Investigation of the genetic basis of insulin resistance and cardiovascular disease

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Declaration of Thesis

The work described in this thesis is my own except for the following; the routine biochemical assays were measured at the RISC study centres.NEFA, total cholesterol, triglycerides, LDL, HDL were undertaken by Mr. Peter Gaffney, Clinical Chemistry Laboratory, Adelaide and Meath Hospitals (Incorp. the National Children's Hospital), Dublin, Ireland.Insulin, C-peptide, glucagon, glucose, pro-insulin and insulin split products were performed by Ms Charlotte Olsen, Odense University Hospital, Denmark.Urine samples were performed by Ms A Kok, department of Clinical Chemistry, Amsterdam, the Netherlands. Plasma adiponectin measurements were carried out by Allan Flyvbjerg and Jan Frystyk. Medical Research Laboratories, Clinical Institute and Medical Department (Diabetes and Endocrinology), Aarhus University Hospital,Denmark.

I have undertaken the clamp studies and oral glucose tolerance test for the 90 subjects recruited at Newcastle centre and other centres were responsible for their own assessments. I extracted 1078 DNA samples and genotyped these samples for PPARγ and SNP+45 of ADIPOQ gene. Sequencing of DNA for the PPARγ and adiponectin polymorphisms and DNA extraction of 200 samples and genotyping of these samples for PPARγ and SNP+45 of the adiponectin gene was carried by Dr. Sheila Patel, School of Clinical Medical Sciences, University of Newcastle upon Tyne, UK. Initially I genotyped 2 of the adiponectin promoter gene (11377 &11391) using the Sequenom method.The repeat genotying of these 2 polymorphisms in addition to SNP 11246 of the adiponectin promoter gene was carried by Dr. Sheila Patel, School of Clinical Medical Sciences, University of Newcastle upon Tyne, UK.

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ABBREVIATIONS

T2DM Type 2 diabetes

CVD Cardiovascular disease

c IMT Carotid intima media thickness

HDL High density lipoprotein

TG Triglycerides

LDL Low density lipoprotein

IR Insulin resistance

HOMA-IR Homeostasis assessment model of insulin resistance

IGT Impaied glucose tolerance

FFA Free fatty acid

TNFα Tumour necrosis factor

NO Nitric oxide

FSIVGTT Frequently sampled intravenous glucose tolerance test

BMI Body mass index

ECG Electrocardiograph

NEFA Non estrified fatty acid

γ GT Gama glutamerase transferase

BSA Body surface area

W Weight

H Height

LBM Lean body mass

OGTT Oral glucose Tolerance Test

Abstract

Introduction

Cardiovascular disease (CVD) is the major cause of morbidity and premature mortality in type 2 diabetes (T2DM). Carotid artery intima-media thickness (cIMT) is a marker of cardiovascular disease. Carotid artery IMT heritability estimates are increased in both healthy and type 2 diabetic families, providing support for the role of genetic factors. This close link between CVD and T2DM raises the possibility that common gene variants might increase susceptibility to both conditions, the "common soil" hypothesis. Common variants of the genes encoding PPARγ and adiponectin have been found to increase susceptibility to type 2 diabetes. The hypothesis to be investigated is that these susceptibility genes exhibit pleiotropy and increase the risk of CVD.

Methods/Results:

Subjects aged 30-60 years were recruited at 19 centres in 14 European countries. Each subject underwent anthropometric and metabolic assessment including euglycaemic hyperinsulinaemic clamp to study insulin sensitivity and β-mode ultrasound scan for the measurement of carotid IMT.The study cohort consists of 1278 subjects. Pro12 Ala was genotyped by restriction fragment length polymorphism (RFLP-PCR). SNP T-45G and SNPs (A-11426G, G-11391A and C-11377G) of the ADIPOQ gene were detected by Sequenom and TaqMan assay respectively.

Results/Conclusions:

In this study we confirmed that the Pro12Ala of the PPARγ variant influences insulin sensitivity in the healthy population. Specifically, subjects homozygous for the Ala allele are more insulin sensitive compared to the rest of the population independent of measures of adiposity. However in this cross sectional data, there was no significant association between cIMT and the genetic variation of the Pro 12 Ala variant.

We also found that SNP +45 of the ADIPOQ gene influences insulin sensitivity independent of serum adiponectin in this healthy population. Subjects homozygous for the G allele are less insulin sensitive compared to the T allele carriers, have high waist circumference and fasting NEFAs. However, there was no association of this polymorphism and c IMT, a measure of CVD.

