncal fat was preserved whereas limb and gluteo-femoral fat were strikingly reduced. Acanthosis nigricans was present in the axillae and nuchal crease. She had no evidence of diabetic complications or macrovascular disease. Biochemical findings are listed in table 1.

In summary, she had features of the HAIR-AN syndrome as well as all the features of the metabolic syndrome despite the absence of obesity. Genetic

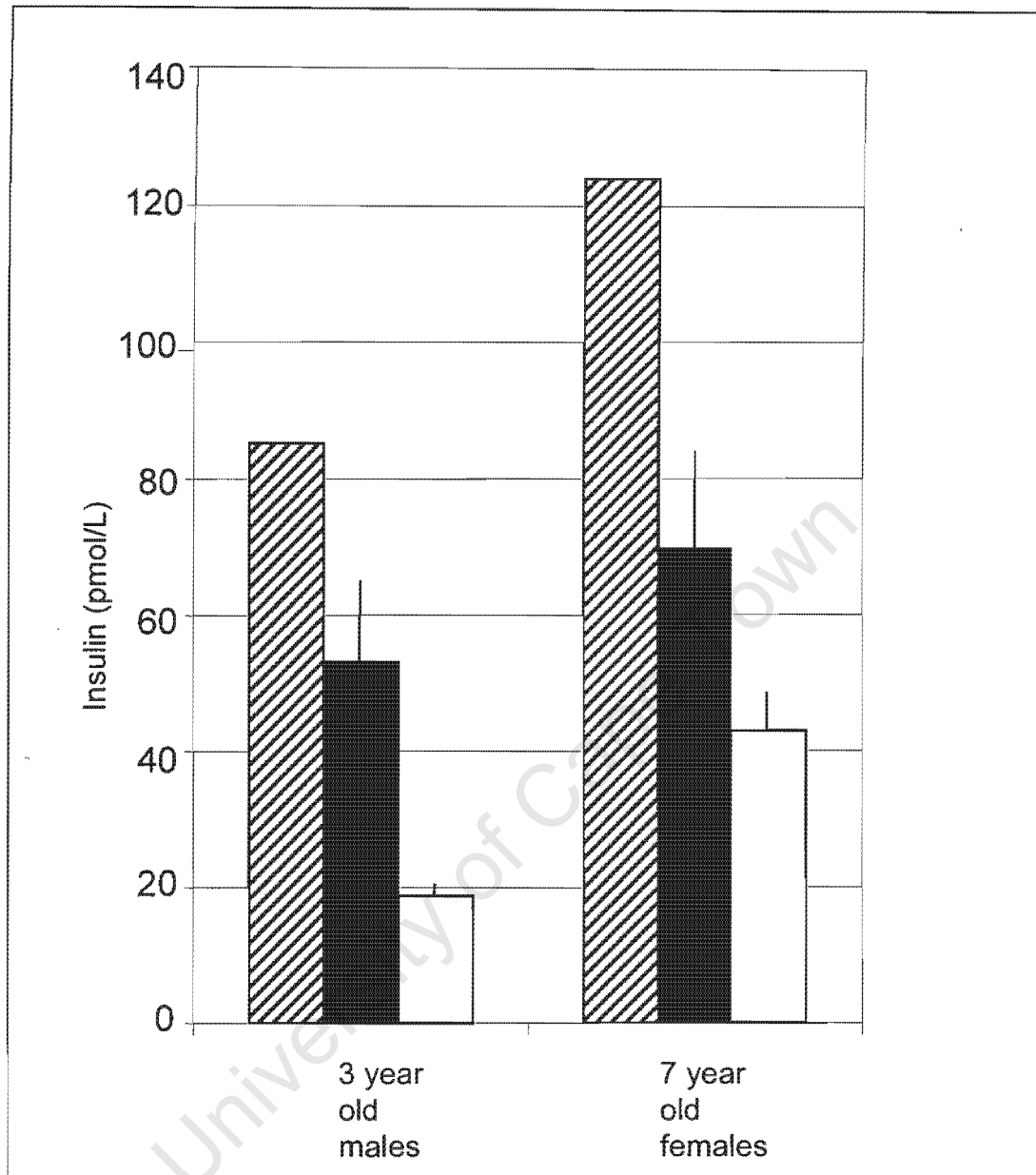


Figure 3

Fasting hyperinsulinaemia in children with the P467L PPAR γ mutation. Hatched bars represent P467L carriers, black bars represent mean $\pm$ SE of 10 age- and sex-matched obese children (BMI standard deviation scores > 4 ; unpublished observations IS Farooqi, S O'Rahilly) and white bars represent mean $\pm$ SE of healthy, age-matched Caucasian children ($n = 14$ three-year-olds and $n = 50$ seven-year-olds respectively).

studies in her family failed to reveal any additional carriers of the V290M mutation. Her mother was wild type at the PPAR γ locus; her father was deceased and there were no other family members available for study.

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Methods

In the light of a large body of *in vitro* data indicating the importance of PPAR γ in adipogenesis (reviewed in (Rosen and Spiegelman, 2001)) and the clinically apparent abnormalities in fat distribution detailed studies of body composition and fat distribution were undertaken.

Body composition

Body composition studies were undertaken at the Medical Research Council Human Nutrition Research centre in Cambridge, UK in collaboration with Dr S. Jebb. Historically, measurements of body composition were based on a two compartment model, in which the body was assumed to be composed of fat and fat-free mass (FFM) alone. These methods included measurements of total body water, total body potassium and body density. However the realisation that inaccuracies of these classical methods related to limitations of the two compartment model rather than errors in the measurements of the physical properties of the body prompted the development of more sophisticated models of the body. Four compartment models include measurements of body weight, volume which is measured in the Bodpod by air-displacement, bone mineral by dual energy X-ray absorptiometry (DXA) and total body water by deuterium dilution. Whilst body density can be very precisely measured, deviations in body density can occur as a result of changes in hydration status or the proportion of bone mineral. Hence, incorporating measurements of total body water improves the accuracy of

body composition measurements. Deuterium is a stable, non-radioactive tracer which can be administered orally and then measured in saliva. This four-compartment body composition model is theoretically one of the most accurate methods available for assessing body composition *in vivo* (Fuller et al., 1992). All the adult subjects described above underwent four compartment body composition analysis.

Fat distribution

Whilst DXA provides a crude impression of regional fat distribution, imaging techniques such as computer tomography (CT) scanning and magnetic resonance imaging (MRI) can provide direct measures of fat mass at specific body sites. Furthermore cross-sectional imaging enables one to discriminate between visceral and subcutaneous abdominal fat. Adipose tissue distribution was studied in all three subjects by T1-weighted MRI and abdominal visceral: total fat ratios were calculated from a single cross-sectional image at the level of the umbilicus.

Intramyocellular triglyceride determination

Ectopic lipid accumulation is increasingly etiologically implicated in both obesity- and lipodystrophy-associated insulin resistance (Friedman, 2002). In particular, intramyocellular triglyceride levels appear to be increased in insulin resistant states. Magnetic resonance spectroscopy has allowed investigators to accurately measure intramyocellular lipid content. The technique is based upon localised proton magnetic resonance spectroscopy (1H-MRS)

(Szczepaniak et al., 1999). Many nuclei in nature behave as miniature bar magnets, hydrogen ("protons") being a typical example. Cellular metabolites rich in protons, such as water and fat, have unique, identifiable magnetic resonance frequencies by which they can be individually identified. Fortuitously, the ultra-structural organization of triglycerides within skeletal muscle alters bulk magnetic susceptibility whereby signals arising from the extramyocellular lipids can be separated from the intramyocellular pool by a 0.2 ppm chemical shift. Whereas methylene resonances at 1.5 ppm represent extramyocellular lipid, those at 1.3 ppm represent intramyocellular lipid droplets situated in close proximity to mitochondria (Szczepaniak et al., 1999). Intramyocellular triglycerides were measured in the soleus muscle of subjects 1 and 2 at the Hammersmith Hospital, Robert Steiner MRI Unit, London, UK in collaboration with Drs E.L. Thomas and J.D. Bell.

Insulin sensitivity measurements

Insulin resistance is generally defined as a reduced ability of a given plasma insulin concentration to lower serum glucose. Measurements of insulin resistance include simple quantification of fasting plasma insulin and glucose, and several indices derived from these measurements e.g. homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI)(Katz et al., 2000). As these measures have not been well validated in states of severe insulin resistance, two of the subjects in this study underwent hyperinsulinemic-euglycemic clamps, which remain the gold standard measurement of insulin sensitivity. Medication was stopped 36

hours before the studies and subjects were fasted for 12 hours prior to – and throughout the clamp. Normoglycemia (5-7mmol/L) was maintained overnight (20h00-08h00) with a variable rate insulin infusion. 6,6 $^2\text{H}_2$ -glucose infusion was commenced at 05h00 and maintained until the end of the study. Insulin (Actrapid) was infused at 10mU/kg/min between 08h00 and 10h00.

Blood glucose measurements were carried out at 5 minute intervals throughout the study period and euglycemia (5mmol/L) maintained by variable infusion of a 20% dextrose infusion (enriched with 6,6 $^2\text{H}_2$ -glucose). Samples for stable isotope measurements were obtained at 15 minute intervals between 08h00 and 10h00, except during the steady state period (09h30-10h00) when sampling occurred at 5 minute intervals. Glucose enrichment was determined using the methoxamine-trimethylsilyl ether derivative by selected ion monitoring by GCMS (Hewlett Packard 5971A MSD). R_a (rate of glucose appearance) and R_d (rate of glucose disposal) were measured according to calculations originally derived by Steele and subsequently modified for stable isotopes (Powrie et al., 1992).

Adipose tissue morphology

Small adipocytes are said to be more insulin sensitive than larger adipocytes. Furthermore a number of rodent studies have suggested that PPAR γ agonists lead to a reduction in adipocyte size and thereby improve insulin sensitivity. One might therefore expect carriers of dominant negative mutations in PPAR γ to have particularly large adipocytes. Subject 2 consented to an open biopsy

of subcutaneous adipose tissue for morphological and molecular analysis. Adipose tissue morphology was studied by Professor S. Cinti in Ancona, Italy as described previously (Cinti et al., 2001).

Examination of *in vivo* adipose tissue function

White adipose tissue metabolism was assessed by measurement of arterio-venous substrate fluxes across subcutaneous abdominal adipose tissue in subject 2 (subject 1 was deemed medically unfit for this procedure and subject 3 elected not to participate). The principle of this technique is as follows: differences in the composition of blood samples from the arterial supply to the tissue and venous drainage from the tissue reflect the net metabolic activity of the tissue. The net uptake of any substrate is then given by: Net uptake = Arteriovenous difference * Blood flow.

Blood flow is determined by washout of ^{133}Xe , following subcutaneous injection in the anterior abdominal wall. "Arterial samples" are taken from a retrogradely inserted cannula in a dorsal hand vein. The hand and forearm are then placed in a hot-box (65 degrees) and arterialisation of the sample is confirmed by checking that oxygen saturation is greater than 95%. Obtaining venous blood from adipose tissue is difficult as very few veins, large enough to be catheterised receive blood from adipose tissue alone. However, there are some sites such as the anterior abdominal wall where the contribution of adipose tissue seems to predominate, probably because other tissues such as skin are less metabolically active. This study was undertaken in Keith Frayn's laboratory in Oxford, UK in collaboration with Fredrik Karpe and Garry

Tan. The subject was fasted overnight prior to the study. Following insertion of cannulas baseline measurements were made for 30 minutes prior to consumption of a standardised mixed meal. The subject then relaxed on a bed while further measurements were made for six hours. The principal measurements included glycerol, non-esterified fatty acids and triglyceride fluxes (for details of this technique see "Assessment of white adipose tissue metabolism by measurement of arteriovenous differences." KN Frayn & SW Coppack. Adipose tissue protocols – Edited by G. Ailhaud 2001 Humana Press Inc.).

Monocyte isolation and culture

Human PBMCs (peripheral blood mononuclear cells) were isolated by ficoll gradient centrifugation. Peripheral venous blood (20 – 40 mls) was collected into 20 ml universal containers inoculated with 50 μ L of preservative-free heparin. The blood was then diluted in a 1:1 ratio with sterile phosphate buffered saline (PBS) and carefully layered onto ficoll (10 – 15 mls blood per 8 ml ficoll). Samples were then centrifuged for 20 minutes at 20 000 g. The white cell layer was aspirated and recentrifuged for 10 minutes at 1200 rpm. Following removal of the supernatant, PBMCs were resuspended in 10 mls PBS and centrifuged for 10 minutes at 1200 rpm. The supernatant was removed and the dilution and centrifugation repeated three times ("washing"). PBMCs were then counted, resuspended in RPMI 1640 (Sigma) with 1% stripped FBS and 3×10^6 cells/ well were transferred to 6-well plates. After 1hr at 37 degrees in a 5% CO₂ humidified incubator, non-adherent cells were removed and the monocytes washed twice with PBS before resuspension in

RPMI 1640 with 1% stripped FBS. Rosiglitazone or GI262570 (a non-thiazolidinedione PPAR γ agonist) (gift from Smith-Kline Beecham), dissolved in DMSO (Sigma), were then added to each well at the desired concentrations.

Microarray methodology

Pre-adipocytes harbouring the dominant negative P467L PPAR γ mutation were isolated from subject 2. Pre-adipocyte isolation and culture from subcutaneous abdominal adipose tissue were performed as previously described (Sewter et al., 2002). Briefly, pre-adipocytes were cultured in 150 cm² dishes and sub-cultured for 4 passages before being allowed to reach confluence in multiple flasks. Confluent cells were left in the confluent state for four days prior to addition of differentiation cocktail. In our experience this improves the ensuing response to differentiation cocktail (unpublished observations).

RNA was isolated from several flasks immediately prior to addition of differentiation cocktail. This RNA was pooled and is hereafter referred to as T0 mRNA. Following the addition of differentiation cocktail (Sewter et al., 2002), which included rosiglitazone 100nM, further RNA samples were isolated 6- (T6) and 48 (T48) hours later. RNA was isolated using Qiagen RNeasy reagents and its quality was verified on 2% agarose gels containing ethidium bromide.

After the isolation of messenger (poly-A) RNA, samples were labelled with Cy3 or Cy5 dye and hybridised to a microarray chip, Unigem 1 (Incyte), which contains cDNA elements from 10176 human genes (Yue et al., 2001). Changes in gene expression between T0 and T6/ T48 mRNA samples were evaluated using a relational database programme based on Filemaker Pro 5, developed for microarray analysis by G. Denyer. The programme allowed the formation of clusters based on mRNA intensity and fold changes. Gene expression at 6hrs or 48hrs was initially compared with that in T0 mRNA in the PPAR γ wild type cells. Genes in these sets were then compared with similar gene sets derived from cells harbouring the P467L mutation.

The entire array experiment was performed twice using the same mRNA pools, on two **independent** occasions. Only those genes which fulfilled the selection criteria in **both** experiments are listed in tables 4 and 5. The fold changes listed in tables 4 and 5 represent the mean of the two independent experiments. In the present study, ~80 % of the top 50 altered genes identified by the first microarray were also changed more than 2-fold in the second independent experiment using the same RNA.

In vivo studies in rodents

Murine white adipose tissue samples were isolated from epididymal fat pads of healthy C57/BL6 mice fed, fasted for 24 hours or fasted (24 hours) and then refed for 24 hours. PDK4 mRNA levels were measured using realtime RT-PCR (see appendix A for probe/ primer sequences).

Real time reverse-transcriptase polymerase chain reaction (RT-PCR (also known as Taqman)) methodology

Total RNA was isolated using Qiagen reagents (Qiagen, West Sussex, UK). RNA samples were quantified spectrophotometrically and RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining.

Following denaturation at 65 degrees for 5 minutes, total RNA (100ng) was reverse transcribed for 1 hour at 37 degrees in a 20 μ L reaction containing 1* RT buffer (50 mmol/L Tris-HCL, 75 mmol/L KCL, 3 mmol/L $MgCl_2$ and 10 mmol/L dithiothreitol), 150 ng random hexamers, 1.25 mmol/L dNTP, and 200 units M-MLV RT (all reagents were from Promega). Reactions in which RNA was omitted served as negative controls. A reaction containing 500 ng of total RNA was also included as a standard. After first-strand cDNA synthesis this was serially diluted 1:2 in DNase-free water to generate a standard curve for the PCR analysis.

Oligonucleotide primers and Taqman probes were designed using Primer Express, Version 1.0 (Perkin-Elmer Applied Biosystems, Foster City, California) (see Appendix 1 for all primer/ probe sequences). In every case the amplicons were designed to span intron-exon boundaries so as to eliminate the risk of PCR amplification from genomic DNA. Probes were labelled at the 5' end with the reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA).

Oligonucleotide primers and a Taqman probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Perkin-Elmer.

PCR was carried out in duplicate or triplicate for each sample on a ABI 7700 sequence detection system (Perkin-Elmer) and all reactions were performed on at least two occasions. Each 25 μ L reaction contained 2-4 μ L of first-strand cDNA, 1 * PCR master mix, 300 nmol/L of each forward and reverse primer and 150 nmol/L Taqman probe (the concentrations of primers and probes varied slightly for some sets of primers and probe depending on the results of prior optimisation experiments). All reactions were carried out using the following PCR cycling parameters: 50 degrees for 2 minutes and 95 degrees for 10 minutes, followed by 40 cycles of 95 degrees for 15 seconds and 60 degrees for 1 minute. After the PCR, standard curves were constructed from the standard reactions for each target mRNA species and GAPDH by plotting values for Ct (the number of PCR cycles at which the fluorescence signal exceeds background) versus log cDNA input (in nanograms (arbitrarily assigned)). The Ct readings for each of the samples were then used to calculate the amount of mRNA for each target gene relative to the standard. For each sample, results were normalised by dividing the amount of target gene mRNA by the amount of GAPDH mRNA.

Biochemical assays

Interleukin-6 (IL-6) and Acrp30 assays were performed in the laboratories of Prof. J-O. Jonson and P. Scherer respectively. All other biochemical assays

including plasma leptin and insulin measurements were undertaken in the University of Cambridge Clinical Biochemistry Department.

Statistical analysis

Statistical analysis was performed using the Windows Excel Statistical package.

University of Cape Town

Results

Body composition and fat distribution

In vitro and rodent data strongly suggests that PPAR γ is a key regulator of adipogenesis (Lowell, 1999). Subjects 1 and 2 (P467L carriers) had body mass indices (BMI) within the healthy range, whereas subject 3 (V290M) was overweight on the basis of BMI (table 2). However, formal measurements revealed that all three subjects with dominant negative PPAR γ mutations had a total body fat content substantially lower than predicted from their BMI (table 2). In addition, magnetic resonance imaging of fat distribution revealed a consistent and striking paucity of subcutaneous limb and buttock fat (figure 2), whereas both visceral and subcutaneous abdominal fat were preserved. This is reflected by the increased waist: hip ratios in both female patients, where the loss of gluteal and femoral fat was particularly striking (figure 2 and table 2). Although the detection of partial lipodystrophy in children is particularly difficult, both total and regional body fat measurements in the 7-year-old P467L carrier (total body fat: 15%; individual regional fat range 12 – 16.3%) were similar to those predicted according to her height and weight (height: 1.23 m; weight: 25.2 kg; predicted total body fat 17.4% (Fomon, 1982)). Lipodystrophy in rodents and humans is believed to result in “ectopic” lipid accumulation in liver and skeletal muscle, a phenomenon increasingly implicated in the pathogenesis of insulin resistance in these syndromes (Friedman, 2002). On ultrasonographic study all three subjects had hyperechoic livers, consistent with fatty infiltration. This radiological

impression was confirmed histologically in subject 1 (data not shown), whose liver disease had in fact progressed to cirrhosis. Surprisingly however, intramyocellular triglyceride (IMTG) levels were within the normal range in two subjects (table 2).

Metabolic studies

The presence of acanthosis nigricans and fasting hyperinsulinemia suggested that all affected subjects were severely insulin resistant (Barroso et al., 1999). To confirm this, subjects 2 and 3 underwent hyperinsulinemic–euglycemic clamp studies. It should be noted that very few studies have included people with insulin resistance of the magnitude seen in the subjects of this work. The choice of high dose insulin infusion rate was based upon previous French studies in patients with total lipodystrophy, a group predicted to display similar levels of insulin resistance (Vantyghem et al., 1999). High dose insulin infusion (10mU/kg/min) failed to stimulate peripheral glucose disposal normally (glucose disposal (R_d , mg/kg/min): S2 - 7.53; S3 – 2.91; healthy adult range – 13-15 (Vantyghem et al., 1999)) or to completely suppress hepatic glucose output (HGO (R_a mg/kg/min): S2 – 1.07; S3 – 0.31) in either subject (table 3).

In addition to insulin resistance all the subjects with dominant negative PPAR γ mutations had early onset hypertension and elevated serum triglycerides with low HDL cholesterol levels (table 1). Hyperuricemia and steatohepatitis, which are increasingly recognised as additional elements of the metabolic syndrome

Table 2: Body composition details.

	56-yr-old ♀ P467L (S1)	32-yr-old ♂ P467L (S2)	21-yr-old ♀ V290M (S3)	Healthy adult ranges
Height (m)	1.53	1.72	1.64	
Weight (kg)	57.1	73.8	75.8	
Body mass index (kg/m ²)	24.4	24.9	28.1	18.5 – 24.9 (WHO, 1998)
Predicted total body fat (%)*	29.0	22.0	34.5	
Measured total body fat (%)	17.6	10.6	25.5	S1: 23 – 34% S2: 8 – 20% S3: 33 – 39% (Gallagher et al, 2000)
Waist: hip ratio	1.1	0.91	0.99	Female < 0.85 Male < 1.0
Visceral fat: total abdominal fat ratio	0.28	0.44	0.39	Female 0.25 ± 0.14 Male 0.42 ± 0.11 (Dixon, 1983)
Fatty liver†	+	+	+	
Intramycellular triglycerides (IMTG) expressed as IMTG: creatine ratio for soleus muscle	13.7	9.7	-	Mean ± SE in 76 controls = 13.6 ± 6.6 (unpublished observations)

*Predicted body fat was calculated as follows (Black et al, 1983): Men % fat = (1.281*BMI) – 10.13; Women % fat = (1.48*BMI) – 7.00

†The diagnosis of fatty liver (+) was made on the basis of an echo-bright ultrasound picture in the absence of a history of alcohol consumption in excess of 40g per week. Steatosis and cirrhosis were confirmed histologically in subject 1.

♀, female; ♂, male

Table 3: Response to PPAR γ agonist therapy (rosiglitazone 4mg b.i.d.)

	P467L (Subject 2)				V290M (Subject 3)				Reference
Months of treatment	0	1	3	6	0	1	3	6	
Weight (kg)	73.8	-	-	76.8	75.8	-	-	76.6	
Fat mass (kg)	6.3	-	-	9.8	17.6	-	-	21.6	
Leptin (ug/L)	0.7	1.5	1.9	1.9	11.2	13.8	14.4	14.4	*
Acrp30 (U/mL)	0.25	-	-	0.5	0.17	-	-	0.24	2.23±0.66 (Combs et al, 2002)
Blood pressure (mm Hg)	140/97	147/84	153/85	156/99	136/84	114/82	135/88	140/85	
Glucose (mmol/L)	10	10.9	12	9.3	9.6	7.3	6.4	7.2	3.5-6.3
Insulin (pmol/L)	85	107	73	114	411	265	414	410	<80
HbA1C (%)	8.7	8.8	8.3	5.6	7.3	6.9	6.7	7.1	4.9-6.3
Glucose disposal (mg/kg/min)	7.53	-	-	14.9	2.91	-	-	3.21	13-15 (Vantyghem et al, 1999)
Hepatic glucose output (mg/kg/min)	1.07	-	-	0	0.31	-	-	0.52	0

Triglycerides (mmol/L)	5.7	6.6	7.6	6.3	4.5	7.1	3.5	6.9	<2.0
HDL-cholesterol (mmol/L)	0.58	0.72	0.66	0.79	0.58	0.71	0.72	0.52	>0.9
NEFAs (mmol/L)	503	365	510	325	558	915	852	643	280-920
ALT (U/L)	85	87	108	82	39	48	79	58	0-50
GGT (U/L)	68	75	86	67	147	100	102	122	0-50

*Leptin adult reference values (unpublished observations Department of Clinical Biochemistry, University of Cambridge, mean and 95% confidence intervals):

♀; BMI 25-30 kg/m²; n=348 adults; 21.1 (8.6-38.9). ♂; BMI <25 kg/m²; n=278 adults; 3.3 (0.4-8.3)

ALT, alanine aminotransferase; GGT, gamma glutamyl transferase

were also present in these subjects. It has been suggested (Ginsberg, 2000) that insulin resistance alone can account for the subsequent development of all the elements of the metabolic syndrome, although the absence of these features in some of our subjects with severe insulin resistance together with their absence in many severely insulin resistant rodent knockout models suggests that this relationship is a tenuous one. We therefore believe that the subjects described herein represent the first human examples of monogenic syndrome X. This notion is supported by the documented ability of PPAR γ agonists to improve all the features of the metabolic syndrome. Hypertension was a particularly striking clinical feature in all the adult carriers of dominant negative PPAR γ mutations and clearly preceded the onset of diabetic nephropathy in subject 1. It was associated with significant left ventricular hypertrophy in all the subjects. The expression of several components of the renin-angiotensin system in adipose tissue (Engeli et al., 2000) suggested the possibility that PPAR γ mutations might lead to dysregulation of this system, thereby contributing to the development of hypertension. However, normal serum potassium levels coupled with normal plasma renin activity and aldosterone levels (data not shown) suggest that abnormal regulation of aldosterone metabolism may not be involved in the pathogenesis of hypertension in these subjects. While the consistent finding of severe early-onset hypertension appears to distinguish PPAR γ deficiency from other genetic causes of severe insulin resistance including "insulin receptoropathies" and inherited lipodystrophies the mechanisms underlying the link with hypertension remain obscure.