However, it was established that variation in the ADIPOQ gene promoter was directly associated with carotid IMT, and this was independent of circulating adiponectin levels and classical CVD risk factors. This observation needs to be replicated in other cohorts, and further evidence is needed to explore the potential mechanisms by which variation in adiponectin influence IMT and CVD.

PUBLISHED RESULTS FROM THESIS

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1. Introduction

1.1 Cardiovascular disease and type 2 diabetes

Cardiovascular disease is a major cause of morbidity and premature mortality in patients with type 2 diabetes. Indeed, cardiovascular disease mortality is two to three fold higher in type 2 diabetic patients compared to the background non-diabetic population (1, 2). As the worldwide prevalence of type 2 diabetes inexorably increases (predicted to reach 221 million by 2010) (3), it is anticipated that there will be a parallel increase in the associated cardiovascular disease.

It is reported that type 2 diabetic patients with no established cardiovascular disease have a similar risk to those who have had a stroke or myocardial infarction without diabetes (4-6). Cardiovascular disease is the most common complication of type 2 diabetes and accounts for about 80% of all cause mortality in type 2 diabetic patients (7). Furthermore, patients with type 2 diabetes tend to have a worse outcome after a cardiovascular event compared to the non diabetic patients. (8-10).

The aetiology of this excess cardiovascular disease mortality appears to be heterogeneous. Insulin resistance is the key predictor for the development of type 2 diabetes and appears to be important in the pathogenesis of cardiovascular disease. The Insulin Resistance Syndrome is a cluster of cardiovascular risk factors associated with premature cardiovascular mortality (11, 12). Following the original description of the insulin resistance syndrome, other cardiovascular risk factors have been recognised as part of the cluster. However the excess in cardiovascular disease in type 2 diabetes is not fully

explained by the increased prevalence of classical cardiovascular disease risk factors (13), emphasising the fact that other factors contribute to the increased cardiovascular disease risk. The pathogenesis of both type 2 diabetes and insulin resistance are largely unknown as they comprise complex traits in which multiple gene effects and environmental factors combine to contribute to the overall pathogenesis and this make it difficult to search for underlying susceptibility genes.

1.2 Insulin Sensitivity

1.2.1 Measurements of Insulin sensitivity:

Insulin resistance is defined as the reduced ability of insulin to control blood glucose by promoting glucose uptake in target tissues and by inhibiting glucose production by the liver (14). The effect of insulin on tissues can be assessed by different methods; this includes peripheral glucose clearance and suppression of glucose output from the liver. When measuring insulin sensitivity it should be considered that different tissues express different insulin sensitivities (15).

One of the methods, which has been widely used in clinical research to assess insulin sensitivity is the homeostasis assessment model of insulin resistance (HOMA-IR). It is obtained from fasting glucose and insulin concentration using a computer model (16). The fasting concentration of glucose (expressed as mg/dL) and insulin (expressed as mu/ μL) is divided by a constant. HOMA-IR is expressed as a percentage of normal. It must be noted that it measures basal rather than stimulated insulin resistance and hence it is rather a predictive than an accurate measure.

Fasting insulin is often used as a surrogate marker for insulin sensitivity. However, plasma insulin levels are only an approximate estimate of in vivo insulin sensitivity. There is interlaboratory variability and high coefficient variance of insulin measurement, which can result in up to three-fold variation in insulin concentrations and hence affect this method as a sensitive measure of insulin sensitivity (17). Furthermore, it is not known whether hyperinsulinemia alone, insulin resistance or both contribute to the observed association with cardiovascular disease.

Insulin sensitivity can be determined from intravenous glucose tolerance tests (IVGTT). The minimal model described by Bergman and Cobelli gives an estimate for both insulin resistance and beta cell function (18).

The minimal model depends on glucose disappearance rate in response to insulin. The pitfall of this model is that it assumes an intact endogenous insulin secretion, which is not the case in all non-diabetic healthy individuals. Estimates of insulin sensitivity obtained from the frequently sampled intravenous glucose tolerance test (FSIGTT) have been reported to correlate well with those from the euglycaemic clamp technique. The test requires up to 3 hours and a computer program for analysis. It also lacks the standardization in methodology, which makes it difficult to compare with other studies (19).

The insulin sensitivity test (IST) involves intravenous infusion of glucose load and a fixed rate of insulin over 3 hours. Somatostatin can be given to inhibit gluconeogensis, prevent insulin secretion and to delay the counter regulatory hormone secretion. Insulin sensitivity is derived from the mean plasma glucose over the last 30 minutes. A simpler version of IST is the insulin tolerance test. It measures the decline in serum glucose after an intravenous bolus of insulin is administered. Depending on the protocol used, it measures insulin-stimulated uptake of glucose into skeletal muscles (20).