Adipose tissue function and morphology

The nature of PPAR γ target genes in adipose tissue led to suggestions that PPAR γ may be involved in the regulation of “trapping” of non-esterified fatty acids (NEFAs) within adipocytes, and that this may, at least in part, explain the insulin sensitising effects of pharmacological PPAR γ agonists (Martin et al., 1998). *In vivo* adipose tissue studies in subject 2, demonstrated the following:

- 1) The usual postprandial increase in plasma triglyceride clearance across the tissue was not seen; in fact the clearance was very low both in the fasting state and postprandially (figure 4).
- 2) Interestingly, adipose tissue lipolysis, as assessed by glycerol output, was also low both in the fasted and postprandial states (data not shown). This apparent failure of adipose tissue to regulate lipolysis is reflected in figure 5, where subcutaneous abdominal adipose tissue NEFA output remained low throughout the study in the P467L subject, whereas in both normal and obese subjects it is suppressed postprandially (Coppack et al., 1992).

Both light and electron microscopy of subcutaneous abdominal adipose tissue from subject 2 revealed normal morphological features. The adipocyte mean cell diameter, area and weight based upon individual cell morphometric analysis were $81.56 \pm 1.48 \mu\text{m}$, $5734.67 \pm 194.54 \mu\text{m}^2$ and $0.33 \pm 0.02 \mu\text{g}$ lipid per cell respectively. These measurements are comparable to those obtained from healthy subjects (personal communication S. Cinti, University

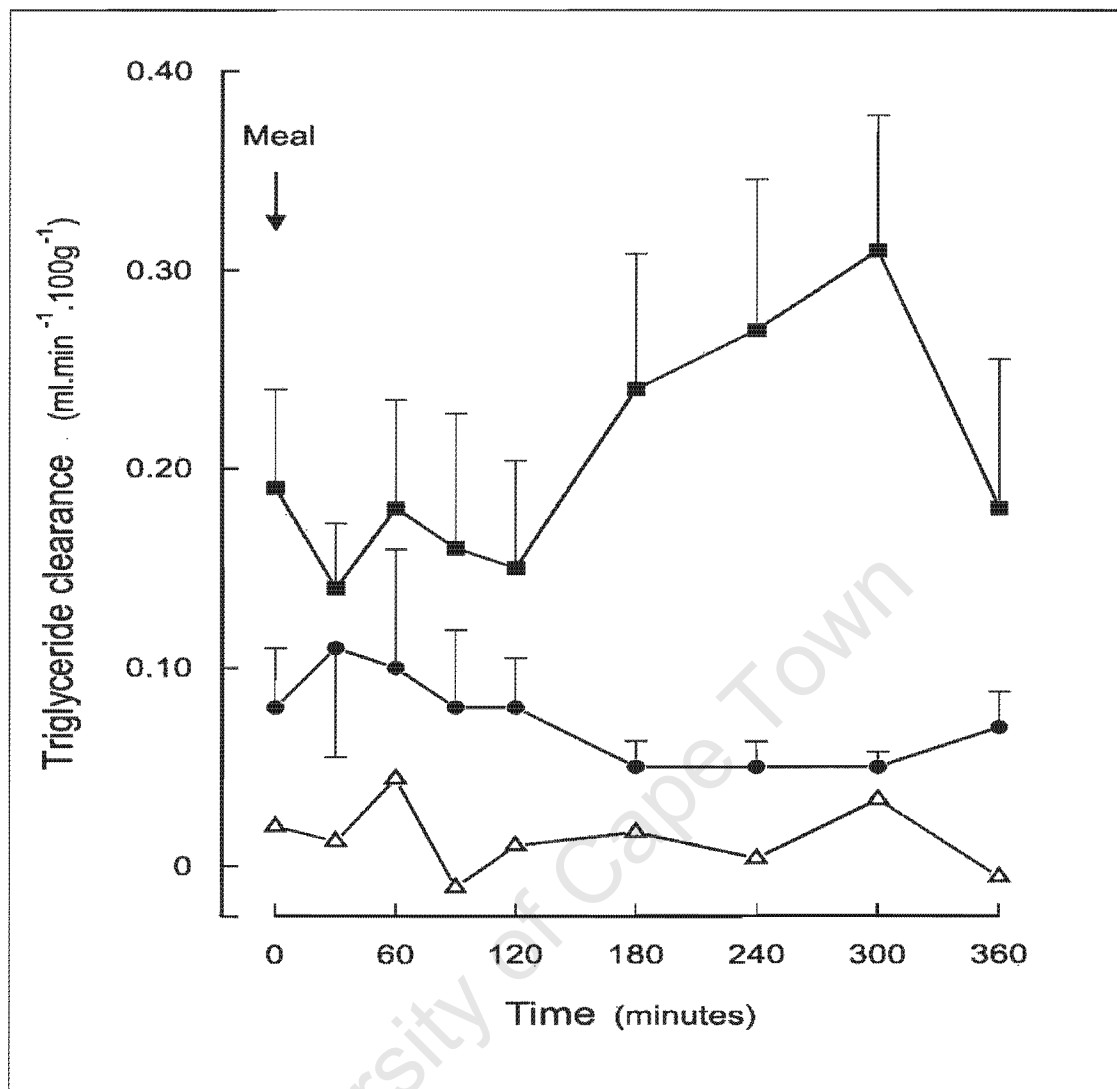


Figure 4

Adipose tissue clearance of plasma triglycerides in mL of plasma. min⁻¹. (100g adipose tissue)⁻¹ in subject 2 (P467L) (open triangles), 10 normal (solid squares) and 8 obese (solid circles) subjects (Coppack et al, 1992).

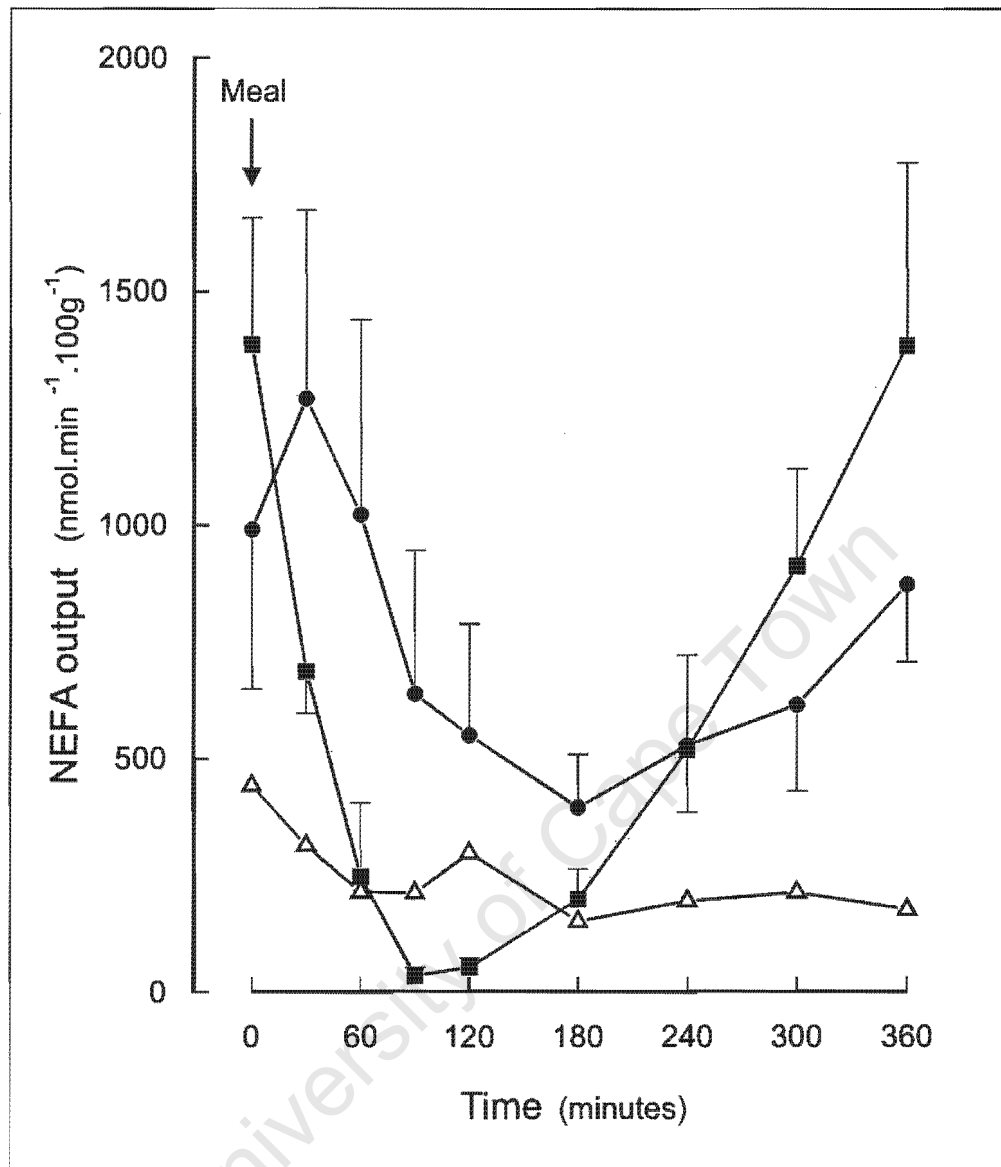


Figure 5

Adipose tissue non-esterified fatty acid (NEFA) output in $\text{nmol} \cdot \text{min}^{-1} \cdot (100\text{g adipose tissue})^{-1}$ in subject 2 (P467L) (open triangles), 10 normal (solid squares) and 8 obese (solid circles) subjects (Coppack et al, 1992).

of Ancona, Italy). Electron microscopy also showed standard ultrastructural features of adipocytes and all other tissue elements.

Adipokine measurements

Resistin

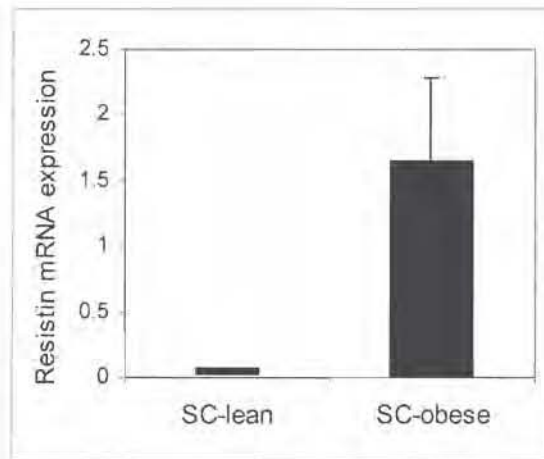
A growing body of evidence indicates that adipocytes secrete proteins ("adipokines") with paracrine and/or endocrine effects, several of which have the ability to influence insulin action, either positively or negatively. During the course of these studies Steppan et al (Steppan et al., 2001) reported a novel cysteine-rich secreted protein, which they termed resistin, the expression of which was markedly decreased by treatment of a murine adipocyte cell line with a PPAR γ agonist. Serum levels of resistin were elevated in obese mice and immunoneutralisation of circulating resistin in these animals improved insulin sensitivity. Administration of recombinant resistin impaired *in vivo* insulin action in mice and *ex vivo* in an adipocyte cell line. These observations led the authors to conclude that resistin might represent an adipocyte-derived mediator of the link between obesity and insulin resistance. They also suggested that the suppression of resistin expression by PPAR γ agonists might explain the beneficial effects of these compounds in insulin resistant states. Contrasting conclusions were reached by Way et al (Way et al., 2001a) who found reduced resistin mRNA levels in white adipose tissue of several obese rodent models. These discrepant results were difficult to reconcile and indicated the need for further studies. Real-time RT-PCR was

used to examine resistin mRNA expression in human samples (see appendix A for primer and probe sequences).

Whilst human resistin mRNA was readily detectable in peripheral blood mononuclear cells (n=8, four men and four women, BMI < 30 kg/m²), it was undetectable in whole adipose tissue of lean subjects (n=6, four men and two women, BMI 22 - 26 kg/m²) and also in cultured pre-adipocytes, *in vitro* differentiated pre-adipocytes, vascular endothelial and vascular smooth muscle cells (all of which are present in whole adipose tissue). In contrast, resistin mRNA was detectable in adipose tissue obtained at surgery from morbidly obese subjects (n=6, five women and one man, BMI 43 - 63 kg/m²) with similar levels in subcutaneous and visceral depots (figure 6, p>0.1). While this relationship to obesity appeared to be consistent with the reported murine data (Steppan et al., 2001), there was no discernible relationship between adiposity and resistin expression in isolated fat cells obtained from 14 subjects ranging in body mass index from 22-59kg/m² (ten women and four men) (figure 7). The finding of predominant expression of resistin in mononuclear cells in humans with very low level expression in adipocytes was supported by a bioinformatic search for resistin sequences in >1 375 human cDNA libraries. Resistin cDNA was only found in ten of these libraries, four of which were of lymphoid/ monocyte origin (personal communication I. Barroso of Incyte Genomics).

PPAR γ agonists were reported to suppress resistin expression in murine adipocytes (Steppan et al., 2001). As resistin was undetectable in cultured

a,



b,

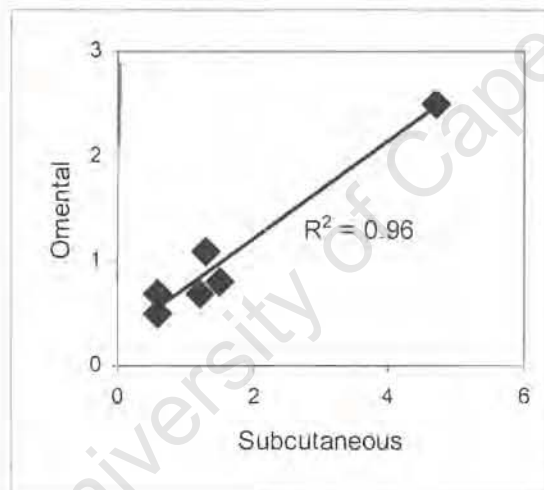


Figure 6

Resistin mRNA expression in whole adipose tissue. Resistin mRNA levels were measured by real-time RT-PCR and expressed relative to levels of the housekeeping gene GAPDH mRNA (arbitrary units). a) Resistin mRNA levels (means $\pm$ SE) in subcutaneous (SC) whole adipose tissue from six lean subjects compared with subcutaneous samples from six morbidly obese subjects ($p < 0.01$). b) correlation between resistin mRNA levels in subcutaneous and omental whole adipose tissue from six morbidly obese subjects.

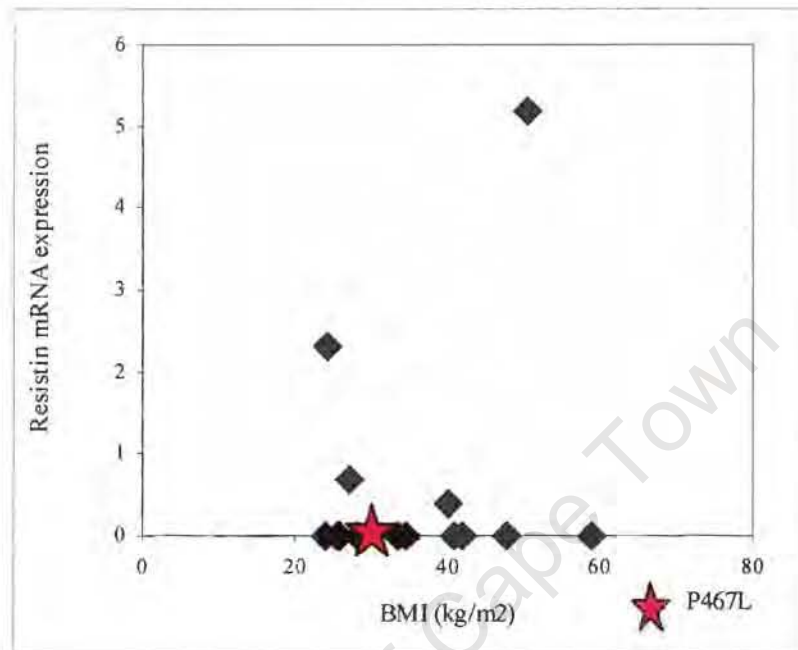


Figure 7

Effect of increasing adiposity on adipocyte resistin mRNA expression. Resistin mRNA levels were measured by real-time RT-PCR and expressed relative to levels of GAPDH mRNA (arbitrary units). Resistin mRNA levels in isolated adipocytes from 14 subjects and from subject 2, who has the P467L PPAR γ mutation (represented by the red star).

human adipocytes (48hrs, n=5 morbidly obese individuals) the effects of two independent PPAR γ agonists were examined in human mononuclear cells. Whereas expression of fatty acid-binding protein 4 (FABP4, human homolog of rodent aP2), a known PPAR γ target, was strikingly increased by a 24 hour treatment with PPAR γ agonists, resistin mRNA levels were unchanged (figure 8). In keeping with the apparent failure of PPAR γ agonists to modulate resistin expression in mononuclear cells, resistin mRNA was undetectable in subcutaneous abdominal adipocytes from subject 2.

Thus, PPAR γ did not appear to be a major controller of human resistin mRNA expression in adipocytes or monocytes, and increased resistin expression did not appear to explain the insulin resistance of human PPAR γ deficiency. While it is as yet not possible to quantitate human resistin protein accurately, adipocyte secreted proteins usually show a strong correlation between mRNA and protein levels (Montague et al., 1998), (Cianflone and Maslowska, 1995).

Acrp30/ adiponectin

Adipocyte complement-related protein of 30 kilodaltons (Acrp30, adiponectin or AdipoQ) is a fat-derived secreted protein present at significant concentrations in plasma (Weyer et al., 2001). Plasma Acrp30 levels are reduced in both obesity and type 2 diabetes in proportion to insulin sensitivity (Weyer et al., 2001). Caloric restriction in obese mice leads to weight loss and improved insulin sensitivity as well as an increase in Acrp30 levels (Berg et al., 2001). PPAR γ agonists increase Acrp30 levels in both mice and humans. Plasma Acrp30 levels in subjects 1 - 3 were within a very low range (0.17 -

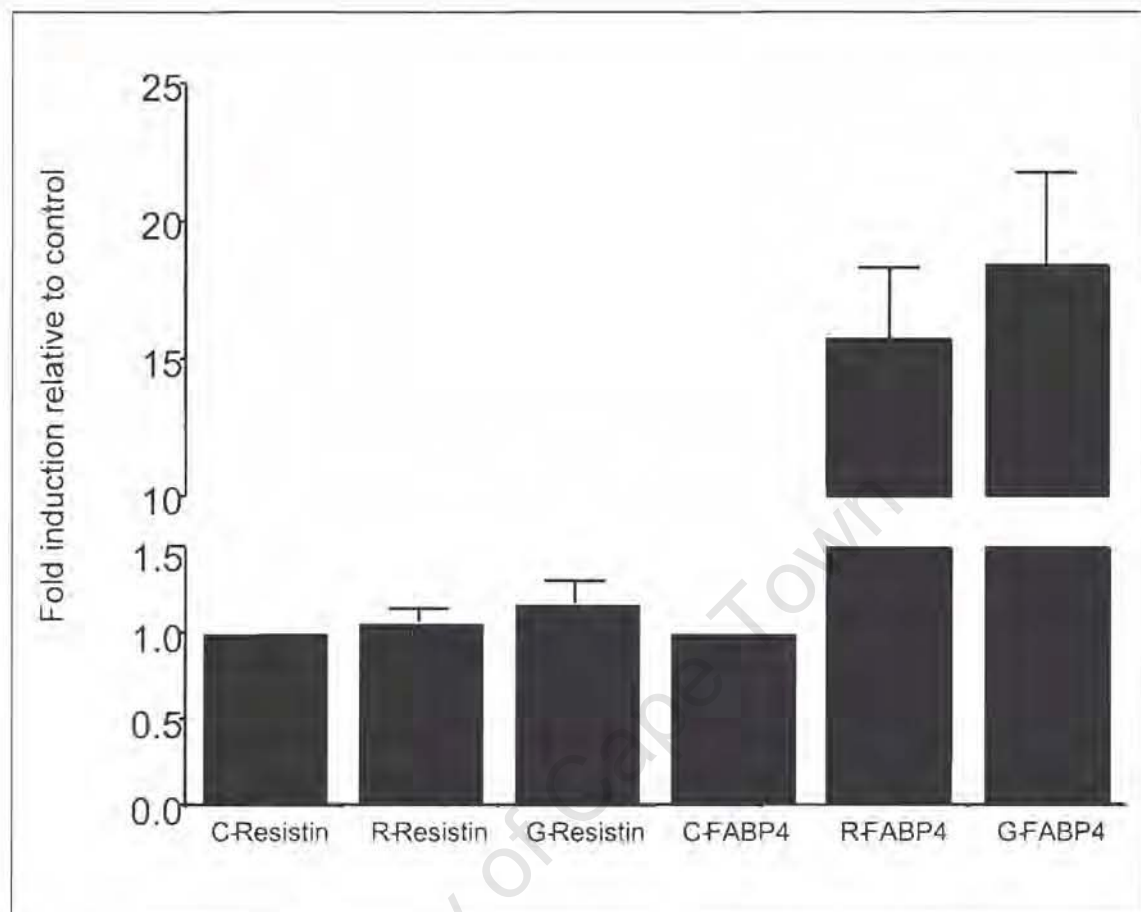


Figure 8

Effect of PPAR γ agonists on resistin and FABP4 mRNA levels in mononuclear cells. Human mononuclear cells were exposed to 100 nM rosiglitazone (R), 100 nM GI262570 (G), or vehicle (control (C)) for 24 hours. Resistin and FABP4 mRNA levels (means $\pm$ SE) were measured by real-time RT-PCR and normalised to the control samples (n=3).

0,71 U/ ml) compared with values measured at the same time in three unrelated normal control subjects (1.75 - 3.46 U/ ml). Mean values in these subjects (0.38 ± 0.05 U/ ml) were also significantly lower (5-fold) than the mean values (1.93 ± 0.6 U/ ml) measured in samples from eight unrelated patients with severe insulin resistance in whom no mutations in the PPAR γ coding region were detected (a subset of the SIR cohort) (Combs et al., 2002). This data provides strong evidence that physiological levels of PPAR γ expression modulate Acip30 levels in humans. It does, however, not prove that reduced Acip30 expression is responsible for the severe insulin resistance seen in carriers of dominant negative PPAR γ mutations.

Leptin/ IL-6/ TNF α

Plasma levels of leptin and interleukin-6 (IL-6) were unremarkable in all the subjects (table 3 and data not shown), and TNF α mRNA was barely detectable in isolated adipocytes from subject 2 (data not shown).

Ex vivo response of patient cells to PPAR γ agonists

PPAR γ is predominantly expressed in adipocytes, but is also known to be expressed at relatively high levels in mononuclear cells, where it is thought to modulate lipid trafficking. We sought to evaluate the *ex vivo* impact of a dominant negative PPAR γ mutation on transcriptional activity in peripheral blood mononuclear cells from subjects 1 and 2; and on gene expression during early pre-adipocyte differentiation in cells isolated from subject 2. Expression of fatty acid-binding protein-4 (FABP4) is known to be induced in

peripheral blood mononuclear cells (Pelton et al., 1999). Realtime RT-PCR measurements of FABP4 mRNA levels in mononuclear cells exposed to PPAR γ agonists (rosiglitazone or GI262570 (a non-thiazolidinedione PPAR γ agonist kindly provided by Dr T. Willson, Glaxo-Smithkline) demonstrated a substantially right-shifted dose response in P467L cells compared to the response seen in wild type control cells, providing a direct demonstration of PPAR γ agonist resistance (figure 9).

Expression profiling in P467L pre-adipocytes

Microarrays were used to explore gene expression in pre-adipocytes from subject 2. As the amount of RNA available for these studies was limited, the experiments were designed to look at early changes in gene expression with the expectation that differences observed at early time points were more likely to be directly attributable to the P467L mutation than those seen at late time points. PPAR γ is expressed in human pre-adipocytes and was detected in all the RNA samples.

1) General effects of PPAR γ mutation on gene expression patterns

Whereas mRNA levels of 86 and 66 genes were at least 2-fold¹ upregulated 6 and 48 hours post onset of differentiation in the control pre-adipocytes, considerably fewer genes were upregulated in cells carrying the P467L mutation (32 and 48 genes respectively). In a similar vein far more genes

¹ The number of false positives at a cut-off of 2-fold is < 0.5% (Yue et al., 2001).

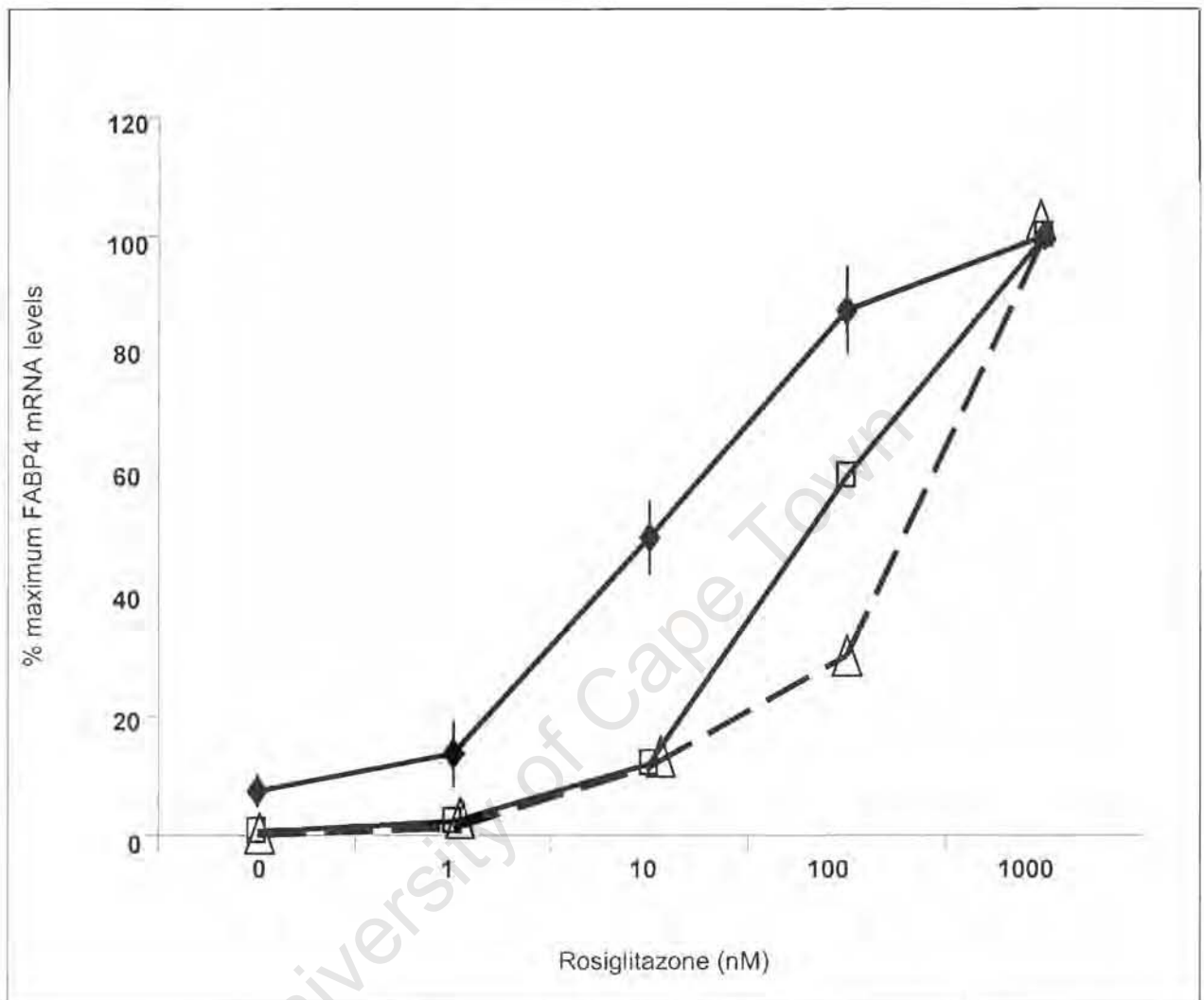


Figure 9

FABP4 mRNA expression in freshly isolated peripheral blood monocytes (PBMCs) from subject 1 (open squares) and subject 2 (open triangles). Control data (filled diamonds) represents the mean $\pm$ SE of FABP4 expression in PBMCs from four healthy individuals (BMI <28 kg/m²; age 28-60y). Results are expressed as a percentage of the maximum FABP4 mRNA levels obtained in each subject.

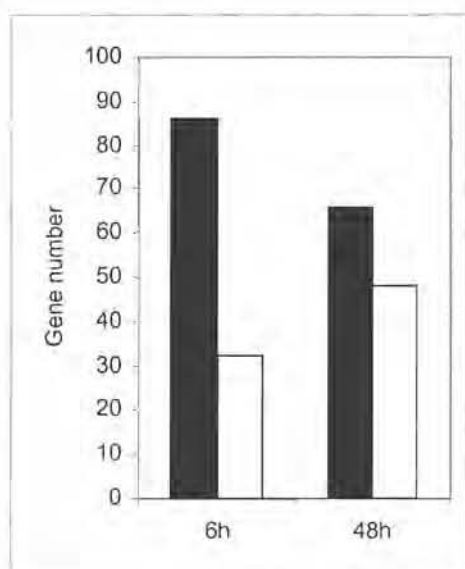
were at least 2-fold down regulated in the control cells than those carrying the P467L mutation (figure 10).

This data suggests that cells harbouring the P467L mutation were globally less responsive to the addition of differentiation cocktail than those of a wild type control. Intriguingly the differences in the number of genes up or down regulated at 48 hours were smaller than the differences noted at 6 hours, suggesting that changes in expression of several genes during pre-adipocyte differentiation may simply be retarded by the mutation rather than prevented altogether. This would be in keeping with the clinical phenotype of P467L carriers as although total adipose tissue mass was significantly reduced, subcutaneous abdominal adipose tissue morphology was normal, indicating that at least some of the adipocytes could differentiate.

2) Gene expression changes unaffected by presence of mutant PPAR γ .

The genes listed in table 4 were at least 2-fold up- or down-regulated at both time points examined in both sets of pre-adipocytes. They therefore firstly represent genes potentially involved in human *ex vivo* adipogenesis and secondly, genes whose transcription does not appear to be significantly altered by a dominant negative mutation in PPAR γ . Four of the genes listed are known to be involved in adipocyte differentiation in rodent cell lines (listed in bold) and served as valuable positive controls. The majority of these genes are involved in either cell cycle regulation or cytoskeleton and cell matrix remodelling. This is consistent with the suggestion by Ross et al that upon differentiation pre-adipocytes are transformed from fibroblast-like cells into

a,



b,

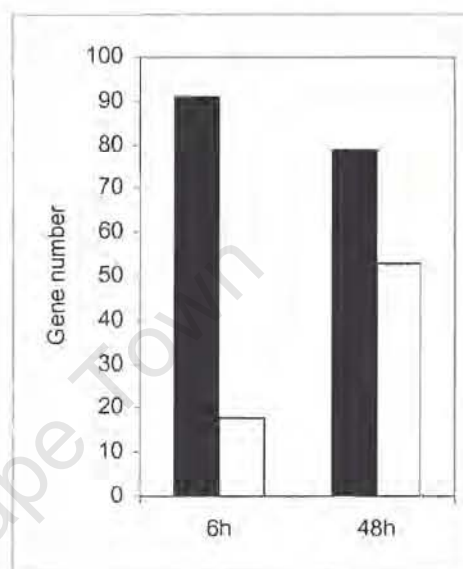


Figure 10

Overview of changes in gene expression 6- (T6) and 48 hours (T48) post addition of differentiation cocktail to confluent human pre-adipocytes. **a**, Number of genes > 2-fold up-regulated in P467L (white bars) and wild type (black bars) pre-adipocytes and **b**, number of genes > 2-fold down-regulated.

Table 4: Genes (A) up- and (B) down-regulated (>2-fold) in both the control and P467L subject. Genes listed in bold are known to change during adipogenesis.

A,

Gene name	Accession number	Function
Enactin (nidogen)	BI870489	Cytoskeleton
IL-1 receptor 1	M27492	Cytokine receptor
Phosphodiesterase 4D, c-AMP specific	U02882	Metabolism
TGF beta receptor III	AJ251961	Cell cycle regulator
Desmoglein 2	AU124258	Cell Adhesion
Delta sleep inducing peptide	AL525317	Unkown
Aldo-keto reductase family 1, member C1	BI759009	Metabolism (steroid)
Aldo-keto reductase family 1, member C4	BG196882	Metabolism (steroid)
Fatty acid CoA ligase, long-chain 2	BE889785	Metabolism (lipid)
Metallothionein	BF130769	Stress response (marker of differentiation)

B,

Keratin 18	BI160503	Cytoskeleton
BRCA1	AW804509	Cell cycle regulator
Cardiac ankyrin repeat protein	X83703	Nuclear protein
Connective tissue growth factor	AL547439	Matrix remodelling
Immediate early response 3	AI911657	Cell cycle
FGF2	NM_002006	Cell cycle
Hepatocellular carcinoma novel gene-3 protein	NM_016651	Cell cycle
Thrombospondin 1	X14787	Matrix/ Cell adhesion
Actin related protein 3, yeast (ARP3)	AL040940	Cytoskeleton
Actin, gamma 1	BI550936	Cytoskeleton

adipocytes, which have a very different morphology (Ross et al., 2002). Aldo-keto reductase C1 and C4, are part of the 3α -hydroxysteroid dehydrogenase family. This family of enzymes is believed to modulate the levels of active androgens, estrogens and progestins, all of which are known to influence adipose tissue metabolism. Fatty acid CoA ligase is an essential enzyme in triglyceride synthesis, a key metabolic function of adipocytes. In keeping with our findings, it was also induced early in 3T3L1 pre-adipocyte differentiation and its expression was not altered by thiazolidinediones (Gerhold et al., 2002).

3) Gene expression changes significantly altered by presence of mutant PPAR γ

Table 5A lists those genes whose expression was altered more than 2-fold at T6 and T48 in wild type cells whereas mRNA levels were altered $<1.4^2$ fold at both time points examined in cells harbouring the P467L PPAR γ mutation. This short list includes fatty acid binding protein-4 (FABP4) (Desvergne and Wahli, 1999), phospholipid transfer protein (PLTP) (Tu and Albers, 2001) and pyruvate dehydrogenase kinase-4 (PDK4) (Way et al., 2001b), all of which have been reported to be potential PPAR γ targets. In fact, PPAR γ was originally identified as a result of its ability to bind to an enhancer in the 5' flanking region of the aP2 gene (murine homologue of FABP4) (Tontonoz et al., 1994). The precise role of this protein in adipocyte biology is still to be clarified, but its expression is consistently and strikingly induced by PPAR γ agonists in pre-adipocytes (Nugent et al., 2001a) and other cell lines

Table 5: Genes whose expression was altered by the P467L PPAR γ mutation.

Genes listed in bold are genes whose expression was already known to be altered by PPAR γ agonists. **A**, Genes up- or down- (-) regulated in wild type pre-adipocytes (>2 fold) at 6- and 48 hours, BUT not up or down (>1.4 fold) at 6- or 48 hrs in pre-adipocytes harbouring the P467L mutation i.e. potential PPAR γ targets which increase or decrease in early pre-adipocyte differentiation.

B, Genes whose expression differed in PPAR γ wild type (WT) and P467L pre-adipocytes 6 hours post addition of differentiation cocktail (<1.4 fold change in P467L cells and >2 fold change in WT cells) whereas by 48 hours changes in expression in P467L cells were >1.4 fold.

A,

	Accession number	6hrs Control	48hrs Control	6hrs P467L	48hrs P467L	Function
FABP4	BG287253	8.2	8.1	1	1.1	Metabolism (lipid)
PDK 4	U54617	5	5.8	1	1.3	Metabolism (glucose)
Phospholipid transfer protein	AU139503	2.6	2.1	1	1.2	Metabolism (lipid)
Proline arginine-rich end leucine-rich repeat protein	BE836107	5.5	4.4	1.1	1.2	Unknown
Chemokine (C-C motif) receptor 1	D10925	2.3	2.3	1.0	1.0	Chemokine
Cytochrome b5	BG740758	2.6	2.3	1	1.2	
TNF α -induced protein 6	U23070	- 3.6	- 3.6	1	1	Cytokine
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (Id 1)	S78825	- 3	- 3	- 1.1	- 1.2	Cell cycle*
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (Id 2)	AI299309	- 2.7	- 2.7	- 1.1	- 1.2	Cell cycle*

B,

LPL	X54516	8.6	6.3	1.1	2.2	Metabolism (lipid)
ApoD	BG478155	4.7	4.1	1	1.9	? Metabolism
IGF2	S77035	5.4	4.2	1	2.0	Growth/ metabolism
IGF1	M11568	2.6	2.2	1.1	2.2	Growth? metabolism
MAO	M69226	8.7	5	1	2.6	Metabolism (catecholamine)
Endothelin receptor B	AL571798	2.6	2.7	1.1	1.9	Vascular tone
PLA2, group IIA	R64075	3.7	2.9	1.1	2.1	Metabolism (lipid)
Alcohol dehydrogenase 1C (gamma)	NM_00066 9	13	10	1	4.3	Metabolism (alcohols)
Alcohol dehydrogenase 1A (alpha)	M12271	18	14	1.1	3.3	Metabolism (alcohols)
Alcohol dehydrogenase 1B (beta)	BG535501	8.6	8.4	1.1	2.8	Metabolism (alcohols)
Matrix Gla protein	AW999947	15	15	1.1	17	Cell matrix
MMP 10	BE740153	4.7	3.4	1.1	1.7	Matrix remodeling
Somatostatin	BI713774	- 2.6	- 2.5	- 1.1	- 1.6	Metabolism

* Id1 and Id2 are known to be down-regulated during adipogenesis (Zilbepfarb et al., 2001).

expressing PPAR γ , such as human mononuclear cells (Savage et al., 2001). Therefore the absence of any change in expression of FABP4 in P467L cells provided very useful corroborative evidence for the experimental approach employed in this report.

The remaining genes listed in table 5B were also clearly differentially regulated in wild type and P467L cells 6 hours post onset of differentiation, but in contrast to the genes in table 5A, those in table 5B were induced - or repressed >1.4 fold by 48 hours in P467L cells. The fact that this list includes lipoprotein lipase (LPL), a gene whose expression is known to be altered by PPAR γ agonists, suggests that PPAR γ agonists may directly or indirectly influence expression of other genes listed in table 5B. The robustness of the microarray data was enhanced by the fact that the results represent consistent findings across two independently undertaken experiments at two early time points during differentiation. However, additional independent confirmatory experiments were undertaken using real-time RT-PCR for three different genes (figure 11). Phospholipase A₂ (PLA₂) is responsible for the release of arachidonic acid, an eicosanoid precursor, from cellular phospholipids. Two recent publications suggest that the cytosolic isoform may be a PPAR α target (Jiang et al., 2001),(Han et al., 2002). Our data indicates that the secreted isoform is expressed during human pre-adipocyte differentiation and may be a PPAR γ target. Nugent et al have also previously shown that arachidonic acid alters glucose uptake in 3T3L1 adipocytes (Nugent et al., 2001b) and that this effect appeared to be mediated, in part,

² 1.4 is considered to be the minimum detectable fold change for statistically significant differential

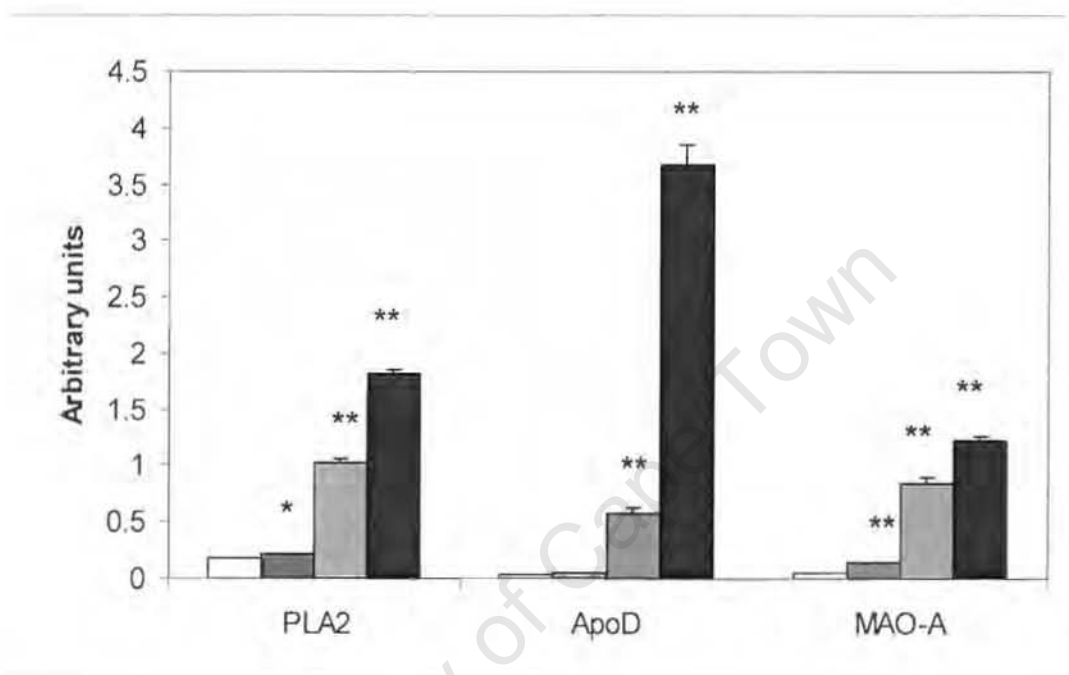


Figure 11

mRNA levels of PLA₂, ApoD and MAO-A, expressed as a fraction of GAPDH mRNA levels in confluent human pre-adipocytes (white bars) or in pre-adipocytes 6 hours (cross-hatched bars), 48 hours (hatched bars) and 15 days (black bars) post addition of differentiation cocktail. Data points represent means $\pm$ SE of three separate PCR reactions. * $p < 0.05$; ** $p < 0.005$.

by PPAR γ . PLA $_2$ may therefore have a role in increasing levels of arachidonic acid and its derivatives, some of which may modulate PPAR γ activity.

Apolipoprotein D (ApoD) is a ubiquitously expressed protein with an as yet ill-defined biological role. Its expression is induced by growth arrest in several cell types including fibroblasts and human astrocytoma cells (Do Carmo et al., 2002). Its induction early in human adipogenesis is in keeping with this data.

Monoamine oxidase-A (MAO-A) is a key enzyme in catecholamine metabolism. Although MAO-A mRNA levels did increase in the P467L cells, the change in expression was substantially reduced in these cells. Whilst the precise role of MAO-A in adipocytes is unclear it is plausible that this depot contributes to local catecholamine clearance from the circulation and may therefore modify catecholamine-induced effects on adipocytes, such as lipolysis. Together with the ability of PPAR γ agonists to reduce expression of the β 3-adrenergic receptor (Bakopanos and Silva, 2000), this is likely to substantially reduce catecholamine-stimulated lipolysis.

4) PDK4 in human adipocytes.

PDK4 is one of four recognised isoforms of PDK, and is expressed in several key insulin target tissues, namely muscle, fat, liver and pancreatic islets (Sugden et al., 2001). PDKs phosphorylate and inactivate the pyruvate dehydrogenase complex (PDC) which regulates oxidation of glycolytic pyruvate, an essential step in glucose oxidation (Sugden et al., 2001). In

expression (Yue et al., 2001).

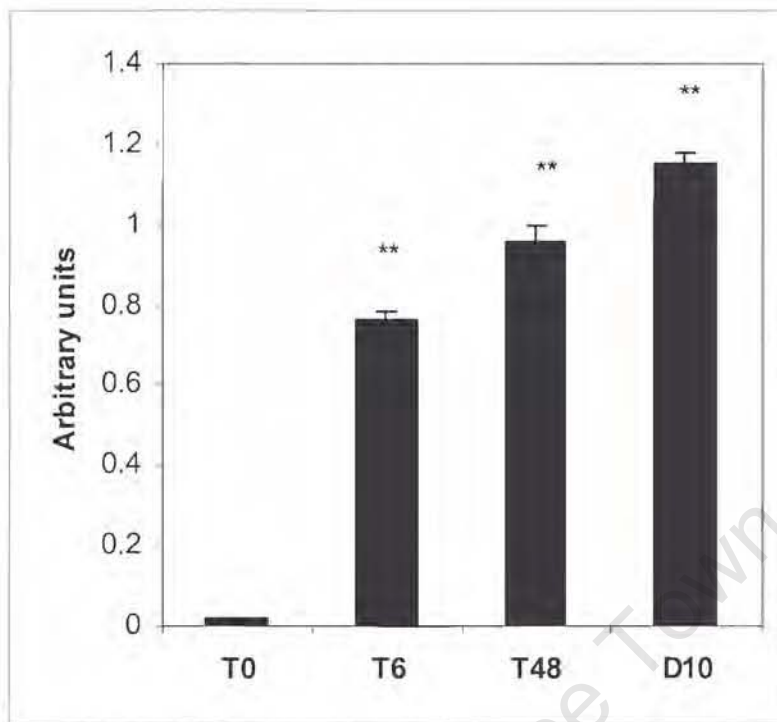
contrast to PDK 1-3, PDK4 is known to be substantially up-regulated by fasting and exercise, and to be down-regulated by feeding and insulin (Sugden et al., 2001). These changes in expression occur at both a mRNA and protein level, although on the whole the mRNA changes tend to be more dramatic as the mRNA transcript has a short half-life. Previous papers have suggested that fatty acids can also modulate its expression in muscle (Wu et al., 1999) and liver (Huang et al., 2002), and that this effect may, at least in part, be dependant upon PPAR α . One previous microarray based study reported induction of PDK4 expression in adipose tissue and repression in muscle of mice treated with PPAR γ agonists (Way et al., 2001b). The authors suggested that the fall in muscle PDK4 expression might have contributed to the reduction in blood glucose levels seen in the mice. They did not discuss possible mechanisms for the observed changes in PDK4 mRNA levels.