The Oral glucose tolerance test (OGTT) can be used to assess insulin sensitivity. This is obtained from the logarithm of 2 hours plasma insulin concentration following OGTT (21). A model described by Mari et al, used the OGTT to derive the oral glucose insulin sensitivity index (OGIS). It requires insulin and glucose concentration from a 75 gram standard OGTT to determine whole insulin sensitivity. This method has been shown to correlate well with results from the hyperglycaemic clamp technique and because of its simplicity, it has the potential to be used in large epidemiological studies. (22). However, it has yet to be validated or widely used.

The euglycaemic clamp technique has become the gold standard method for measuring insulin sensitivity. Glucose is clamped at a predetermined level by varying and titrating the glucose infusion rate against a fixed insulin infusion rate. Once a steady state of glucose uptake rate has been reached the degree of insulin resistance is inversely related to the amount of glucose used during the clamp study. It is time consuming, labour intensive and an expensive measure of insulin sensitivity and this makes it difficult to be used in large epidemiological studies. It uses supraphysiological levels of insulin

especially for patients with diabetes (23). Nevertheless it remains the most sensitive method to assess insulin sensitivity in vivo.

In summary, there are number of methods to assess insulin sensitivity depending on the size and the type of the study. Most experts would agree that the euglycemic hyperinsulinaemic clamp technique is the gold standard method of measuring insulin sensitivity in non diabetic healthy volunteers (15).

1.2.2 Insulin sensitivity and Type 2 diabetes:

The worldwide increase in the overall prevalence of type 2 diabetes has been linked to the sedentary life style which leads to obesity and insulin resistance. Type 2 diabetes is a complex metabolic disorder, characterized by a combination of ß-cell dysfunction, peripheral insulin resistance and increased hepatic glucose production (24). Many patients with type 2 diabetes have clinical evidence of insulin resistance. This includes a cluster of cardiovascular risk factors, hypertension, dyslipidaemia (raised triglycerides and low HDL cholesterol) and central obesity (25, 26). Central obesity has been recognised as a key etiological feature in the development of insulin resistance (7). Insulin resistance is thought to be critical in the development of type 2 diabetes. It has been reported that up to three out of four individuals with type 2 diabetes meet the diagnostic criteria for the metabolic syndrome and have evidence of insulin resistance (7).

It has been suggested that the pathogenesis of type 2 diabetes includes primarily insulin resistance, which result initially in hyper secretion of insulin by the pancreatic ß-cells. This leads eventually to beta cell failure and the development of clinical diabetes (27). Prospective studies have demonstrated that hyperinsulinaemia is an independent predictor of type 2 diabetes, the insulin resistance syndrome and cardiovascular disease (28, 29). In a prospective study of non-diabetic first-degree relatives of northern European extract, it has been shown that insulin resistance is an independent risk factor for the deterioration in glucose tolerance (30).

Insulin resistance is reported to be present in about 90% of individuals with type 2 diabetes and impaired glucose tolerance (IGT) individuals (31). However not all individuals with insulin resistance will develop type 2 diabetes which suggests there must be other possible mechanisms in the development of type 2 diabetes.

The genetic susceptibility, which contributes to the clinical and metabolic components of type 2 diabetes, remains largely unknown. There is evidence that multiple gene defect influence overall susceptibility to type 2 diabetes (32).

1.2.3 Insulin sensitivity and CVD:

Insulin resistance is an underlying feature of both the metabolic syndrome and type 2 diabetes (28). It is associated with abnormalities in both glucose and lipid metabolism. These abnormalities are associated with an increased risk of cardiovascular disease and are often present before the onset of type 2 diabetes (29). There is now convincing evidence that cardiovascular disease begins before the onset of clinical diabetes (33). However, it is not known which specific element of the diabetic state is responsible for the increase in cardiovascular risk. As hypertension, hyperglycemia, hemostatic factors, proinflammatory cytokines and changes in circulating lipids tend to cluster in insulin resistance, it is not clear which of these factors can account for the excess risk of cardiovascular disease.