Whereas PDK4 mRNA levels were induced at least 5-fold within 6 hours of onset of differentiation in wild type human pre-adipocytes, they did not appear to change at all in the P467L cells (table 5A). The change in PDK4 mRNA levels early in human pre-adipocyte differentiation was confirmed in an independently derived set of human pre-adipocytes where real-time RT-PCR was used to quantify PDK4 mRNA levels. The rise in PDK4 expression in this set of cells was again substantial and rapid (figure 12a). Additional experiments were done to confirm that PDK4 mRNA levels remain elevated at late time points in adipocyte differentiation. Furthermore, PDK4 expression was increased by the addition of rosiglitazone, a PPAR γ agonist (figure 12b). This may simply reflect enhanced -/ accelerated adipogenesis in the presence

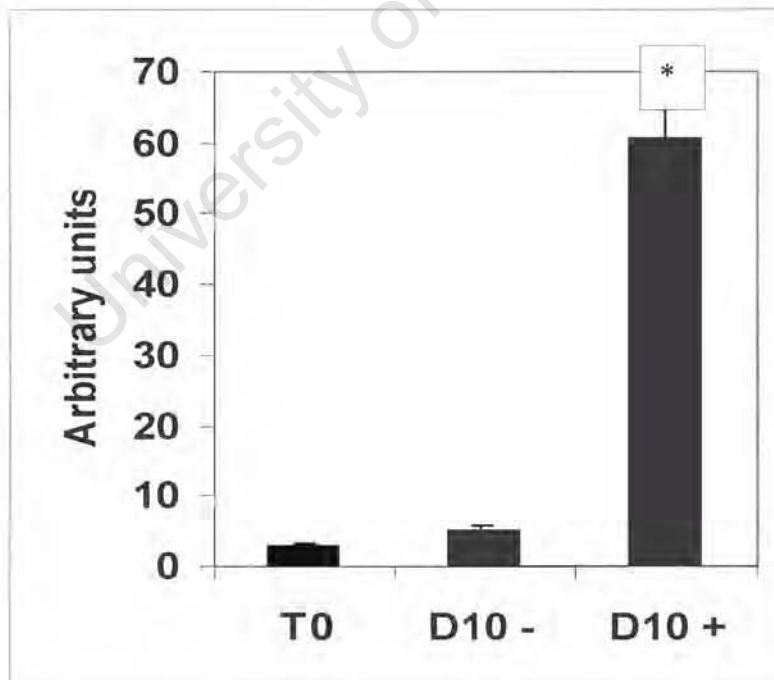
of PPAR γ agonists. In the light of the dramatic increases in PDK4 expression in differentiating pre-adipocytes and its persistent expression in differentiated pre-adipocytes we wondered how PDK4 would be regulated in adipose tissue *in vivo*. In keeping with the pattern seen in other metabolically active tissues, PDK4 mRNA expression was significantly induced by a 24 hour fast in mouse adipose tissue and suppressed by re-feeding (figure 12c). This presumably reflects the metabolic energy requirements of the adipocytes themselves, so that during fasting conditions, as well as releasing free fatty acids into the bloodstream for utilization by other tissues such as liver and muscle, adipocytes oxidise fatty acids in order to meet their own energy requirements. The precise reason for the induction of PDK4 expression during human pre-adipocyte differentiation is unclear although it probably contributes to the conversion of a relatively metabolically inert cell type to a key insulin responsive cell. Muoio et al (Muoio et al., 2002) recently reported similarly dramatic increases in PDK4 expression in human myoblasts undergoing differentiation into myotubes. They also documented significant augmentation of this response by PPAR α agonists.

As far as we are aware this is one of the first studies in which primary human cell cultures harbouring a mutation in a transcription factor have been used to explore gene expression. The major advantage of this approach is the fact that it allows one to assess the molecular impact of a mutant protein expressed at endogenous levels, a situation rarely achieved by exogenous expression systems. This is particularly pertinent to dominant negative transcription factors where massive over-expression of the abnormal protein

a,



b,



c,

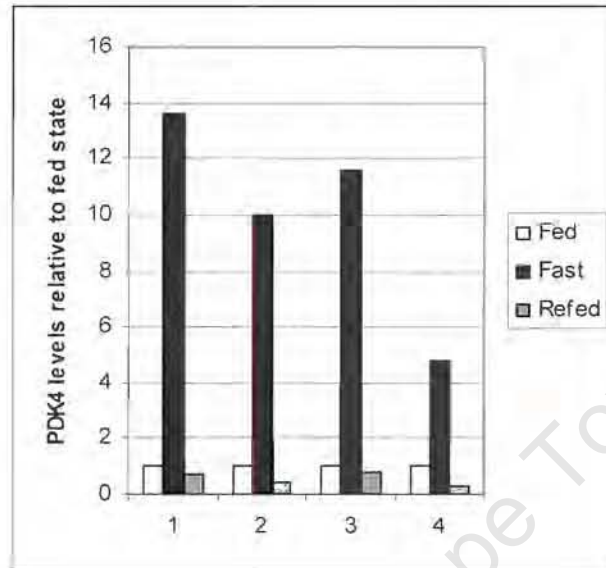


Figure 12

a, PDK4 mRNA levels expressed as a fraction of GAPDH mRNA levels in human pre-adipocytes during differentiation. **b**, PDK4 mRNA levels 10 days after onset of differentiation in cells treated with - (D10 +) or without (D10 -) 100nM rosiglitazone (RSG). **c**, PDK4 mRNA expression in murine WAT ($n = 4$ animals) in the fed, fasted (24hr) and refed (24 hr) state. Data points represent mean $\pm$ SE of two or three independent RT-PCR determinations. * $p < 0.05$; ** $p < 0.005$

raises the possibility that observed effects have more to do with the level of over-expression than *in vivo* biology. Another advantage is the fact that all the cells present are likely to harbour the mutation whereas exogenous expression systems tend to induce over-expression in a subset of the total cell population (the one exception being retroviral expression systems, which are not readily usable in human primary cell cultures).

However, the following caveats warrant consideration when interpreting the data herein:

a) As the cells were isolated from a single carrier of the mutation, additional undetected genetic variants may be present in cells carrying the mutation of interest. They too could conceivably influence gene expression. Given this potential confounding factor we applied particularly tight criteria when extracting information from the data sets, which may of course lead to the loss of some significant information. We were also careful to replicate changes in genes of particular interest and suggest that this is necessary for any subsequent users of this information.

b) The expression profile of cells harbouring the P467L mutation need not be entirely representative of all dominant negative PPAR γ mutations. Although our own work on the molecular characteristics of the P467L and V290M mutants suggests that they behave in a very similar fashion *in vitro* (Barroso et al., 1999a) and the phenotypes of carriers of the mutations are very similar (Diabetes *in press*), it is likely that subtle differences do exist and this may be

reflected in downstream gene expression. Again, this possibility highlights the need for confirmatory experiments.

c) We were unable to provide direct confirmation of the effects on expression in mutant cells by an independent technique as all of the limited material was used for microarray analysis. Nor were we able to conclusively examine the effects of the mutant on cellular differentiation *in vitro* on account of the fact that, unlike 3T3L1 rodent pre-adipocytes, the *in vitro* response of human pre-adipocytes to differentiation cocktail is both much less dramatic than that seen in rodent pre-adipocytes and is also very variable, necessitating multiple experiments in order to reach definitive conclusions.

In conclusion, expression profiling in pre-adipocytes with or without a dominant negative mutation in PPAR γ 1) suggested that changes in gene expression *in toto* during adipogenesis were retarded by the presence of the mutant; 2) identified a number of genes whose expression was apparently not modified by PPAR γ during human pre-adipocyte differentiation; 3) revealed several potentially novel PPAR γ targets. Of particular interest from a metabolic perspective was the emergence of PDK4 as a gene whose expression is modified by PPARs in fat, muscle, liver and pancreatic β -cells. Whilst PDK4 enzyme activity may be influenced allosterically, mRNA levels change dramatically during exercise and fed/ fast transitions. We propose that PPARs are involved in facilitating these rapid changes in gene expression and as such are likely to be important players in modulating "metabolic flexibility" in terms of macronutrient fuel selection.

Clinical response to PPAR γ agonist therapy in subjects 2 and 3

As the P467L and V290M mutant receptors retained some residual ability to respond to PPAR γ agonists *in vitro* (Barroso et al., 1999) and as monocytes harbouring the P467L mutation also demonstrated some *ex vivo* responsiveness to rosiglitazone (figure 9) two subjects were treated with rosiglitazone 4mg twice daily for six months. Subject 1 was considered unfit for such a trial in view of her aortic stenosis and liver disease (thiazolidinediones have been associated with fluid retention and their impact on hepatocellular carcinoma is unknown). Neither of the treated subjects experienced any adverse effects, in particular liver function tests remained essentially unchanged and neither developed any peripheral oedema.

Fat mass increased in all body regions in both subjects (table 3). As changes in fat mass were assessed by DXA scanning alone, no comments can be made regarding any possible changes in the visceral to total abdominal fat ratio. Plasma leptin increased in both patients in keeping with their increased fat mass. The metabolic impact of rosiglitazone (table 3) was very striking in subject 2, with insulin sensitivity and glycemic control being normalised. In contrast, subject 3 remained severely insulin resistant and showed little change in glycated hemoglobin levels. Subject 2 also experienced a much greater percentage increase in serum Acrp30 levels in response to rosiglitazone than did subject 3 (100% vs. 41%). The exact reason for the differences in efficacy of rosiglitazone in subjects 2 and 3 is not known.

However, the following may at least in part have contributed to this observation:

- *In vitro* studies of the dominant negative behaviour of the two mutant receptors appear to mirror the differences in responsiveness to rosiglitazone. When co-transfected with the wild type receptor, the V290M mutant receptor inhibited transcriptional activity of a reporter gene to a greater extent than did the P467L mutant (figure 13). These differences were statistically significant, although extrapolating such *in vitro* data to the *in vivo* situation always necessitates caution.
- Compliance - whilst rosiglitazone was detectable in serum from both subjects during therapy, this does not prove long-term compliance. The fact that subject 3 experienced an increase in fat mass, leptin and Acrp30 levels attests to her taking the medication but once again is not definitive evidence of compliance.
- Differences in pharmacokinetics in subjects 2 and 3 were not explored, but may also be part of the explanation.

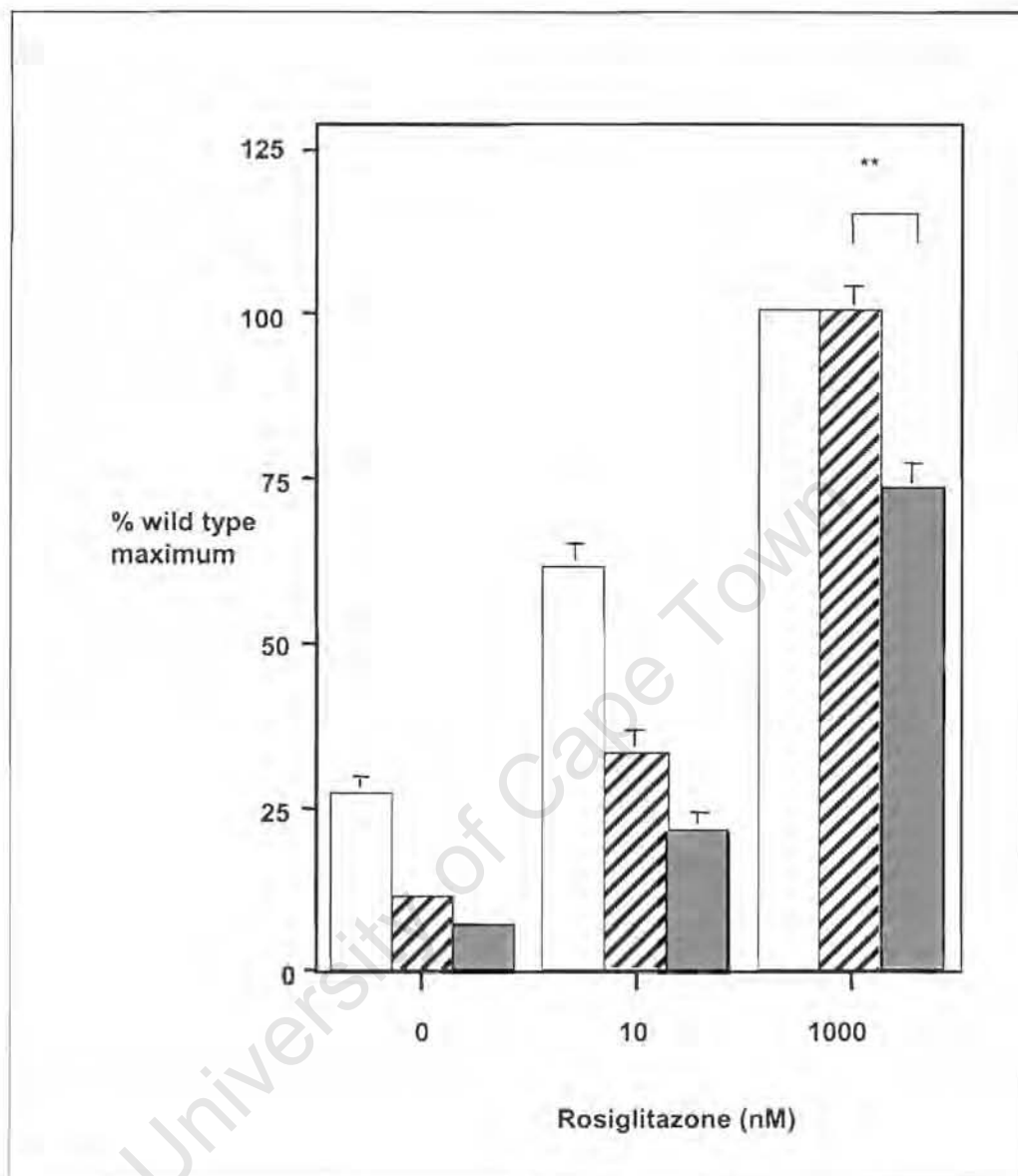


Figure 13

Dominant-negative activity of mutant PPAR γ . 293EBNA cells were transfected with 500ng of reporter construct, (PPARE)3TKLUC, 100ng of wild type (WT) receptor plus an equal amount of either WT (white bars) or mutant (P467L (hatched bars); V290M (grey bars)) expression vectors, and 100ng of control plasmid Bos- β -gal. The transcriptional responses mediated by either 100ng or 200ng of WT receptor at each concentration of ligand did not differ significantly (data not shown). Results are expressed as a percentage of the WT maximum and represent the mean $\pm$ SE of 8 independent experiments each performed in triplicate. ** $p < 0.001$.

Conclusions

Humans with dominant negative mutations in PPAR γ represent a novel subtype of inherited partial lipodystrophy. The paucity of limb and gluteal fat resembles that seen in familial partial lipodystrophy (FPLD, Dunnigan-Kobberling syndrome) and HIV-associated lipodystrophy, but differs from these syndromes in the preservation of normal facial and abdominal (subcutaneous and visceral) fat depots (Jackson et al., 1997), (Carr et al., 1999). The lack of excess facial fat distinguishes people with dominant negative PPAR γ mutations from those with FPLD but also renders recognition of the syndrome more difficult. In fact the lipodystrophy was not initially recognised in our subjects (Barroso et al., 1999). Consistent with our observations two further patients with PPAR γ mutations in the ligand binding domain have recently been identified. Agarwal and Garg reported a heterozygous R425C mutation in PPAR γ in a 64-year-old non-Hispanic white female with limb and facial lipoatrophy, early onset type 2 diabetes (formal measurements of insulin sensitivity were not reported), dyslipidemia and hypertension (Agarwal and Garg, 2002). Although they have still to report on the *in vitro* characteristics of this mutant, studies undertaken in Cambridge by Dr M Agostini in Professor VKK Chatterjee's laboratory suggest that this mutant does display dominant negative behaviour. Hegele et al then described a F388L mutation in a Canadian kindred with partial lipodystrophy, type 2 diabetes and dyslipidemia (Hegele et al., 2002). In contrast to other recently identified causes of inherited lipodystrophy (Shackleton et al., 2000), (Magre et al., 2001) in which the genotype /phenotype relationships are, as

yet, poorly understood, PPAR γ is believed to be "the master regulator of adipogenesis" (Rosen and Spiegelman, 2001). The finding of lipodystrophy in association with dominant negative germline mutations in human PPAR γ is entirely consistent with a key role for this molecule in human adipogenesis. Furthermore microarray expression profiling in pre-adipocytes with or without the P467L dominant negative mutation in PPAR γ suggested that changes in gene expression *in toto* during adipogenesis were retarded by the presence of the mutant and revealed several potentially important PPAR γ targets involved in this process.

One particularly intriguing aspect of the lipodystrophy seen in these subjects is the molecular mechanism responsible for the selective loss of subcutaneous AT in the limb and gluteal depots. The fact that PPAR γ agonists increase subcutaneous AT mass and reduce visceral adipose tissue in people with diabetes is in part consistent with the observed pattern of lipodystrophy, but cannot account for the preservation of subcutaneous abdominal adipose tissue.

The presence of severe insulin resistance from early childhood in carriers of dominant negative PPAR γ mutations highlighted the key role of this molecule in the control of insulin action and the studies undertaken in these patients provided some possible insights into how interference with PPAR γ signalling might result in insulin resistance. Firstly, they have unequivocally demonstrated that these subjects have partial lipodystrophy with a total fat mass much lower than predicted by their BMI. Lipodystrophy, both of the

partial and generalised types, is consistently associated with insulin resistance in animals and man, and thus is likely to contribute to the insulin resistance seen in this disorder (Reitman et al., 2000). In addition to diminished fat mass, physiological studies provided evidence for a major functional disturbance of the residual adipose tissue. In particular the abdominal adipose tissue of subject 2 had both a reduced capacity to increase lipolysis in response to fasting and an inability to suppress fatty acid mobilization. In the postprandial state this inability to "trap and store" NEFAs is likely to expose skeletal muscle and liver to NEFAs in an unregulated manner, a phenomenon which is known to impair insulin sensitivity in these tissues (Shulman, 2000), (Boden, 1997). The hepatic steatosis found in these subjects probably reflects this phenomenon. The absence of a marked elevation of intramyocellular triglyceride (IMTG) was however surprising. It is possible that the accumulation of IMTG may itself be, at least in part, dependent on PPAR γ . While there were no striking alterations in the protein or mRNA levels of adipokines such as leptin, IL-6, resistin or TNF α in serum or adipocytes, serum Acrp30 levels were markedly reduced in all three subjects. This adipocyte-derived circulating protein is capable of potentiating insulin's actions in skeletal muscle and hepatocytes (Yamauchi et al., 2001a) (Berg et al., 2001) and has been shown to be highly responsive to PPAR γ agonists (Maeda et al., 2001). Whether the reduced level seen in the subjects of this study is merely an *in vivo* marker of reduced PPAR γ action or whether low levels of the protein actively contribute to the causation of insulin resistance is yet to be determined.

In addition to insulin resistance, carriers of dominant negative PPAR γ mutations manifest early-onset hypertension, dyslipidemia and hepatic steatosis. Whilst it is conceivable that these features may be secondary to severe insulin resistance *per se* (Ginsberg, 2000), they have not been previously described as co-existing in patients with other forms of hereditary severe insulin resistance nor are they common in monogenic murine models of severe insulin resistance (Kadowaki, 2000). Carriers of dominant negative PPAR γ mutations would thus appear to represent a unique human monogenic model of the metabolic syndrome. As this cluster of metabolic abnormalities is frequently associated with the development of macrovascular disease the limited extent of detectable atherosclerotic disease in subject 1 was particularly surprising. Whilst the role of PPAR γ in modulating lipid fluxes in macrophages within the vasculature is complex (Rosen and Spiegelman, 2001), the right-shifted dose response of FABP4 mRNA levels observed in monocytes may hinder foam cell formation, as was documented in aP2 knockout mice (Makowski et al., 2001).

In conclusion, it is becoming apparent that dominant negative PPAR γ mutations in humans represent a new member of a group of conditions involving an inherited impairment of nuclear receptor function (e.g. androgen insensitivity syndrome, estrogen resistance and hereditary vitamin D resistant rickets). The closest analogy is with thyroid hormone resistance where heterozygous dominant negative mutations in thyroid receptor (beta isoform) are associated with pituitary and peripheral refractoriness to thyroid hormone action. Although the physiological ligand for PPAR γ remains to be identified,

this study has demonstrated *ex vivo* resistance to a pharmacological agonist in both mononuclear cells and pre-adipocytes harbouring the dominant negative PPAR γ mutation and therefore we believe that PPAR γ ligand resistance is almost certainly present *in vivo*. The fact that humans with dominant negative mutations in PPAR γ appear to represent a monogenic model of the metabolic syndrome in association with a stereotyped form of partial lipodystrophy is in keeping with the presence of lipodystrophy and insulin resistance noted by Yamauchi et al (Yamauchi et al., 2001b) in heterozygous knockout mice treated with retinoid X-receptor (RXR) antagonists. Although PPAR γ haploinsufficiency paradoxically improved insulin sensitivity in both aging mice (Miles et al., 2000) and in mice fed a high fat diet (Kubota et al., 1999), the dominant negative mutations identified in our human subjects induce a more severe impairment of PPAR γ function than that seen in PPAR γ haploinsufficiency. The ensuing combination of lipodystrophy of limb and gluteal subcutaneous fat with a major functional disturbance of the remaining adipose tissue are likely to be major contributors to the observed metabolic dysfunction. This may be compounded by the low circulating Acrp30 levels found in these patients. Details of the specific molecular abnormalities responsible for the adipose tissue dysregulation seen in carriers of dominant negative PPAR γ mutations remain to be identified although several hypotheses exist. For example, Masuzaki et al (Masuzaki et al., 2001) recently described a murine model of syndrome X in mice selectively over-expressing 11- β -hydroxysteroid dehydrogenase-1 (HSD-1) in adipocytes. As HSD-1 expression may be indirectly altered by PPAR γ agonists (Berger et al., 2001) it is conceivable that elevated HSD-1 levels in

the adipocytes of our patients could be contributing to the observed phenotype. Finally, reflecting the *ex vivo* ability of the mutant receptors to respond, at least partially, to pharmacological ligands, thiazolidinedione therapy can result in substantial reversal of pathophysiological disturbances together with clinically meaningful therapeutic benefits in some patients with this disease.

University of Cape Town

Chapter 3

Digenic inheritance of severe insulin resistance in a human kindred.

A: Identification and characterization of a novel PPAR γ frameshift/premature stop mutant in a human kindred (Kindred A).

Introduction

In ongoing studies of the PPAR γ gene in subjects in the SIR cohort another individual was found to harbour a different mutation in PPAR γ . Unlike the mutations described in chapter 2, this mutation ((A⁵⁵³ AAAT)fs.185(stop 186) – denoted hereafter as FS PPAR γ) results in a frameshift and premature stop codon within the DNA-binding domain of PPAR γ . It was therefore predicted to induce truncation of the receptor within the DNA-binding domain. Detailed *in vitro* characterization of this mutant was largely undertaken in Professor V.K.K. Chatterjee's laboratory in Cambridge, UK by Drs M. Agostini and M. Gurnell. Their work is summarised below.

***In vitro* characterization of the PPAR γ frameshift mutant**

PPAR γ is a ligand-inducible transcription factor that regulates target gene transcription as a heterodimer with the retinoid X receptor (RXR) (see "Introduction" for additional details)(Rosen and Spiegelman, 2001). It exhibits

a modular structure consisting of a central DNA-binding domain, an amino-terminal activation domain, and a carboxy-terminal ligand-binding domain (figure 1). The frameshift premature stop mutation leads to a mutant receptor which is truncated within the second zinc finger of the DNA-binding domain – a region common to both the $\gamma 1$ and $\gamma 2$ isoforms of the receptor (figure 1), and which is critical in mediating receptor interaction with PPAR-specific response elements (PPAREs) in target gene promoters. Accordingly, the ability of the PPAR γ mutants to bind DNA as heterodimers with RXR was examined in an electrophoretic mobility shift assay as described previously (Collingwood et al., 1994). Unlike their wild type (WT) counterparts, neither FS PPAR $\gamma 1$ (FS $\gamma 1$) nor FS PPAR $\gamma 2$ (FS $\gamma 2$) formed heterodimeric complexes when coincubated with a radiolabelled probe encoding the acyl-CoA oxidase PPARE (figure 2). Consistent with this and in contrast to wild type constructs, neither mutant mediated transactivation when cotransfected with a reporter gene containing a PPARE and increasing concentrations of the thiazolidinedione, rosiglitazone (figure 3a). Moreover, unlike the previously reported naturally occurring missense PPAR γ mutants (P467L and V290M)(Barroso et al., 1999), the truncated mutants did not exhibit dominant negative activity when co-expressed with wild type receptor and a TK-luciferase reporter construct (figure 3b). In short, this work suggested that the PPAR γ FS variant effectively rendered carriers thereof PPAR γ haploinsufficient, although at this stage the possibility that the truncated mutant PPAR γ protein has effects beyond those of a null allele cannot be entirely excluded.

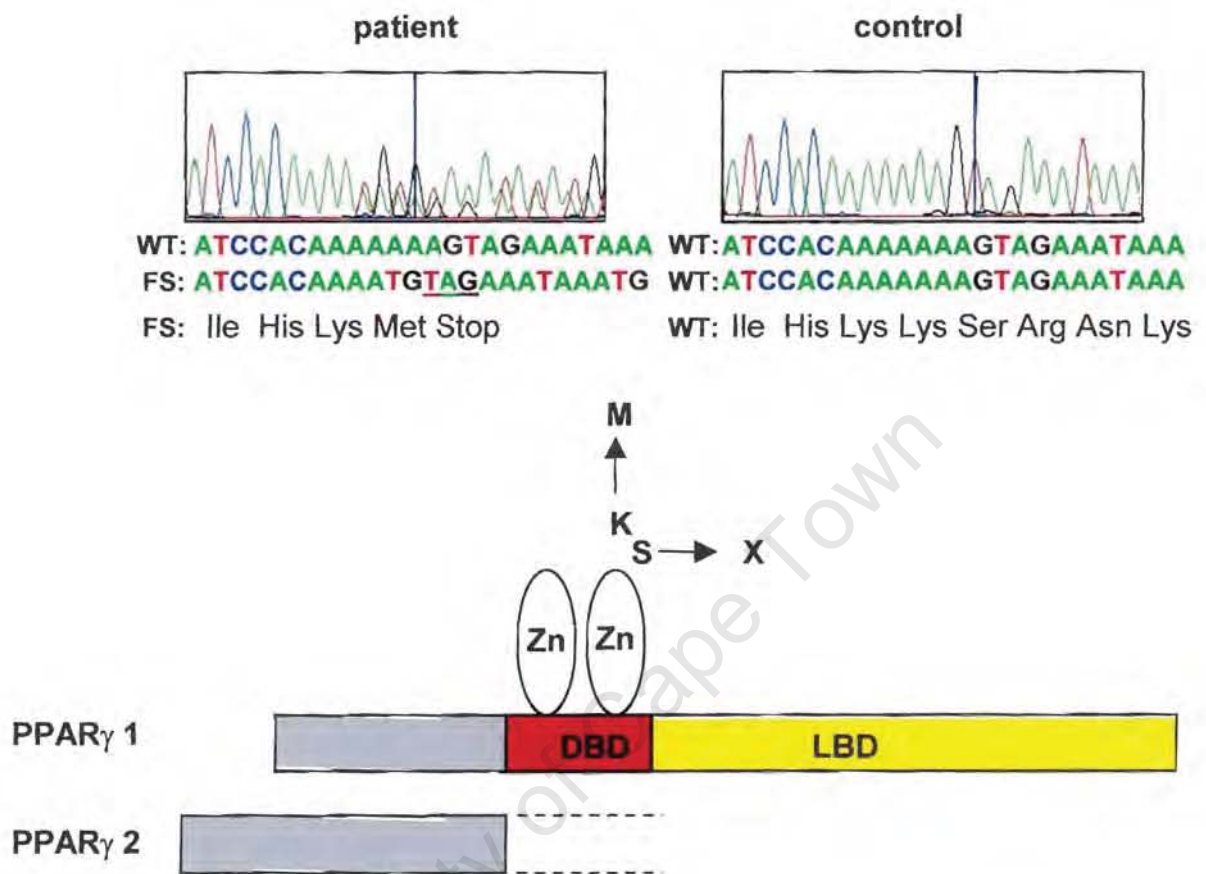


Figure 1

Heterozygous frameshift PPAR_γ mutation detected in kindred A. The frameshift leads to premature truncation of the receptor within the second zinc-finger of the DNA-binding domain (DBD). M – methionine; K – lysine; S – serine; X – stop; LBD – ligand-binding domain.

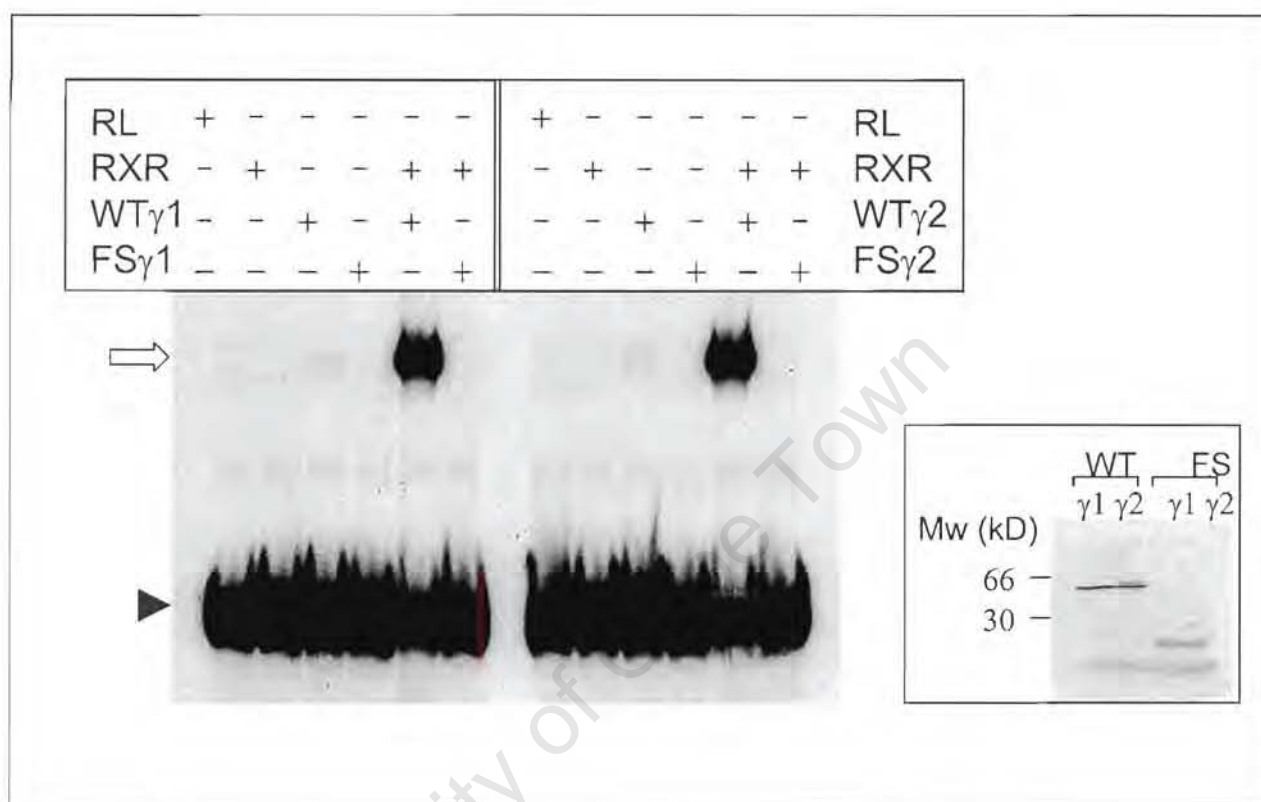


Figure 2

FS PPAR γ f ails to bind to DNA with its heterodimeric partner RXR. Using an electrophoretic mobility supershift assay, *in vitro* translated WT PPAR γ 1 (WT γ 1), WT PPAR γ 2 (WT γ 2), FS PPAR γ 1 (FS γ 1) or FS PPAR γ 2 (FS γ 2) and RXR were coincubated with oligonucleotide duplexes encoding the acyl-CoA oxidase PPARE. Complexes were resolved by PAGE. The open arrow indicates the location of the PPAR γ -RXR heterodimer, whilst the solid arrowhead denotes free unbound probe. Inset, ^{35}S -labelled *in vitro* translated WT and FS mutant PPAR γ 1 and PPAR γ 2 proteins. RL, reticulocyte lysate; Mw, molecular weight.

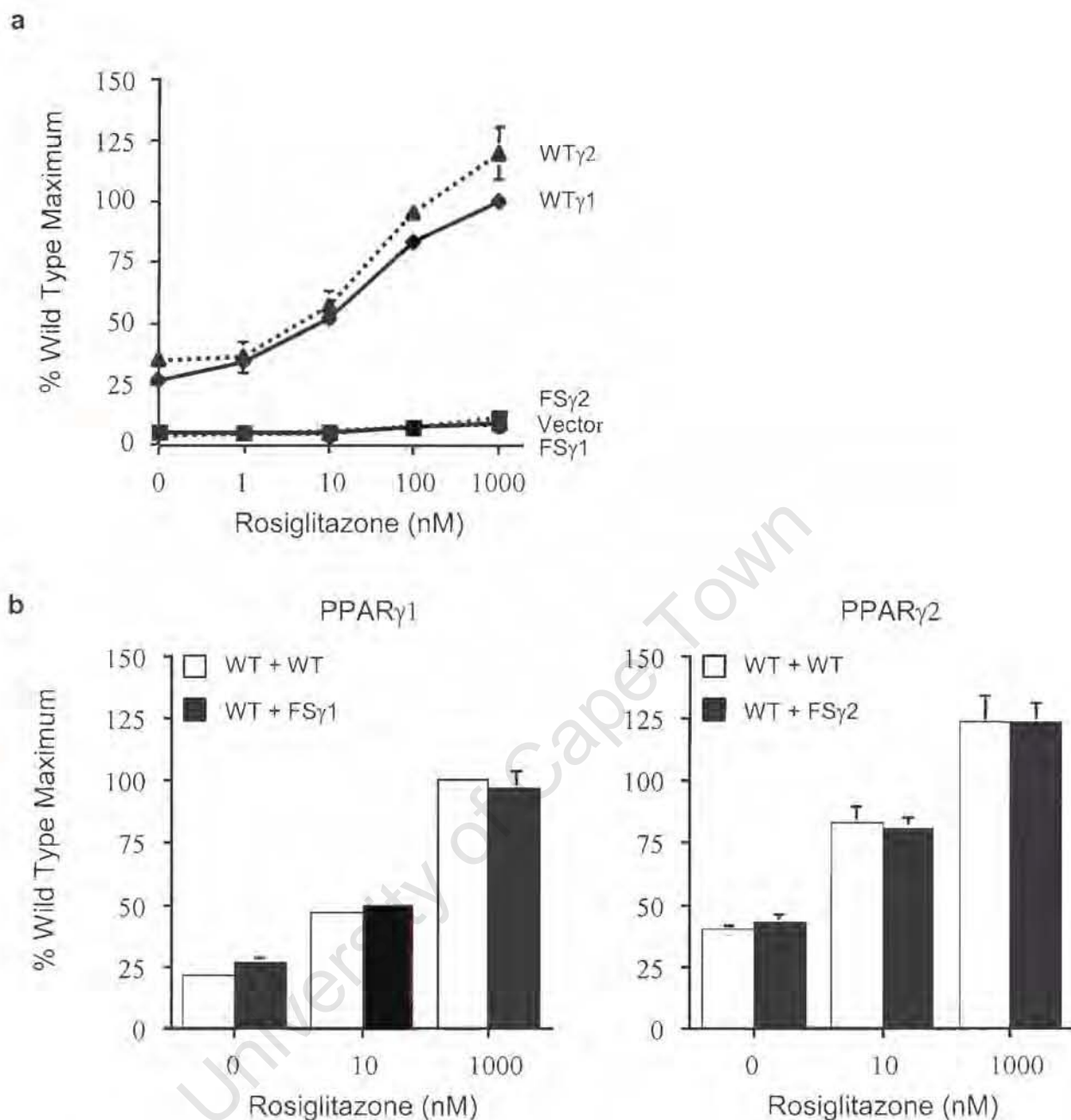


Figure 3

a, The FS PPAR γ mutants are transcriptionally silent in the context of both the γ 1 and γ 2 isoforms. 293EBNA cells were transfected with WT γ 1, WT γ 2, FS γ 1, FS γ 2 or empty expression vectors together with a reporter gene (PPAR γ)₃TKLUC, in the presence of increasing concentrations of the thiazolidinedione (rosiglitazone). Results are expressed as a percentage of the maximum activation obtained with WT γ 1. b, The FS PPAR γ mutants do not exhibit dominant-negative activity when co-expressed with their WT counterparts. 293EBNA cells were transfected with 100ng of WT plus an equal amount of either WT or FS mutant expression vectors, together with the same reporter construct as in a. Results are expressed as a percentage of the maximum activation obtained with WT γ 1. The transcriptional responses to 100ng or 200ng of WT receptor are identical (data not shown).

Proband's (subject IIIiv in figure 4) Case History:

The patient, a 21-year-old female, first came to medical attention at the age of 14 years with secondary amenorrhoea, hirsutism and acanthosis nigricans. Abdominal ultrasound revealed large bilateral ovarian cysts. Although not overtly diabetic she had impaired glucose tolerance (IGT) and striking hyperinsulinemia with serum insulin values as high as 9730 pmol/L after 75 grams of oral glucose. At the time of referral she remained oligo-/amenorrhoeic, hirsute and glucose intolerant. By this time she was also hypertensive (BP 150/110). On examination she had a BMI of 31 kg/m² with no apparent lipodystrophy. She had nuchal and bilateral axillary acanthosis nigricans, as well as facial hirsutism. There was no evidence of micro- or macrovascular disease. Biochemical tests demonstrated striking fasting hyperinsulinemia (346 pmol/L; N < 80 pmol/L), significantly elevated serum triglycerides (10 mmol/L) with a normal HDL fraction (1.04 mmol/L; this may relate to the use of a combined oral contraceptive (Dianette)) and mildly elevated transaminases in keeping with steatohepatitis.

Co-segregation studies and clinical characteristics

All of the proband's first degree relatives consented to further genetic and clinical assessment. Six additional carriers of the PPAR γ FS variant were identified (subjects Ii, IIii-iv, IIvi and IIIiii in figure 4). The proband's father, two of her aunts and her grandmother, none of whom had any features of insulin resistance, were wild type at the PPAR γ locus. Whereas five of the seven

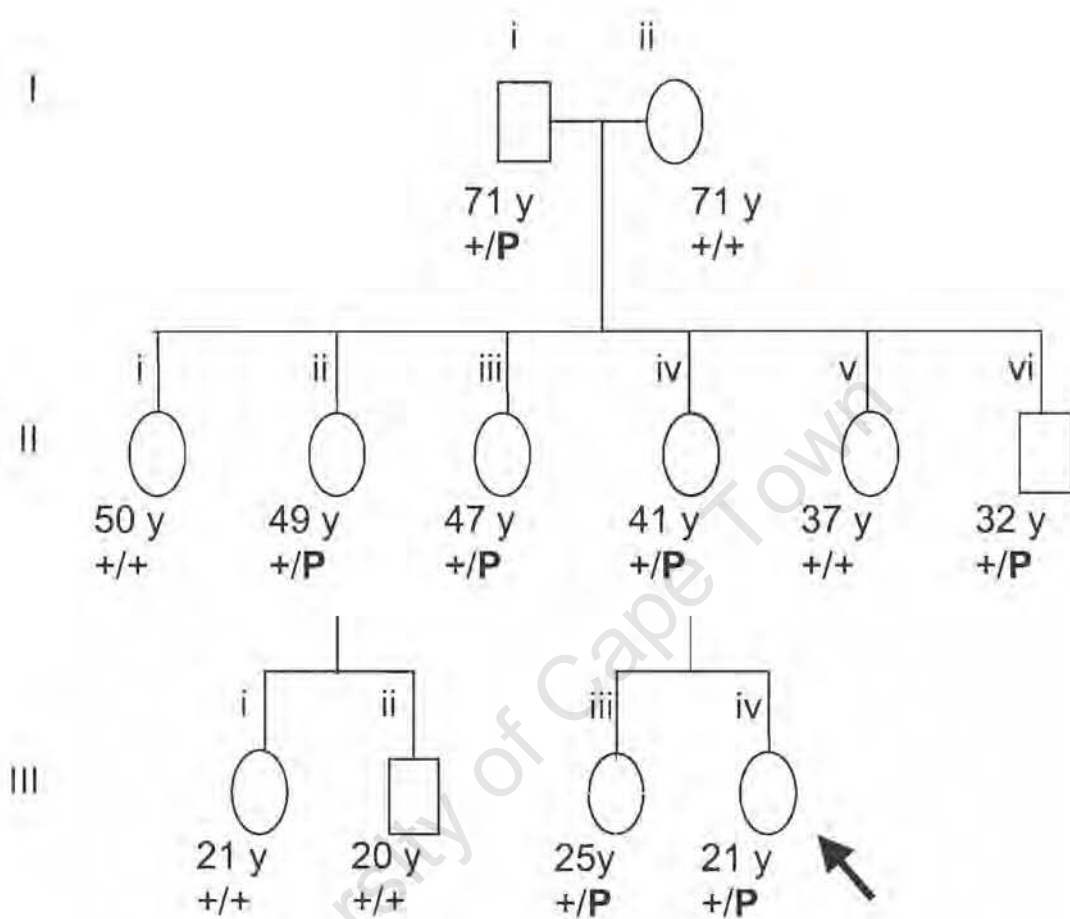


Figure 4

Kindred A pedigree. The age and genotype (+, wild type; P, FS PPAR γ mutant) of members is indicated.

subjects carrying the PPAR γ FS mutation had both acanthosis nigricans and strikingly elevated fasting insulin levels, subjects Ii and IIvi were normo-insulinemic. Subject Ii, the proband's grandfather, was found to be diabetic at the time of screening for this study. He was lean, with a BMI of 24.2 kg/m² and did not have acanthosis nigricans. Subject IIvi was also lean (BMI 25.8 kg/m²) without any features of severe insulin resistance.

In summary, five of seven carriers of the PPAR γ FS variant and no family members without the PPAR γ mutation appeared to be severely insulin resistant. Of the two apparently insulin sensitive carriers, one was diabetic, making interpretation of his insulin levels difficult and the other reported regular physical activity in the form of long-distance running, a state known to improve insulin sensitivity. However, this somewhat tenuous explanation coupled with the knowledge that PPAR γ haplo-insufficient mice were known to be insulin sensitive (Kubota et al., 1999), (Miles et al., 2000) even when given a high fat diet prompted ongoing candidate gene studies in this large kindred. The subsequent identification of a second frameshift/ premature stop mutation in an unlinked gene involved in glycogen metabolism (PPP1R3A), its co-segregation with insulin resistance and *in vitro* characterization are described in the next section of this chapter.

B: Identification of a second frameshift/ premature stop mutation in an independent gene (PPP1R3A) with a key role in glycogen metabolism in the same kindred (Kindred A).

Introduction

(The introduction to this chapter includes background information and a brief literature review of the role played by glycogen targetting subunits in carbohydrate metabolism).

Approximately 50 candidate genes were screened by Incyte Genomics, Cambridge, UK. Genomic DNA from subjects was randomly pre-amplified in a primer extension pre-amplification (PEP) reaction. All coding exons and splice junctions of candidate genes were then PCR amplified from PEP DNA with gene specific primers. PCR products were studied by single-stranded conformation polymorphism analysis and direct sequencing of all abnormal conformers (Thorpe et al., 1999). These studies led to the identification of a second frameshift/ premature stop mutation in phosphoprotein-phosphatase 1, regulatory subunit 3A (PPP1R3A; (C¹⁹⁸⁴ΔAG)fs.662(stop 668); hereafter denoted as FS PPP1R3A) (figure 5).

PPP1R3A is a key molecule in the regulation of glycogen metabolism in skeletal muscle (Newgard et al., 2000). Skeletal muscle which accounts for about 40% of body mass is responsible for the vast majority of insulin stimulated glucose disposal. ¹³C nuclear magnetic resonance (NMR) spectroscopy measurements of intramuscular glycogen content indicate that

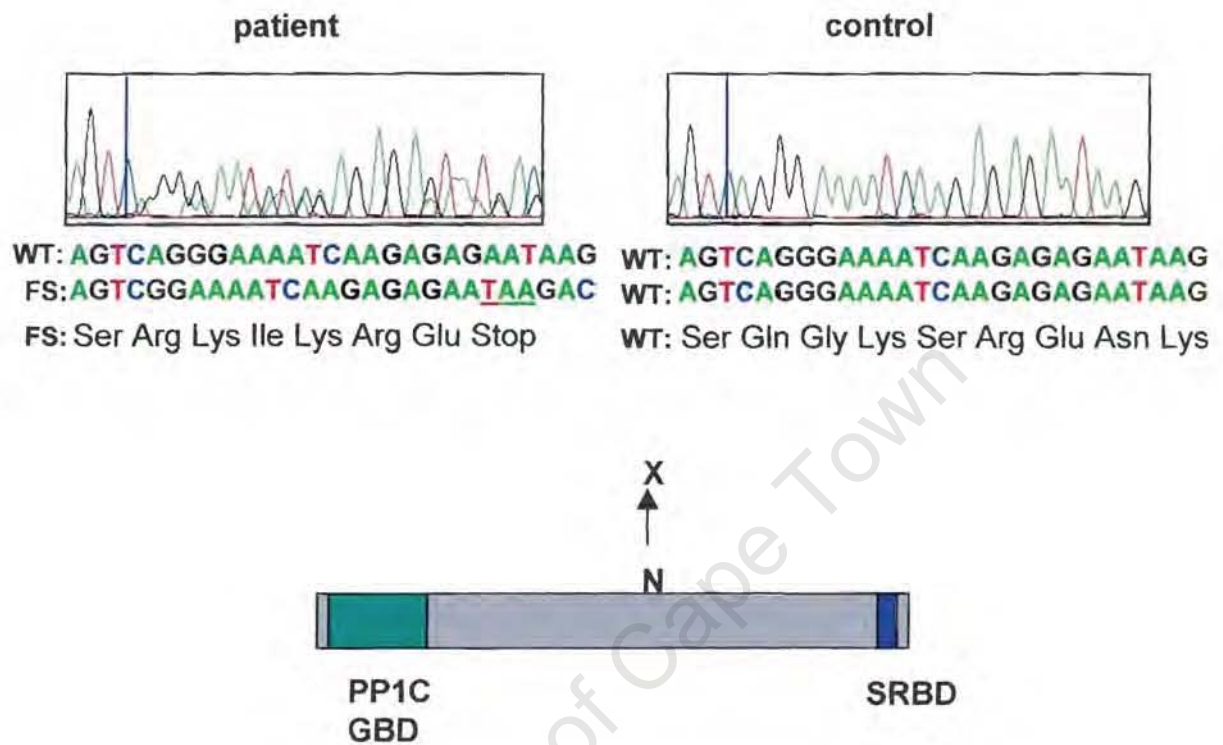


Figure 5

Heterozygous FS PPP1R3A mutation [(C¹⁹⁸⁴del.AG)fs.662(stop 668)]. The frameshift results in a premature Stop codon (X) at position 668 with subsequent loss of the putative sarcoplasmic reticulum-binding domain (SRBD)10. N – asparagine; PP1C/ GBD – PP1C- and glycogen binding domains.

muscle glycogen synthesis accounts for ~90% of whole-body glucose metabolism and all non-oxidative glucose disposal (Roden and Shulman, 1999). Glycogen stores are regulated by two key enzymes, glycogen synthase (GS) and glycogen phosphorylase (GP), both of which are primarily regulated by phosphorylation/ dephosphorylation (Brady and Saltiel, 2001). Although the idea that insulin stimulates glycogen synthesis is now accepted dogma, the mechanism by which this effect is exerted remains controversial. Insulin was originally thought to activate GS by 1) promoting its dephosphorylation, via the inhibition of kinases (glycogen synthase kinase-3 (GSK3) and protein kinase-A (PKA)) and 2) increasing glucose transport and hence glucose-6-phosphate levels which activate GS allosterically (Brady and Saltiel, 2001). Insulin can inhibit GSK3 and induce dephosphorylation of GS in a variety of cell types (Cross et al., 1995), (Cross et al., 1997). Additional evidence to support this mode of activation included the following:

1. Over-expression of GSK3 constructs reduced basal and insulin-stimulated GS activity (Eldar-Finkelman et al., 1996).
2. Lithium, a GSK3 inhibitor, stimulated GS activity (Orena et al., 2000), (Summers et al., 1999).

However, inhibition of GSK3 is not sufficient to mediate GS activation by insulin as GSK3 does not phosphorylate several of the residues on GS that are dephosphorylated by insulin (Lawrence and Roach, 1997) and GSK3 inactivation by other factors does not result in GS activation (Brady et al., 1998). Investigators later suggested that protein phosphatase 1 (PP1)

mediated the active process of dephosphorylating GS (Dent et al., 1990). PP1 also catalyzes the dephosphorylation and inactivation of GP, thereby promoting further increases in glycogen storage. Although PP1 is a ubiquitous enzyme, it was proposed that insulin specifically activates discrete pools of PP1 in the vicinity of glycogen by facilitating binding of the PP1 catalytic subunit (PP1C) to glycogen targeting regulatory subunits (Cohen, 1993). The latter were first identified by workers in Sir P. Cohen's laboratory at the University of Dundee, Scotland (Dent et al., 1990). These glycogen targeting subunits appear to serve as "molecular scaffolds", bringing PP1C together with its substrates glycogen synthase and phosphorylase in a macromolecular complex, and in the process have significant effects on PP1C activity (Newgard et al., 2000). PPP1R3A is a skeletal- and cardiac muscle-specific regulatory subunit (Tang et al., 1991). It differs from the other well-characterised glycogen targeting subunits (PPP1R3B, -C and -D) in having a long C-terminal tail of about 900 base pairs (bp), the function of which is still to be precisely determined (Newgard et al., 2000). The only region in this long tail with an apparent biological role is a short segment (bp 1064-1094) very near the C-terminal end which encodes a hydrophobic domain believed to target the protein to sarcoplasmic reticulum (Hubbard et al., 1990), (Walker et al., 2000). Like the other members of this protein family, PPP1R3A has binding sites for PP1C, GS and glycogen near its N-terminal (Newgard et al., 2000). There is to date no direct evidence for GP binding although this is believed to occur. Finally, and in contrast to the other glycogen targeting subunits, the N-terminal region of PPP1R3A has two hormonally regulated phosphorylation sites, serine 48 (site 1) and serine 67 (site 2). Site 1

phosphorylation appears to be stimulated by insulin and leads to GS activation (Dent et al., 1990). Phosphorylation of site 2, which is located within the PP1C binding domain, promotes dissociation of the catalytic subunit (PP1C) from PPP1R3A, thereby favouring phosphorylation and activation of GP. Adrenaline, which is known to promote glycogenolysis, promotes site 1 and 2 phosphorylation via PKA (Walker et al., 2000). In this setting, site 2 phosphorylation appears to override the glycogen synthesis promoting effect of site 1 phosphorylation (Liu and Brautigan, 2000).

What then is the evidence that these proteins modulate glycogen metabolism *in vivo*? This question has been addressed in at least three ways:

- 1) Over-expression studies: PPP1R3C (also known as protein targeted to glycogen or PTG) over-expression in CHO-IR (Chinese hamster ovarian cells stably over-expressing the insulin receptor) (Printen et al., 1997), hepatocytes (Yang et al., 2002a) and human myotubes (Lerin et al., 2000) dramatically increases glycogen accumulation. Yang et al (Yang et al., 2002a) also overexpressed PPP1R3A and PPP1R3B in hepatocytes using recombinant adenoviruses and as with PTG this dramatically increased glycogen deposition. Adenoviral over-expression of PPP1R3A-C in the livers of living mice similarly increased glycogen accumulation (Gasa et al., 2002). Intriguingly, the whole-body consequences of these alterations in liver glycogen metabolism were most beneficial in mice over-expressing a truncated PPP1R3A mutant (Gasa et al., 2002). The authors suggested that this

reflected the mutant protein's ability to facilitate glycogenolysis as well as synthesis, whilst the other isoforms tended to drive glycogen synthesis alone.

- 2) Loss of function studies: PPP1R3A knockout mice had a 90% reduction in muscle glycogen stores due to reduced glycogen synthase activity and increased glycogen phosphorylase activity (Suzuki et al., 2001). Surprisingly, however, glucose tolerance and insulin sensitivity were normal. More specifically, insulin-stimulated glycogen synthesis within skeletal muscle was normal. This observation prompted the authors to speculate about the presence of an unidentified insulin responsive phosphatase. Additional phenotyping of these mice indicated an impairment in exercise stimulated glycogen synthesis (Aschenbach et al., 2001).
- 3) Human genetics: A number of human genetic studies of glycogen targeting subunits have reported associations with type 2 diabetes. Hansen et al (Hansen et al., 1995) identified a common polymorphism at codon 905 in PPP1R3A. This variant was thought to be associated with insulin resistance and hypertension, although despite the allele being present at high frequency in the Japanese population, no linkage could be established in this population (Shen et al., 1998). *In vitro* characterization of this variant also yielded conflicting data regarding its potential impact on glycogen turnover (Rasmussen et al., 2000), (Ragolia et al., 1999). A second mutation in the 3' non-coding region of

PPP1R3A has been linked to insulin resistance and diabetes in Native American populations (Hegele et al., 1998), (Xia et al., 1999). This variant appears to alter PPP1R3A protein levels (Xia et al., 1999), (Permana et al., 2000). Lastly an Arg883Ser mutation in PPP1R3A has been associated with diabetes, but again functional data failed to identify any detectable effects on glycogen metabolism (Permana et al., 2000). It is worth noting that both of the coding mutations described above result in amino acid substitutions within regions of the C-terminal part of PPP1R3A without any known function, so that the failure of investigators to identify any *in vitro* consequences of these mutations is not particularly surprising. In contrast to these mutations, the FS PPP1R3A mutation was predicted to result in a truncated protein (figure 5), lacking the C-terminal sarcoplasmic reticulum-binding domain. All other known domains involved in protein-protein interactions and glycogen binding were expected to be intact. As the precise reason for PPP1R3A's targeting to sarcoplasmic reticulum is unknown further predictions regarding the metabolic impact of this mutation remain speculative.

Methods

Clinical studies

Local ethics committee approval was obtained for all studies involving human subjects and informed consent was provided by every participant. Initial clinical characterization included a physical examination, fasting blood tests as described for subjects in chapter 2 and MRI/ NMR studies of fat mass and distribution, and intramyocellular triglycerides.

Site directed mutagenesis

(This was done with Stratagene's "Quickchange XL Site-directed Mutagenesis Kit".)

A plasmid expressing human PPP1R3A (Rasmussen et al., 2000) was kindly provided by Professor P. Cohen, University of Dundee, Scotland. Primer sequences used to generate the FS PPP1R3A mutant were as follows: forward - 5'-GGA ATG TTC TGG AAA GTC GGA AAA TCA AGA GAG- 3'; reverse - 5'-CTC TCT TGA TTT TCC GAC TTT CCA GAA CAT TCC-3'. The PCR sample reaction contained 5 μ L of 10* reaction buffer; 10 or 25 ng of plasmid DNA; 125 ng of forward and reverse primers; 1 μ L of dNTP mix (10mM); 3 μ L of Stratagene Quicksolution; 1 μ L of *PfuTurbo* DNA polymerase (2.5 U/ μ L) and distilled water to a final volume of 50 μ L. Cycling parameters were as follows: 1 cycle of 1 minute at 95 degrees; 18 repeats of 95 degrees for 50 seconds, 60 degrees for 50 seconds and 68 degrees for 2.5 minutes; and 1 cycle of 7 minutes at 68 degrees. The reaction was then cooled on ice

for 2 minutes prior to digestion with the *Dpn* I restriction enzyme (1 μ L per reaction, incubated at 37 degrees for 1 hour). XL-10 Gold ultracompetent cells (Stratagene) were then transformed with 2 μ L of the *Dpn* treated DNA and plated overnight. Transformed colonies were subsequently amplified and plasmid DNA was isolated using a Qiagen Midiprep Kit. Mutagenesis was then confirmed by Big Dye sequencing.

Cell culture

All cells were grown in monolayer cultures at 37 degrees in a humidified incubator with 5% CO₂. In all cases medium contained 2 mM glutamine and a 1:100 dilution of a penicillin/ streptomycin solution.

1) CHO cells were grown in Hams F12 medium with 10% foetal calf serum (FCS).

2) Rat L6 myoblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% FCS. Cells were always passaged at between 50 and 70% confluence in order to optimize ultimate cell differentiation. Prior to differentiation myoblasts were allowed to reach confluence in 24-well plates. The medium was then changed to MEM alpha medium (MEM α , Gibco) with 2% horse serum. The culture medium was replaced on alternate days and differentiation was assessed microscopically by the development of multinucleated myotubes. Cells were used for subsequent experiments 7 - 9 days after the medium was changed to MEM α .

Transient transfection of CHO cells

Cells were plated in 6-well plates at a density sufficient to produce approximately 50 - 60% confluence 24 hours after plating. Plasmid DNA (1 ug/ well in 6 well plate) was co-incubated with Optimem (46 μ L per well) (Sigma) and Eugene reagent (3 μ L per well) (Roche) for 45 minutes prior to its being added to the cell culture medium. Subsequent experiments were performed 24 to 48 hours later.

Adenoviral infection of L6 myotubes

Recombinant adenoviruses were generated by Galapagos plc according to standard protocols. Differentiated L6 myotubes (day 7 - 9 post addition of differentiation medium) in 24 well plates were infected with recombinant virus by addition of 2×10^9 viral particles in 100 μ L of medium per well. The cells were subsequently gently rocked in a humidified incubator at 37 degrees for 1 hour prior to the addition of a further 400 μ L of medium. They were then left on the rocker in the incubator overnight. 16 hours later cell medium containing free virus was removed and replaced with fresh medium. Viral infection was confirmed in every experiment by western blotting for human PPP1R3A.

Protein analysis

Protein extraction from cells

Cells were washed twice in ice-cold PBS before the addition of 100 μ L/ well of a 24-well plate of RIPA lysis buffer (1% NP40; 1% sodium deoxycholate;

0.1% SDS; 0.9% NaCl; 25 mM Tris (pH 6.8); 1 mM EDTA; protease inhibitors and water). The lysed cells were left on ice for 30 minutes before being scraped off and centrifuged at 13000 rpm for 5 minutes at 4 degrees. The supernatant was then removed and either stored or used for subsequent experiments. For SDS-PAGE 24 μ L of the cell lysates was mixed with 6 μ L of 5 * sample loading buffer (50% w/v glycerol, 10% w/v SDS, 500mM DTT, 50 mM Tris pH 6.8, 0.5% w/v bromophenol blue), boiled for 5 minutes and then loaded onto the gels.

SDS-PAGE and Western blotting

SDS-PAGE was carried out as previously described (Laemmli, 1970), using 6% gels for PPP1R3A and 10% gels for PP1C. Band sizes were determined using broad range markers (New England Biolabs, Hertfordshire, UK). Gels were run at 150V for approximately 1 hour. The Bio-Rad semi-dry transfer apparatus was used to transfer protein from the gels to a PVDF mebrane (Millipore, MA, USA). Transfer took place at 180 mA for 1 hour, in a buffer containing 1.3 mM SDS, 48 mM Tris base, 39 mM glycine (pH 9.2) and 20% methanol. Membranes were then placed in a blocking buffer (5% milk in Tris-buffered saline (TBS), 0.05% Tween-20) for 1 hour at room temperature, followed by overnight incubation with primary antibody at 4 degrees ((i) sheep anti-PPP1R3A binds an N-terminal epitope on human PPP1R3A; this antibody was a gift from Dr P. Cohen, University of Dundee, Scotland (ii) polyclonal goat anti-PP1C α (Santa Cruz)). The membranes were then washed in TBS, 0.05% Tween-20 (TBS-T) (6 * 5 minute washes) before being

incubated for 1 hour at room temperature in secondary antibody (anti-sheep for PPP1R3A and anti-goat for PP1C) diluted 1:10000 in blocking buffer. Following a further 6 washes in TBS-T, bound antibody was visualised by enhanced chemiluminescence (ECL kit, Amersham Biosciences Ltd., Buckinghamshire, UK).

Immuno-precipitation

20 μ L of a 1:4 slurry containing monoclonal mouse anti-HA antibody (Santa Cruz) cross-linked to agarose beads was added to 100 μ L of cell extract (from 1 well of a 12-well plate) and mixed for 1 hour in lysis buffer at 4 degrees. The resulting immunoprecipitate was washed three times in lysis buffer and one times in PBS before being resuspended in 20 μ L of 1 X sample buffer and boiled for 5 minutes before loading onto an SDS polyacrylamide gel.

Immunofluorescence Microscopy

CHO cells were transiently transfected (Fugene) with N-terminal HA-tagged wild type (WT) or PPP1R3A FS (FS) mutant expression vectors (pACCMV.pLpA-HA-PPP1R3A). 24 - 36 hours later, cells were fixed in 3% paraformaldehyde/0.05% glutaraldehyde in 100 mM K-Hepes/3 mM $MgCl_2$ buffer (pH 7.5) for 15 min, treated with 0.5% borohydride/PBS for 10 min, and then blocked and permeabilised in 1% BSA/0.1% saponin for 20 min. The CMV promoter generally induces massive over-expression of downstream genes so some cells were permeabilised prior to fixation, a step expected to

lead to loss of unbound cytosolic proteins. The latter cells were incubated for 5 min in 0.05% saponin in 80 mM K-Pipes/5 mM EGTA/1 mM MgCl_2 (pH 6.8) at room temperature prior to fixation. Cells were labelled with a rat anti-HA antibody (Boehringer; 1:100) followed by Texas Red goat anti-rat (Molecular Probes; 1:200). Confocal images were collected using a Leica TCS SP system and processed using Adobe Photoshop software (Adobe Systems). This study was undertaken in collaboration with Dr G. Ihrke, Cambridge, UK.

Glycogen synthesis

Glycogen synthesis was assessed by quantifying ^{14}C -glucose incorporation into cellular glycogen with-/without insulin stimulation. Approximately 36 hours post infection with adenoviruses differentiated L6 myotubes were serum starved in 0.5 ml MEM α with 0.2 % bovine serum albumin (BSA) for 3 hours at 37 degrees and then in 0.5 ml fresh MEM α / 0.2 % BSA for another hour. The serum starvation was modified as described in the results section in cases where cells were placed in glucose free medium (Sigma) in order to deplete glycogen stores prior to measuring glycogen synthesis. Medium was then replaced with 0.25 ml fresh MEM α / 0.2 % BSA. Insulin (100 nM human actrapid) and D[U- ^{14}C] glucose (0.3 μCi) were immediately added and cells were incubated at 37 degrees for a further 2 hours. Cells were then washed in ice cold phosphate buffered saline (5* 0.5 mL washes) before being lysed in 100 μL per well of 20% KOH (left at room temperature for 1 hour). 100 μL of 1 M HCL was added to neutralise the KOH prior to scraping of each well to ensure release of the cell monolayer. Lysates were transferred to tubes and

the wells scraped again in 200 μ L water, which was then added to the lysates. 300 μ L of these samples were then transferred to tubes containing 30 μ L of 240 mg/ mL oyster glycogen. Ice - cold ethanol (900 μ L per sample) was added to prompt glycogen precipitation. Following a 30 minute incubation on ice glycogen was pelleted by centrifugation and the supernatant removed. Following the addition of 1 M formic acid (50 μ L per sample) and water (200 μ L per sample), samples were boiled to resuspend the glycogen. Radioactivity was measured by scintillation counting.

Glycogen content

Total cellular glycogen content was assessed by a modification of the method of Lust et al (Lust et al., 1975). Glucose is liberated from cellular glycogen using amyloglucosidase (Sigma A-3514). Hexokinase (Roche 1426362) catalyses the conversion of glucose to glucose-6-phosphate (G6P) and G6P-dehydrogenase (Roche 127663) oxidises G6P to gluconate-6-phosphate, with the subsequent reduction of nicotinamide dinucleotide phosphate (NADP). NADPH production is measured by changes in fluorescence.

Differentiated L6 myotubes were cultured in 24 well plates. Following four washes in 0.5 mL ice cold PBS, 100 μ L of 0.2 M sodium acetate (pH 4.8) was added to each well. Wells were then scraped and lysates carefully transferred to tubes. Extracts were briefly sonicated (Soniprep 150), prior to addition of 250 mU amyloglucosidase per sample (30 μ L out of original cell lysate). Samples were then incubated at 37 degrees for 2 hours with regular

vortexing. Duplicate 9 μ L aliquots from each sample were transferred to a 96 well plate and 200 μ L of assay cocktail (0.1 M Tris-HCL, pH 8; 0.3 mM ATP; 6 mM MgCL₂; 5 mM dithiothreitol, 1 mM NADP, 2.5 U/mL hexokinase and 1 mg/mL G6P-dehydrogenase) was added to each sample. Following a 30 minute incubation at room temperature, changes in fluorescence were determined using an excitation wavelength of 350 nM and an emission wavelength of 460 nM. Reaction blanks were determined as the fluorescence of samples prior to enzymatic treatment with amyloglucosidase. Fluorescence readings were made on a Fluoroscan Ascent FL fluorimeter and were compared to a standard curve.

Statistical analysis

Statistical analysis was performed using the Windows Excel Statistical package.

Results

Cosegregation studies and basic phenotypic characterization

The FS PPP1R3A mutation was present in the proband and six of her relatives, four of whom also carried the FS PPAR γ mutation (figure 6). All five doubly heterozygous individuals had significantly elevated fasting insulin levels, whereas as was seen in the case of the PPAR γ variant, individuals harbouring the PPP1R3A variant alone were not hyperinsulinemic (figure 7). Further clinical evidence of insulin resistance, in the form of acanthosis nigricans, was also present in the doubly heterozygous subjects. In contrast to what was seen in carriers of the dominant negative PPAR γ mutations described in chapter 2, hypertension and other features of the insulin resistance syndrome were only present in some of the double heterozygotes, and were also present in other family members, suggesting that these features did not require the presence of both mutations (table 1).

Whole body adipose tissue mass was slightly lower than predicted and leptin levels were below the 25th percentile for age- and sex-matched individuals in all double heterozygotes and one PPAR γ FS heterozygote (table 1). However, magnetic resonance imaging of body fat did not reveal an obvious lipodystrophy in these subjects. The paucity of detailed reference data and the absence of tight definitions of lipodystrophy made it impossible to definitively ascertain whether or not subjects carrying the FS PPAR γ variant were mildly lipodystrophic. However, they clearly retain significantly more

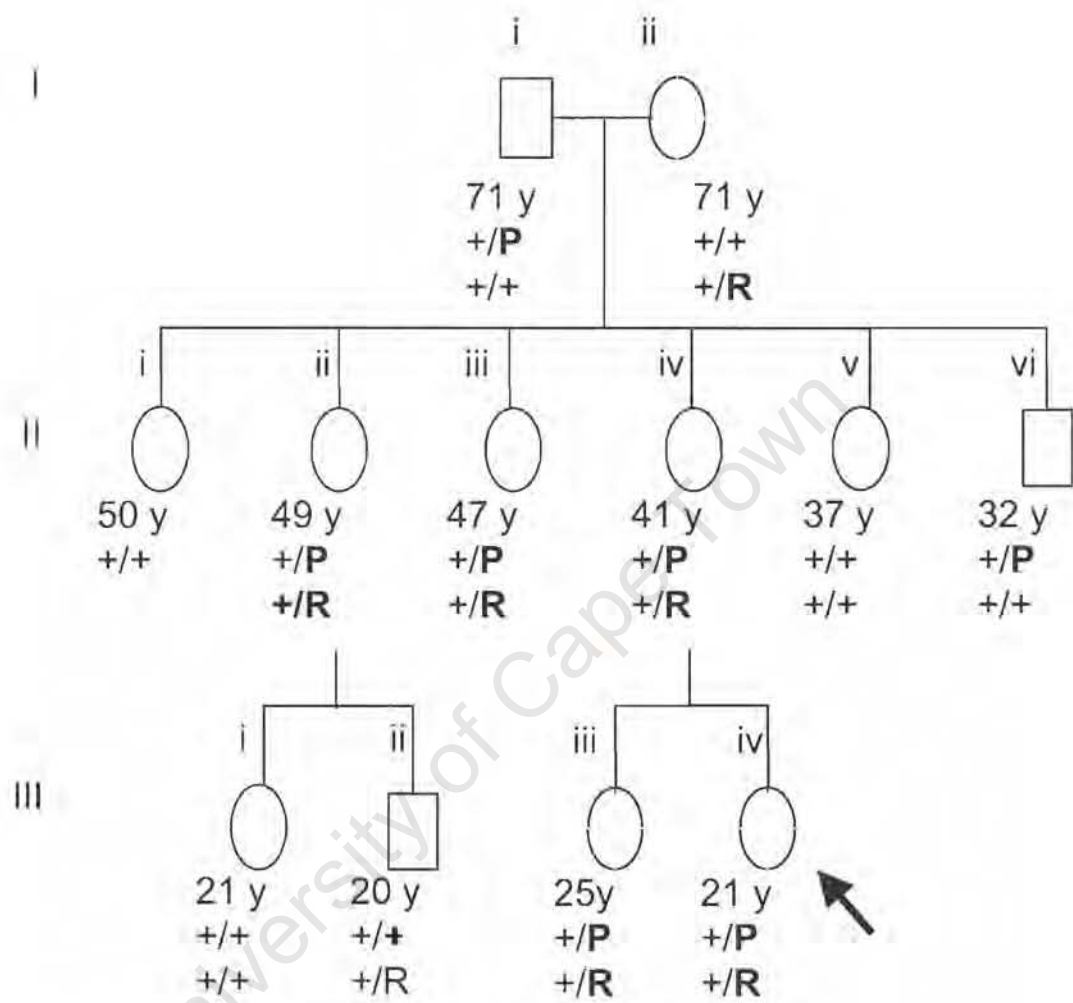


Figure 6

Kindred A pedigree. The age and genotype (+, wild type; P, FS PPAR γ mutant; R, FS PPP1R3A) of members is indicated.

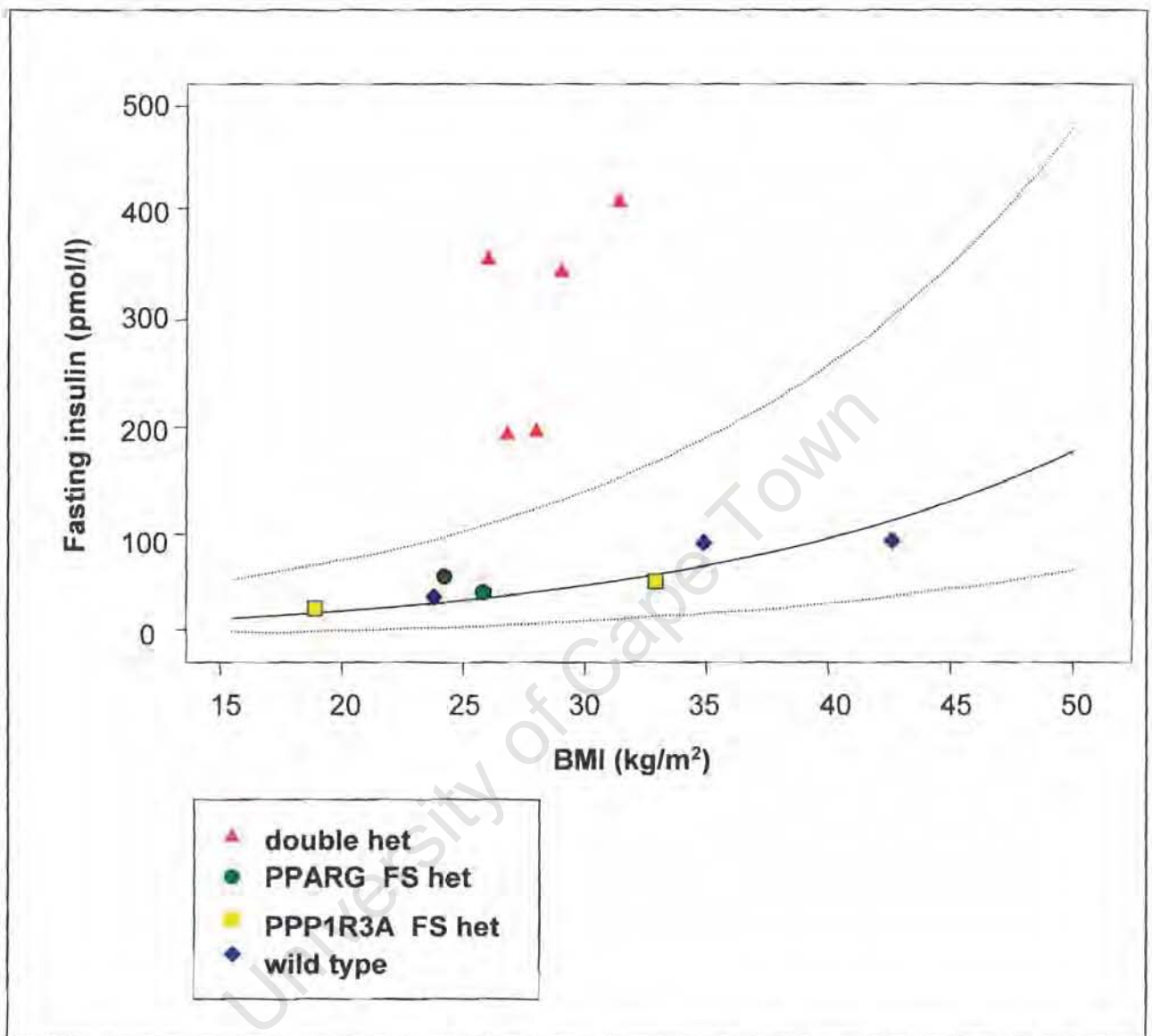


Figure 7

Fasting insulin measurements plotted against BMI in 'digenic family' members. The solid line represents the log-linear regression line between fasting insulin and BMI in 1211 participants in the MRC Ely population-based cohort study. The 95% confidence intervals (broken lines) include 95% of individuals at any given BMI.

Table 1: Clinical and biochemical characteristics of mutant allele carriers.

Fig.4 reference	Kindred A										Kindred B	
	Doubly heterozygous subjects					FS PPAR γ mutant heterozygotes		FS PPP1R3A mutant heterozygotes				Normal values
	IIii	IIiii	IIiv	IIiii	IIiv	Ii	IIvi	Iii	IIiii	IIi	Ii	
Age	49	47	41	25	21	71	32	71	20	20	65	
Gender	F	F	F	F	F	M	M	F	M	M	M	
BMI (kg/m ²)	26.8	26	28	31.4	29	24.2	25.8	32.9	18.9	36.5	30	
Blood pressure	190/ 110	140/ 80*	130/ 84	130/ 70	150/ 110	170/ 90*	125/ 90	170/ 105*	105/ 69	135/ 82*	172/ 93*	
Measured body fat as percentage of predicted body fat†	84.3	63.5	83.7	46.8	79.4	n/a	75.2	n/a	n/a	n/a	n/a	100%
Glucose	5.6	6.4	4.4	9.2‡	3.9	12‡	4.6	4.5	5.2	4.4	6.2	3.5-6.3 mmol/L
Insulin	195	359	197	411	346	61	46	56	31	437	178	<80 pmol/L
% insulin sensitivity (HOMA) **	27	15	28	14	20	87	115	95	168	13	30	100%
TG	6.1	2.1¶	3.4	34.6‡	10.1	6.6	1.5	1.1	0.7	1.5	2.4	Desirable <2.0 mmol/L
HDL	0.82	0.63¶	0.81	0.52	1.04	0.71	1.02	1.84	1.36	0.7	0.91	Desirable >0.9 mmol/L
NEFA	1442	202¶	526	2532	867	1219	584	933	n/a	n/a	n/a	280-920 μ mol/L
Uric acid	0.31	0.24	0.23	0.23	0.28	0.35	0.31	0.23	0.32	0.17	0.44	0.15-0.35 mmol/L
Leptin	12.1	4.4	8.2	17.3	12.4	1.2	0.9	13.2	0.6	14.6	19.8	ug/L
IMCL/ creatine ratio (soleus)	19.8	19.1	25.5	28.3	44.9	n/a	28.3	n/a	n/a	n/a	n/a	13.6 $\pm$ 6.6 [#]

All samples were obtained following an overnight fast. *, Measurements affected by anti-hypertensive therapy; †, Body fat was quantified by magnetic resonance imaging (MRI) as described previously. Predicted body fat (Black et al, 1983): for women = $(1.48 \times \text{BMI (kg/m}^2)) - 7.00$; for men = $(1.281 \times \text{BMI (kg/m}^2)) - 10.13$; ‡, Abnormalities detected at the time of screening; ¶, Measurements affected by lipid lowering therapy; **, HOMA (homeostasis model assessment) is likely to be influenced by the diabetic status of some individuals; #, IMCL reference values represent mean and SD of measurements from 76 control subjects (unpublished observations EL Thomas and JD Bell); TG, triglycerides; HDL, high-density lipoprotein; NEFA, non-esterified fatty acids; IMCL, intramyocellular lipid.

subcutaneous limb and gluteal fat than adult carriers of the dominant negative PPAR γ mutations.

Subcutaneous abdominal adipose tissue physiology was studied in subject (II vi), a 32-year-old male with the FS PPAR γ variant alone (see chapter 2, page 52 for details of methods). Although this subject did not appear to be insulin resistant these studies demonstrated some subtle abnormalities in adipose tissue function. In particular, NEFA levels in adipose tissue venous blood were surprisingly low in the fasting state. They fell appropriately in the postprandial period and then returned to a surprisingly low level (figure 8). This pattern was reminiscent of that seen in the P467L carrier and suggests that the metabolic flexibility of lipid metabolism in adipose tissue may be altered. However, given the subtlety of the abnormalities and the fact that only one subject could be studied, definitive conclusions cannot be drawn as yet.

Identification of a second kindred (Kindred B) with the PPP1R3A frameshift mutation

One other Caucasian subject in the SIR cohort had the same heterozygous frameshift mutation in PPP1R3A that was found in Kindred A. This subject (III, Kindred B) presented with acanthosis nigricans at age 20 years. At that age he had a body mass index of 36.5 kg/m² and a fasting insulin of 437 pmol/L (N<80 pmol/L). He inherited the mutation from his moderately obese father (BMI 30 kg/m²) who also had marked hyperinsulinemia (fasting insulin 178 pmol/L) (figure 9). The two other wild type family members were clinically and

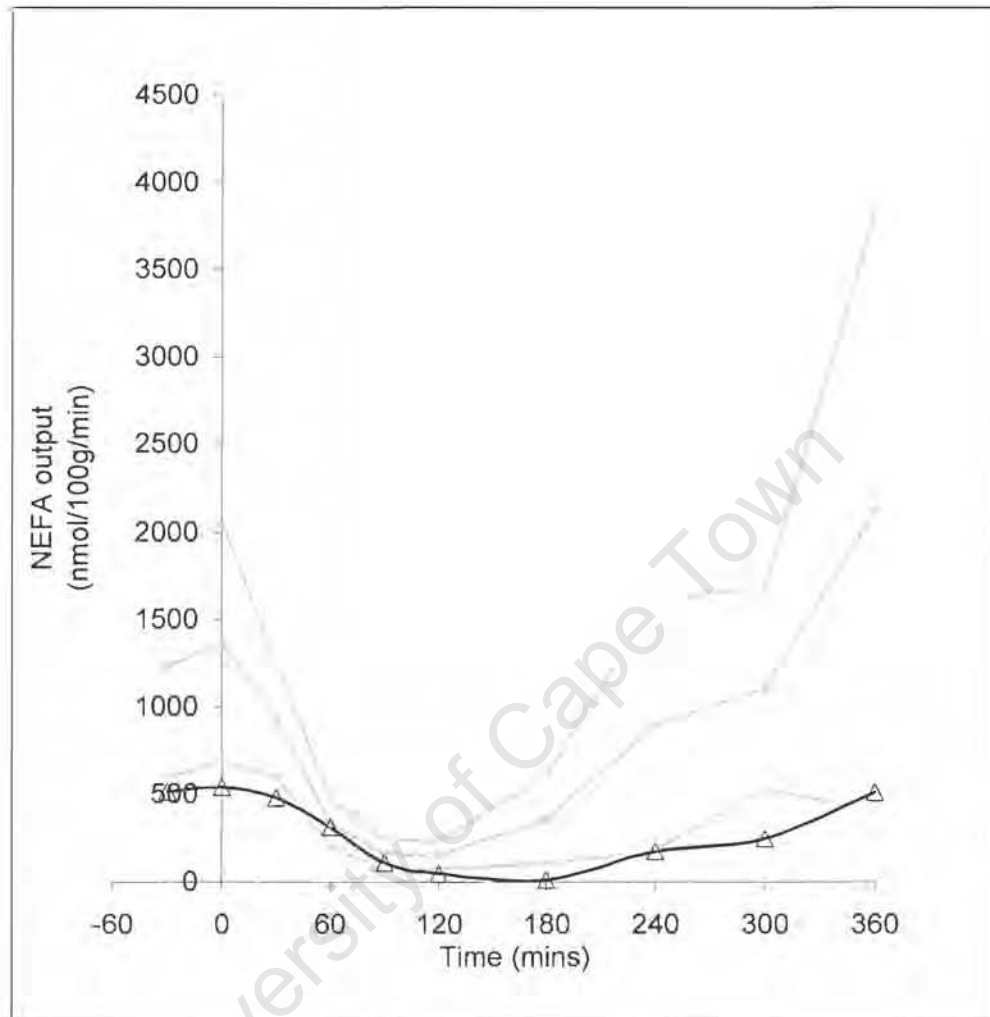


Figure 8

Adipose tissue non-esterified fatty acid (NEFA) output in $\text{nmol} \cdot \text{min}^{-1} \cdot (100\text{g adipose tissue})^{-1}$ in 32 year-old male carrier of the FS PPARG variant (open triangles). Grey lines represent mean and 95% CIs of measurements made in 10 healthy subjects (Coppack, 1992).

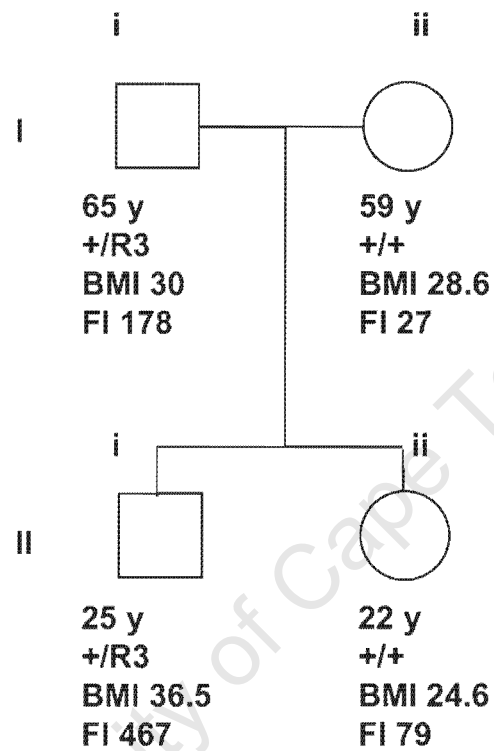


Figure 9

Kindred B pedigree suggests that carriers of the PPP1R3A frameshift variant develop fasting hyperinsulinaemia when obese. The age, BMI (kg/m²), fasting insulin (FI, pmol/L) and genotype (+, wild type; R3, PPP1R3A mutation) are indicated.

biochemically normal. Of note, the proband subsequently lost 40 kg and reduced his BMI to 27 kg/m². At that stage his fasting insulin level fell dramatically to 93 pmol/L.

***In vitro* characterization of PPP1R3A FS mutant**

Transient transfection studies

As some prematurely truncated proteins are immediately degraded, expression of the truncated protein was initially checked in transiently transfected CHO cells. The FS PPP1R3A mutant vector produced a detectable protein of approximately 83 kDa, which is about half the size of the wild type protein, in keeping with the predicted truncation at position 668 (figure 10). Furthermore PP1C co-precipitated with an HA-tagged version of the truncated protein with an efficiency similar to wild type PPP1R3A (figure 10). However, confocal microscopy revealed strikingly different intracellular distributions of the wild type and mutant PPP1R3A. As transfected cells tend to massively over-express exogenous proteins (see figure 11), cells were briefly permeabilised with saponin prior to fixation and subsequent immunostaining. This technique leads to the release of cytosolic proteins whilst membrane attached proteins are retained, thereby facilitating visualization of their precise intracellular localisation. A significant fraction of over-expressed wild type PPP1R3A localised, as expected, to intracellular membranes, whereas mutant PPP1R3A was almost exclusively cytosolic (figure 11). The precise nature of the intracellular membrane network to which

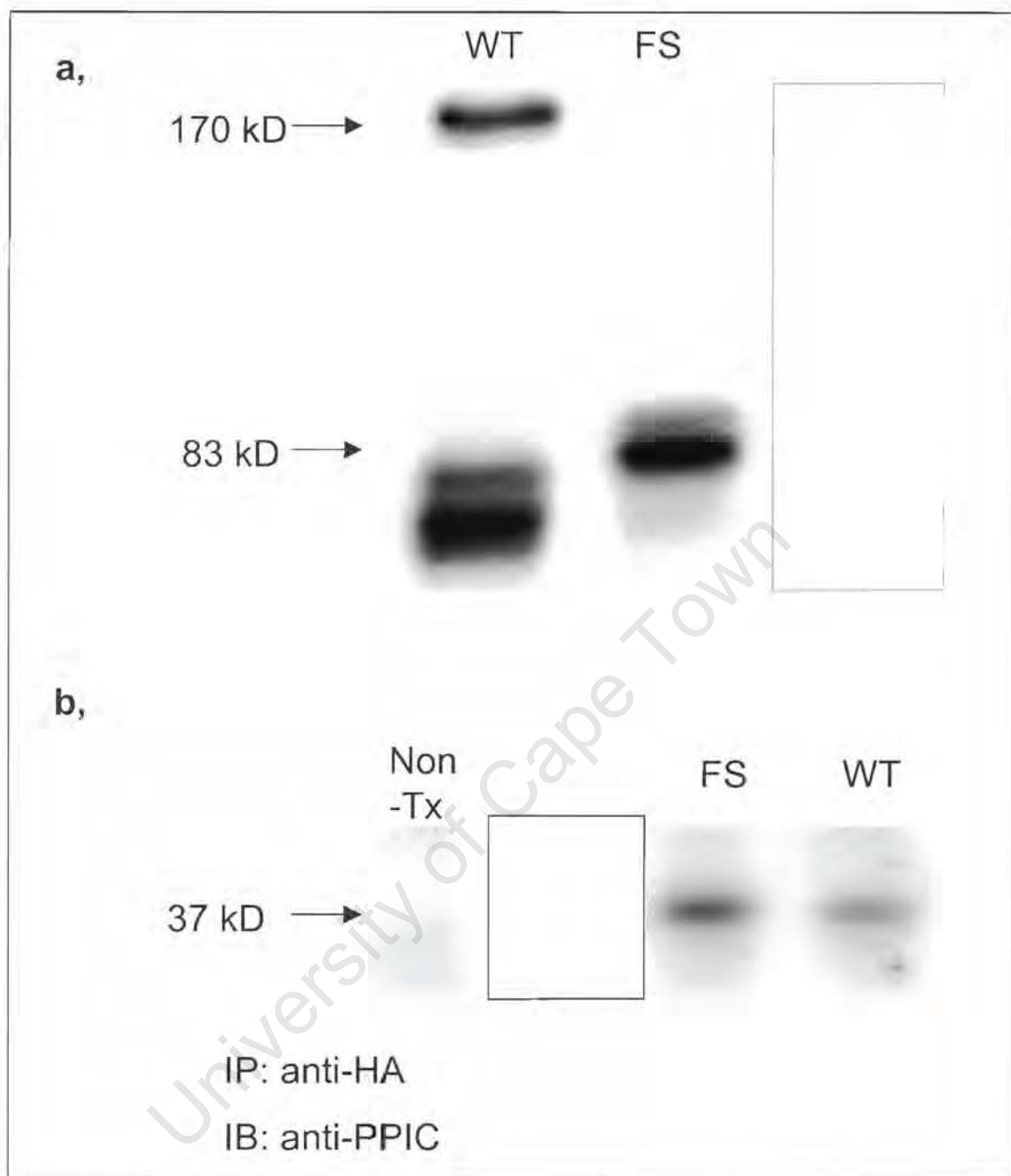


Figure 10

CHO cells were transiently transfected (Fugene) with HA-tagged wild type (WT) or PPP1R3A FS mutant expression vectors (pACCMV.pLpA-HA-PPP1R3A, gift from P. Cohen).

a, Western blotting of whole cell lysates using a sheep monoclonal N-terminal PPP1R3A antibody (gift from P. Cohen). Note that PPP1R3A undergoes rapid proteolysis (Tang et al, 1991) and one of the proteolytic fragments is of similar size to the frameshift and stop mutants. b, Whole cell lysates from non-transfected (Non-Tx) and transfected CHO cells were immunoprecipitated with an anti-HA antibody (Santa-Cruz) and Western blotted with a PP1C antibody (Santa- Cruz).

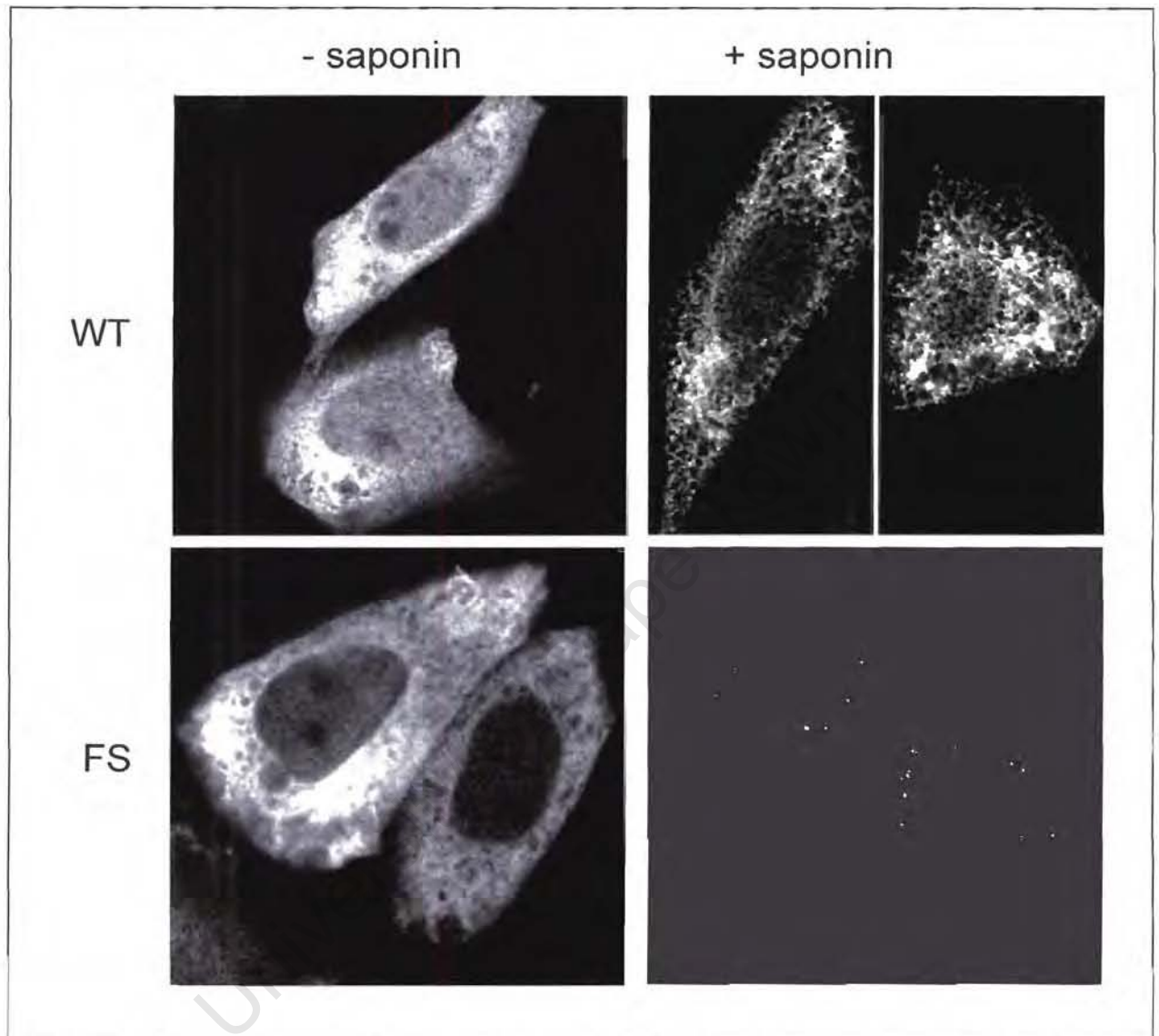


Figure 11

Confocal microscopy of wildtype PPP1R3A (WT) and FS PPP1R3A (FS) proteins. Cells transiently expressing HA-tagged versions of PPP1R3A were fixed either without (left panel) or with prior permeabilisation with saponin to release cytosolic PPP1R3A (right panel), then fully permeabilised and labelled with an anti-HA antibody. The loss of mutant PPP1R3A in cells permeabilised with saponin prior to fixation indicates that the protein is largely cytosolic whereas WT PPP1R3A is associated with intracellular membranes.

wild type PPP1R3A was bound was subsequently confirmed by co-localisation studies in which CHO cells were transfected with both PPP1R3A and the IP3 receptor, a protein known to be bound to the endoplasmic reticulum (data not shown). Prior reports on the association between PPP1R3A and endoplasmic reticulum were based upon differential ultracentrifugation alone (Hubbard et al., 1990), (Walker et al., 2000).

While previous studies had demonstrated that intracellular localisation influences GS activity, the precise functional consequences of the mislocalisation of PP1 were still to be determined. As transient transfection typically results in fewer than 5% of plated cells expressing the desired protein and myotubes are a particularly difficult cell type to transfect, recombinant adenoviruses were used to explore the metabolic consequences of the frameshift/ premature stop PPP1R3A variant.

Adenoviral studies in L6 myotubes

Expression of human PPP1R3A in L6 myotubes

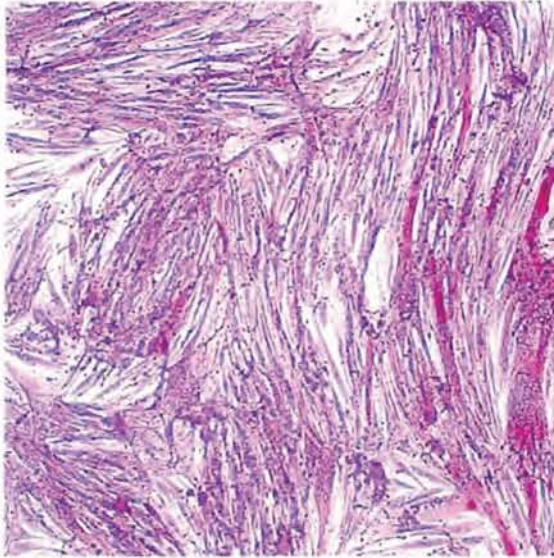
In order to evaluate glycogen-targeting subunit expression, an anti-human PPP1R3A sheep antibody was used for western blotting. The antibody, which recognises an N-terminal epitope of PPP1R3A, binds to both the full length PPP1R3A protein and several smaller proteolytic PPP1R3A fragments (see figure 10), which are believed to be the result of *in vitro* proteolysis (Tang et al., 1991). These fragments were not detected in cells infected with

adenoviruses containing an empty vector and hence do not represent non-specific antibody binding. In comparing expression of the wild type and mutant proteins one is therefore obliged to compare the mutant band with the combined signal from all bands of greater size (figure 10). This approach indicated that equal viral titres led to equal levels of protein expression. All subsequent work was undertaken using cells infected with 2×10^9 viral particles per well of a 24-well plate and western blots were performed to document consistent protein expression in every experiment.

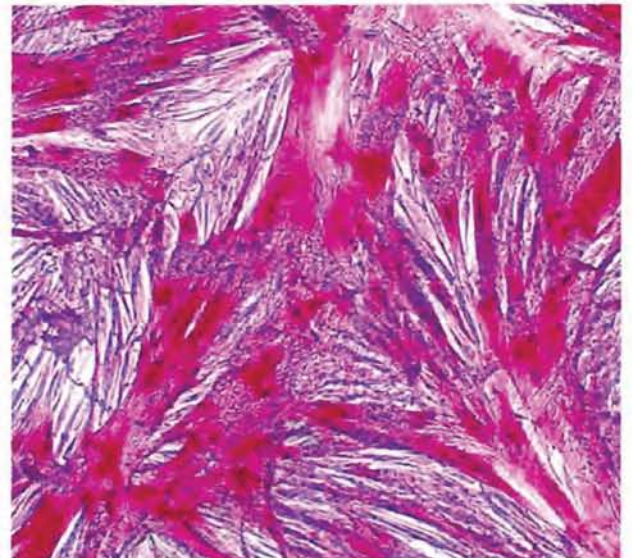
Efficiency of adenoviral infection

As the principal endpoints examined in this study were of a metabolic nature an attempt was made to infect as many cells as possible in each well, hence the use of recombinant adenoviruses. The expressed PPP1R3A proteins were untagged in order to avoid potential interference with the function of targeting subunits by the "tag". This meant that quantifying the number of infected cells could only be indirectly determined by staining for glycogen with Periodic Acid Schiff (PAS; Sigma kit), which stains all carbohydrates pink. As uninfected L6 myotubes produce very small amounts of glycogen in the basal state, infected cells were readily distinguishable from uninfected cells. This data suggested that approximately 60% of cells infected as described above with 2×10^9 viral particles/ well of a 24-well plate were over-expressing glycogen-targeting subunits (figure 12).

Total cellular glycogen content



'Empty'



PPP1R3A

Figure 12

PAS staining in L6 rat myotubes infected with adenoviruses lacking an expression cassette ('empty') or expressing PPP1R3A constructs.

Similarly to what was seen in hepatocytes (Yang et al., 2002a), a denoviral over-expression of both wild type and the frameshift mutant PPP1R3A substantially increased glycogen content (> 3 fold compared to cells infected with the empty vector) in L6 myotubes (figure 13). Even when L6 myotubes are incubated in high glucose containing medium (20 mM) and insulin 100 nM, intracellular glycogen content is substantially lower than that obtained by over expressing PPP1R3A (data not shown).

Glycogen synthesis

Glycogen content is a major determinant of glycogen synthesis both *in vitro* (Yeaman et al., 2001) and *in vivo* (Laurent et al., 2000). This was readily apparent in the L6 myotubes as although radioactivity counts do vary between experiments, counts of ¹⁴C-glucose incorporation into glycogen were dramatically increased in glycogen depleted cells (average counts in cells constantly exposed to 5 mM glucose range from 200 to 500 dpm/ well; in cells starved of glucose for 4 hours and then re-incubated in 5 mM glucose at the time of the addition of ¹⁴C-glucose, counts range from approximately 1500 to 2500 dpm/ well; and in cells starved of glucose for 18 hours prior to re-introduction of glucose containing medium, the counts range from 2500 to 6500 dpm/ well). Interestingly, when measured in cells starved of serum and glucose for four hours prior to the re-addition of glucose +/- insulin, glycogen synthesis did not differ significantly in cells infected with "empty" adenoviral constructs compared to those over-expressing either the wild type or mutant PPP1R3A (figure 14). However, when depleted of glucose for 18 hours,

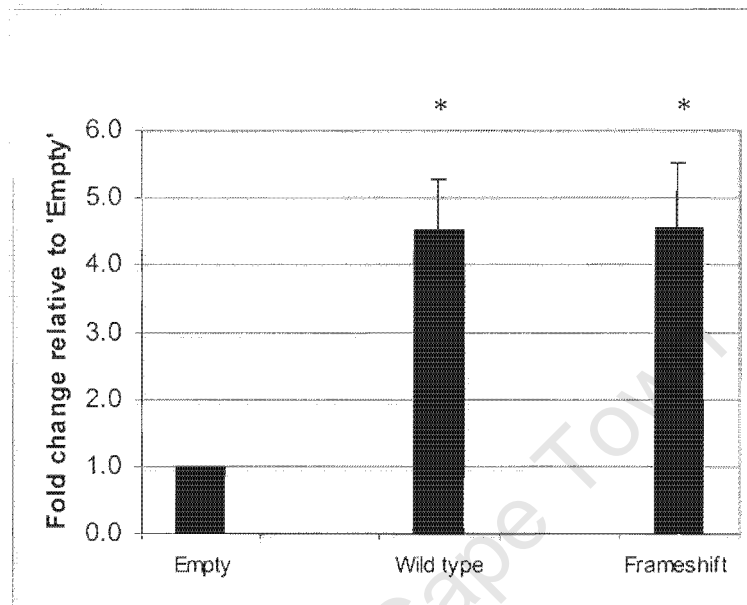


Figure 13

Glycogen content in L6 myotubes over-expressing either human wild type PPP1R3A or the PPP1R3A frameshift/ premature stop mutant. Cells infected with adenoviruses without an insert (empty) were used as the control. The data represents the mean $\pm$ SE of three independent experiments in each of which triplicate wells of a 24 well plate were infected with each adenoviral construct. * $p < 0.05$

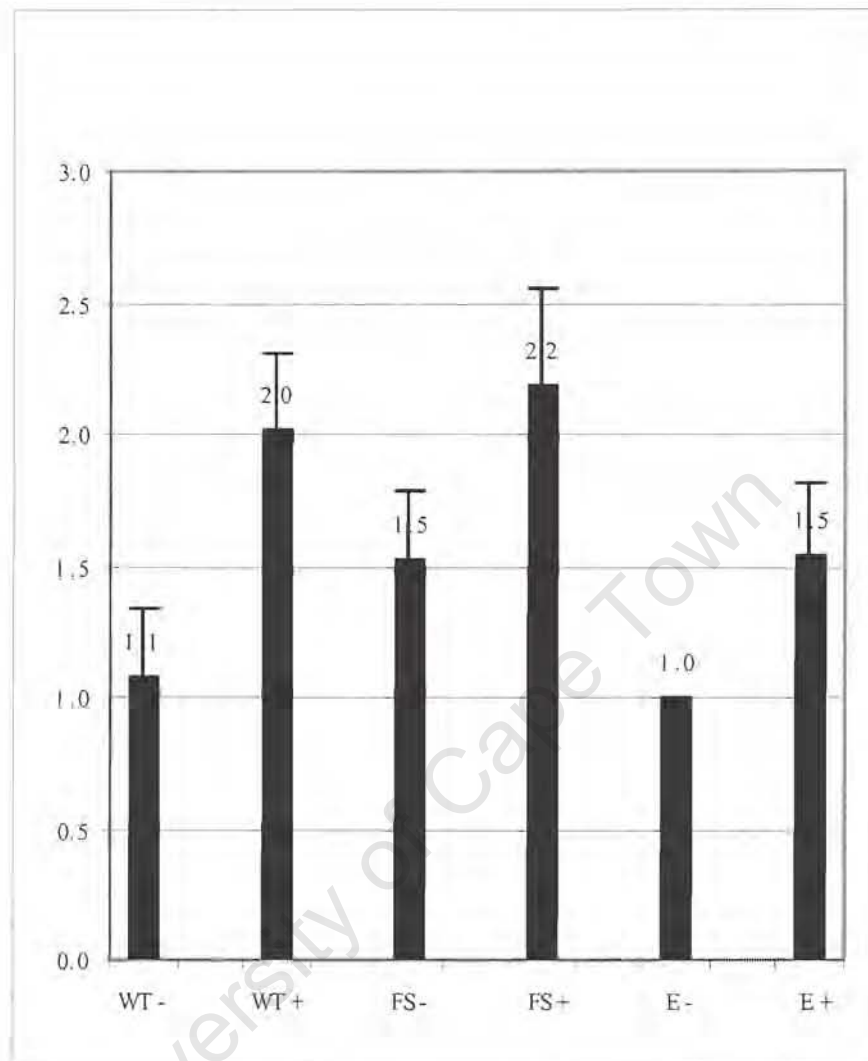


Figure 14

Glycogen synthesis in L6 myotubes over-expressing either wild type (WT) PPP1R3A or the frameshift/ premature stop PPP1R3A mutant (FS). Control cells were infected with an empty vector (E) adenoviral construct. Following a 4 hour incubation in serum and glucose free medium, cells were incubated in 5.5 mM glucose (spiked with C14-glucose) containing medium with (+) or without (-) 100 nM insulin for 2 hours. The data is normalised to E - and is the mean $\pm$ SE of triplicate measurements in three independent experiments. The differences between the different constructs were not statistically significant in either the presence or absence of insulin.

glycogen synthesis was significantly increased in the presence of over-expressed PPP1R3A (figure 15). Again this effect was seen with the wild type and mutant constructs. These observations suggest 1) that in cells deprived of glycogen for only 4 hours, the elevated glycogen content induced by PPP1R3A acts as the predominant regulator of rates of glycogen accumulation, effectively negating the stimulatory effect of the over-expressed targeting subunits, 2) that when glycogen levels are substantially depleted by depriving cells of glucose for 18 hours, over-expression of PPP1R3A does stimulate increased glycogen synthesis and 3) that loss of the C-terminal tail of PPP1R3A has no demonstrable effect on glycogen synthesis in cells massively over-expressing the protein.

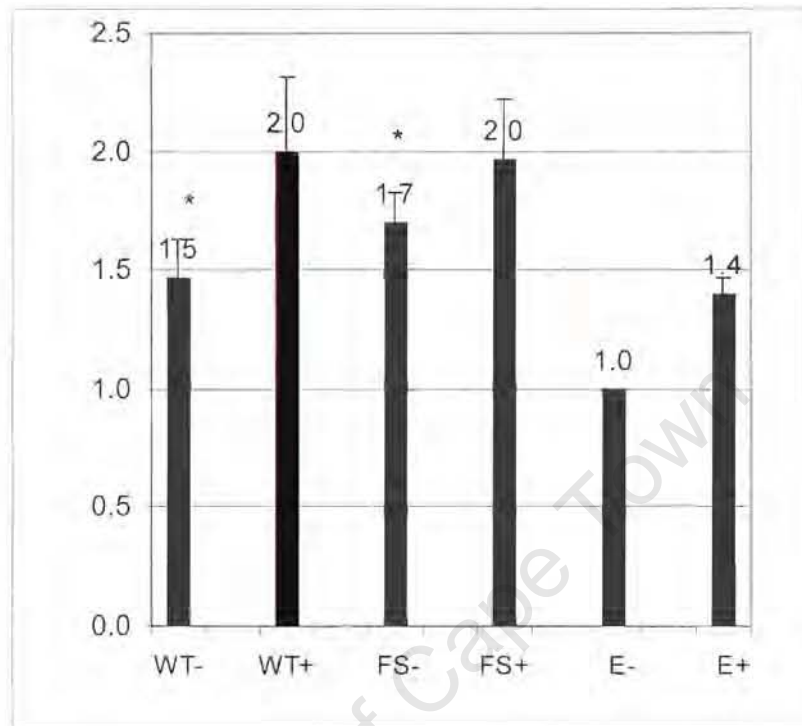


Figure 15

Glycogen synthesis in L6 myotubes over-expressing either wild type (WT) PPP1R3A or the frameshift/ premature stop PPP1R3A mutant (FS). Control cells were infected with an empty vector (E) adenoviral construct. Following a 18 hour incubation in serum and glucose free medium, cells were incubated in 5.5 mM glucose (spiked with C14-glucose) containing medium with (+) or without (-) 100 nM insulin for 2 hours. The data is normalised to E - and is the mean $\pm$ SE of triplicate measurements in three independent experiments. Only the differences between E- and WT-, and between E- and FS- were statistically significant (* $p < 0.05$).

Conclusions

As a result of 1) the *a priori* knowledge that both PPAR γ and PPP1R3A are intimately involved in insulin action 2) the fact that the mutations found result in truncated proteins with clear abnormalities in their function or localisation and 3) the observation that only the five doubly heterozygous members of kindred A and not the other seven members had unequivocal severe insulin resistance, it appears highly likely that the extreme insulin resistance phenotype seen in this family is the result of an interaction between the two mutations. Thus, familial extreme insulin resistance can now be added to the short list of human inherited conditions in which digenic inheritance has been described. These include some forms of retinitis pigmentosa (Goldberg and Molday, 1996) and junctional epidermolysis bullosa (JEB) (Floeth and Bruckner-Tuderman, 1999). Katsanis et al (Katsanis et al., 2001) have recently suggested that Bardet-Biedl syndrome (BBS) may be a complex trait requiring three mutant alleles in at least two genes to manifest the phenotype. While no previous human examples of digenic inheritance of human insulin resistance or type 2 diabetes have been described, a number of experimental genetic manipulations in murine models have established the principle that such gene-gene interaction might result in metabolic disorders. For example, Bruning et al (Bruning et al., 1997) demonstrated that while mice heterozygous for insulin receptor or IRS-1 (insulin receptor substrate-1) knockouts had minor metabolic abnormalities, doubly heterozygous animals were markedly insulin resistant and had a high incidence of diabetes. Similarly Terauchi et al (Terauchi et al., 1997) crossed IRS-1 and glucokinase

knockout mice, and produced a digenic model of type 2 diabetes. How might the mutations in kindred A interact to result in extreme insulin resistance? Of note, all previous well-documented examples of human digenic disease involve direct protein-protein interactions between the two mutant gene products. The subjects of this study differ strikingly from this paradigm as, in their case, the genes concerned are predominantly expressed in different tissues, namely skeletal muscle and fat. Whilst PPAR γ is detectable in many tissues in addition to fat including skeletal muscle, expression levels in other insulin sensitive tissues are very low making it most likely that the interaction occurs through a subtle amplifying effect of a metabolic derangement in one tissue (fat or muscle) on the other. This hypothesis is further supported by the recent generation of muscle specific PPAR γ knockout mice which are insulin sensitive and in which microarray analysis of gene expression revealed no changes in genes directly involved in insulin signalling (Norris et al., American Endocrine Society Meeting Abstract (OR11-2), 2002). Both skeletal muscle and adipose tissue are key players in "buffering" the postprandial influx of carbohydrate and lipids (Frayn, 2002), a response chiefly orchestrated by insulin. The recently acquired credibility of the notion of "metabolic dialogue" between insulin sensitive tissues is based upon several observations: i) the recognition that adipose tissue, not only responds to hormonal inputs from insulin, catecholamines etc. but also secretes a number of proteins (adipocytokines) with the capacity for endocrine, paracrine and/ or autocrine signalling (Kahn and Flier, 2000); ii) the demonstration that adipokines such as Acrp30 can induce metabolic changes in muscle (Yamauchi et al., 2001a) and liver (Berg et al., 2001), although these observations await definitive

confirmation and identification of the Acrp30 receptor; and iii) the development of muscle insulin resistance in fat-specific GLUT4 knockout mice (Abel et al., 2001). Whilst dominant negative mutations in PPAR γ demonstrably alter free fatty acid and triglyceride fluxes across adipose tissue, the precise mechanism by which loss of a single PPAR γ allele might contribute to maladaptive metabolic cross talk awaits elucidation. Although PPAR γ haploinsufficient mice appeared to be protected from high-fat diet and aging- induced insulin resistance (Kubota et al., 1999), (Miles et al., 2000), the PPAR γ frameshift mutant described herein may have properties beyond those of a null allele. Furthermore the ability of PPAR γ haploinsufficient mice to "cope with" the specific metabolic challenges posed by a defect in glycogen metabolism are still to be thoroughly explored.

The observed relationship between the presence of obesity and severe insulin resistance in kindred B suggests that in this kindred the expanded fat mass of obesity produces the "second hit" by altering adipose tissue function. This notion is supported by the dramatic effect of weight loss on fasting hyperinsulinemia in subject III in kindred B.

In the light of the relatively subtle metabolic disturbances induced by the two mutations described in this chapter, a collaborative effort was made to ascertain their frequency in the general population (this component of the study was performed in collaboration with Dr NJ Wareham, Dr A Meirhaeghe and Incyte Genomics). Whereas, the FS PPAR γ mutation was not detected in 1034 UK Caucasian subjects (517 diabetics and 517 controls) and is

therefore likely to be "private" to the index pedigree, the FS PPP1R3A mutation was found in two independent case-control studies in a total of 20/1029 UK Type 2 diabetics and 8/1033 normoglycemic controls (weighted Mantel-Haenszel odds ratio 2.53 (95% confidence limits 1.06 – 6.70, $p = 0.03$), suggesting that this mutation may predispose to type 2 diabetes in the general UK population. Given the rarity of the mutation further large multicentre population genetic studies will be required to robustly test this hypothesis. The latter are currently in progress.

Efforts to understand the effects of the PPP1R3A FS upon glycogen metabolism are also ongoing. The truncated variant is clearly mistargeted, but how and why this might alter glycogen metabolism *in vivo* remains to be clarified. Over-expressing wild type and mutant PPP1R3A in a rat myotube cell line (L6) promotes substantial glycogen accumulation, but there were no apparent differences between the full length and truncated proteins. A number of plausible explanations for this apparent similarity exist:

1. The FS PPP1R3A variant retains the domains necessary for interaction with PP1C (in fact, the capacity to bind to PP1C has been demonstrated herein), GS and glycogen; and should therefore be capable of promoting glycogen synthesis. The key question being, how mislocalisation of this "scaffolding protein" alters glycogen metabolism? As the precise reason for binding to sarcoplasmic reticulum is unknown it remains difficult to discern the consequences of loss of this binding.

2. Expression of the PPP1R3A constructs was driven by the human CMV promoter, which tends to induce massive over-expression of the downstream protein, as was apparent in figure 11. This may make identification of subtle changes between the proteins difficult.
1. Immunofluorescence studies of the wild type PPP1R3A protein in CHO cells suggest that a significant fraction of the protein is located within the cytosol (as represented by the reduction in signal after prepermeabilisation with saponin (see figure 10)), making it difficult to detect changes in metabolism specifically mediated by endoplasmic reticulum targeting.

Whilst we cannot entirely discount the possibility that the "digenic inheritance" of severe insulin resistance in five members of kindred A is a chance finding, at this stage we believe that the apparent failure to demonstrate significant *in vitro* differences between FS PPP1R3A and wild type PPP1R3A is a reflection of the limitations of this relatively crude *in vitro* approach. Therefore we remain confident that these findings provide the most tangible evidence yet available that mutations which, when present alone, have, at most, subtle effects on different, metabolically relevant tissues, can combine to result in extreme disturbances of human insulin action.

Chapter 4

Conclusions and future work

The following conclusions are drawn from the studies described herein:

1) Dominant negative mutations in the ligand binding domain of PPAR γ result in a novel form of partial lipodystrophy, an observation in keeping with the wealth of *in vitro* and rodent data suggesting that PPAR γ is the “master regulator” of adipogenesis.

2) The lipodystrophy is associated with severe peripheral and hepatic insulin resistance and all the features of the metabolic syndrome. It is also associated with triglyceride accumulation in the liver, but not in skeletal muscle.

3) The following factors are likely to be the principal determinants of the insulin resistance syndrome observed in these subjects:

- Reduced body fat mass, a circumstance consistently associated with insulin resistance in humans and rodent models
- Impaired postprandial fatty acid trapping in residual adipose tissue

- Altered adipocytokine production by adipocytes, in particular plasma adiponectin levels were very low

However, a direct role for the low levels of PPAR γ in skeletal muscle and liver cannot be entirely excluded and awaits the results of tissue specific knockout studies.

4) *Ex vivo* studies in mononuclear cells suggest that dominant negative mutations in PPAR γ induce a state of PPAR γ ligand resistance. Subjects with this condition can respond to PPAR γ agonist therapy.

5) Humans, effectively rendered haploinsufficient for PPAR γ , by a mutation which truncates the protein within the DNA binding domain, do not appear to be insulin resistant. However, this mutation does appear to alter metabolism as doubly heterozygous individuals, harbouring a second mutation in a key regulator of glycogen metabolism in skeletal muscle, are severely insulin resistant. At the time of writing, this conclusion warrants cautious interpretation as it is not inconceivable that the truncated PPAR γ mutant has properties beyond those of a purely null allele.

6) Digenic inheritance of severe insulin resistance in carriers doubly heterozygous for a PPAR γ and a PPP1R3A mutation is likely to be the result of some form of subtle metabolic cross-talk between adipose tissue and skeletal muscle as the two proteins are predominantly expressed in adipocytes and myotubes respectively. The ability of mutations which in

isolation produce mild phenotypes, to collectively induce severe phenotypic states may represent the sort of gene-gene interactions responsible for complex conditions such as type 2 diabetes.

Of note the two genes involved, PPP1R3A and PPAR γ have their major roles in the regulation of carbohydrate and lipid metabolism respectively. There has been considerable debate about the relative roles of disturbances of carbohydrate and lipid metabolism as the "prime mover" in the development of insulin resistance, the metabolic syndrome and type 2 diabetes (McGarry, 2002). The illustration that a combination of modest primary defects in both processes can have such catastrophic consequences for insulin sensitivity emphasises the requirement for taking an integrated approach to the search for etio-pathogenic pathways in common metabolic diseases such as type 2 diabetes.

The immediate aim of future work is to precisely dissect the molecular pathogenesis of the insulin resistance seen in this unique paradigmatic kindred (i.e. kindred A, chapter 3) with a view to learning more about the molecular basis for the co-ordination of human fat and carbohydrate metabolism. My principal hypothesis is that the severe insulin resistance observed in the digenic kindred is a consequence of a combination of subtle alterations in energy storage as glycogen and fat. The fact that subjects harbouring the PPP1R3A mutant alone become severely insulin resistant when obese is in keeping with this notion, as obesity might also be regarded as a state in which fat storage capacity is saturated. Of course it is also

possible that muscle and fat "communicate" by as yet unrecognised hormonal signals.

Specific approaches to be taken include:

1) Human physiological studies will address:

- Adipose tissue metabolism in PPAR γ frameshift carriers. I am currently recruiting further carriers of this mutant from kindred A (chapter 3) and plan to examine substrate fluxes across subcutaneous abdominal adipose tissue as was done in subject Ilvi (chapter 3).
- The impact of the PPP1R3A mutant on glycogen turnover will be examined by NMR spectroscopy in single heterozygotes and digenic carriers.
- The capacity for subjects harbouring the PPAR γ frameshift or both mutations to respond to a "fat challenge" will be examined in chamber calorimeters. Subjects are to be admitted to chamber calorimeters for three days on two occasions, during which measurements of resting metabolic rate, total energy expenditure, exercise-induced energy expenditure and macronutrient oxidation rates will be examined. During their stay they will either receive a eucaloric diet (53% carbohydrate, 35% fat, 12% protein) or a hypercaloric diet (130% of total energy expenditure) with excess calories provided as fat

2) The creation of a transgenic mouse harbouring the PPP1R3A mutation is in progress. This will be used:

- To examine the impact of dietary manipulation in this model
- To generate mice doubly heterozygous for the PPP1R3A mutation and PPAR γ haploinsufficiency. The *in vitro* work described in chapter 3 suggests that the PPAR γ frameshift/ premature stop mutant effectively renders carriers haploinsufficient for PPAR γ and as PPAR γ +/- mice are already available we envisage crossing these mice with the PPP1R3A transgenics.

3) Finally, a multinational collaborative project is in progress to evaluate the possibility that the PPP1R3A variant, which is present in approximately 1% of the general population (in the United Kingdom), is a "diabetogene".

Publications arising from work described in this thesis

- 1) Savage DB, Sewter C, Klenk ES, Vidal-Puig A, Considine RV, O'Rahilly S. Resistin/ Fizz3 Expression in relation to Obesity and PPAR γ action in Humans. **Diabetes** 2001; 50:2199-2202.
- 2) Savage DB, Agostini MA, Barroso I, Gurnell M, Luan J, Meirhaeghe A, Harding A-H, Ihrke G, Rajanayagam O, Soos MA, George S, Berger D, Thomas EL, Bell JD, Meeran K, Ross RJ, Vidal-Puig A, Wareham NJ, O'Rahilly S, Chatterjee VKK, Schafer AJ. Digenic inheritance of severe insulin resistance in a human pedigree. **Nature Genetics** 2002; 31:379-384.
- 3) Combs TP, Wagner JA, Berger J, Doebber T, Wang WJ, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DB, Chatterjee K, Weiss S, Larson PJ, Gotesdiener KM, Gertz BJ, Charron MJ, Scherer PE, Moller DE. Induction of adipocyte complement-related protein of 30 kilodaltons by PPAR γ agonists: a potential mechanism of insulin sensitisation. **Endocrinology** 2002; 143(3):998-1007.
- 4) Savage DB, Tan GD, Acerini CL, Jebb SA, Agostini M, Gurnell M, Williams RL, Umpleby MA, Thomas EL, Bell JD, Dixon AK, Dunne F, Boiani R, Cinti S, Barroso I, Schafer AJ, Vidal-Puig A, Karpe F, Chatterjee VKK, O'Rahilly S. The Human Metabolic Syndrome Resulting from Dominant Negative Mutations in the Nuclear Receptor PPAR γ . **Diabetes in press 2003.**

Contributions to textbooks

1) George S, Savage DB, O'Rahilly S

Hereditary and Acquired Syndromes of Severe Insulin Resistance

Textbook of Diabetes, 2002 Edition, Edited by Williams and Pickup

2) Savage DB, O'Rahilly S

Case history (Severe insulin resistance)

Fifty Cases of Diabetes Mellitus, Edited by Betteridge, *in press*

Manuscripts in preparation

1) Agostini M, Gurnell M, Savage DB, Wood EM, Smith A, Rajanayagam, Garnes KT, Levinson SH, Xu HE, Schwabe JW, Willson TM, O'Rahilly S, Chatterjee VKK. Tyrosine agonists reverse the molecular defects associated with dominant negative mutations in human PPAR γ .

- submitted to ***J. Biol. Chem.***

2) Savage DB, Barroso I, Denyer G, Lelliot C, Vidal-Puig A, Schafer AJ, O'Rahilly S. PPAR γ dependant changes in gene expression during human adipogenesis: studies in human pre-adipocytes with a dominant-negative mutation in PPAR γ .

- submitted to ***J. Clin. Endo. Metab.***

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Appendix A: Taqman primer and probe sequences

	Forward primer (5' – 3')	Reverse primer (5' – 3')	Probe (5'FAM and 3'TAMRA fluorescent labelling)
Human resistin	ATCAATGAGAGGATCCAGGAG	TCC AGG CCA ATG CTG CTT A	CGCCGGCTCCCTAATATTTAGGGC
Human fatty acid binding protein 4 (FABP4)	GGAAAATCAACCACCATAAAGAGAA	GGAAGTGACGCCTTTCATGAC	ACGAGAGGATGATAAACTGGTGGTGAATG
Human monoamine oxidase-A(MAO-A)	TGACACCAAGCCAGATGGG	CAGCTTTCGGGCAAGAA	CACTGCCTGCCATCATGGGCTTC
Human phospholipase A2	CTGTGGCGTGGGTGGC	CATGAGTGACACAGCAGCGA	AGGATCCCCCAAGGATGCAACGG
Human apolipoprotein D	CCGGACTGGCTGGCC	GCACTTCCCAAGATGAAATGC	CTTCGGTGCGGCAGAGGGACA
Human pyruvate dehydrogenase kinase 4 (PDK4)	TCCACTGCACCAACGCCT	GCAAGCCGTAACCAAAACCA	ATGGATAATTCCCGGAATGCTCCTTTGG
Mouse PDK4	ACCTTCACCACATGCTCTTCG	GACGGTTTTCTTGATGCTCGA	ACTCTTCAGAATGCCATGAGGGCCAC