Insulin resistance is now believed to underlie the clustering of metabolic abnormalities that mark the onset of cardiovascular disease. It has been hypothesized that insulin resistance per se may promote the development of cardiovascular disease (34). Although it was suggested that insulin resistance might initiate cardiovascular disease, the evidence for the direct role of insulin resistance is not yet available (35). The San Antonio Heart Study showed a link and overlap between hyperinsulinemia, type 2 diabetes, obesity, hypertension and dyslipidemia indicating that the cluster of the insulin resistance syndrome carries an increase risk of cardiovascular disease (31). However, there is evidence that hyperinsulinemia exerts a distinct pathological effect independent of insulin resistance, which implies that hyperinsulinaemia and reduced insulin sensitivity are two separate entities (34, 36).

Table [1] summarize some of the recent evidence of the association between insulin sensitivity and cardiovascular disease, with key points from each study. (33, 34, 37, 38, 39). Both the Verona diabetes complication study and IRAS study showed that insulin resistance was an independent predictor of cardiovascular disease (34, 37). However, in the IRAS study, insulin resistance was not totally independent predictor of CVD as measured by c IMT. This association was reduced but not completely eliminated by the classical CVD risk factors, suggesting that the association is partly mediated by traditional CVD risk factors and this association was not seen in black African Americans. In the Paris Prospective study, fasting plasma insulin level and the fasting insulin-glucose ratio were positively associated with coronary heart disease independent of the other CVD risk factors in middle aged 7246 non diabetic men followed up for 5 years (158)

The results of the large longitudinal study, (RISC) study, to determine whether insulin resistance predicts the development of cardiovascular disease in a healthy non-diabetic population of European descent, are still awaited (40). One of the first conclusions of the RISC study was recently published; this showed that insulin resistance is not the sole driver of the cardiovascular risk with a major contribution of central obesity and high circulating insulin levels (41).

Table 1: Insulin resistance and cardiovascular disease, review of the evidence.

Name of the Study	**Numbers/ Population**	**Measurement of insulin sensitivity**	**Follow up (Yrs)**	**Outcome/Findings**
San Antonio Study Haffner SM. *Diabetes*(1992) 41: 715-722	614 Mexican Americans	Fasting insulin	8	Fasting insulin predicted development of type 2 diabetes, ↓HDL-C, ↑TG & hypertension. Baseline insulin concentrations were higher in subjects who subsequently developed multiple metabolic disorders.
IRAS study Howard G. Circulation. (1996) 93: 1809-1817	1600(Total) 398(Black) 457(Hispanic) 542(non-Hispanic white)	IVGTTwith minimal modelling	N/A	Significant association between insulin resistance and atherosclerosis of the (c IMT), this is partially dependent and mediated by traditional CVD risk factors. No significant association between IR and c IMT in African Americans.

Verona Diabetes Complications Study Bonora E. *Diabetes Care* (2002)25: 1135-1141	1,326 Patients with type 2 diabetes	HOMA-IR	4.5	HOMA-IR was an independent predictor of both prevalent and incident CVD. Improvement of insulin resistance might have beneficial effects on CVD in patients with type 2 diabetes.
Strong Heart Study Resnick HE. Diabetes Care (2003) 26: 861-867	2,283 Non diabetic American Indians	HOMA-IR	7.6	Insulin resistance was associated with established CVD risk factors including BMI, waist circumference, blood pressure, incident diabetes and lipid levels. Insulin resistance on its own did not predict incident CVD.
VA-HIT study Robins SJ. Diabetes Care (2003)26: 1513-1517	2,283 Men with CHD	HOMA-IR	5	Significantly higher risk of CVD event with insulin resistance The rate of new CVD events and the reduction of events with Gemfibrozil were in subjects with IR than in those without.

The close association of type 2 diabetes with cardiovascular disease led to the hypothesis that they both share a common antecedent. This concept has been labelled the 'common soil hypothesis' (42, 43).

The metabolic syndrome is a cluster of metabolic abnormalities. Each of the component which constitutes the metabolic syndrome, is an established cardiovascular risk factor and the risk becomes exponentially higher when these components are combined together. In the Botnia study, the prevalence of coronary heart disease, myocardial infarction and stroke were approximately threefold higher in subjects with the metabolic syndrome than in those without (7). The global epidemic of type 2 diabetes is believed to be driven by the rise in the prevalence of the metabolic syndrome.

The most widely used definitions for the metabolic syndrome come from the World Health Organization (WHO), European group for the study of insulin resistance (EGIR) and the National Cholesterol Education Program – Third Adult Treatment Panel (NCEP ATP III, the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) and the International Diabetes Federation (IDF) (5, 44, 45, 46,159).

The WHO definition of the metabolic syndrome includes insulin resistance as one of the major underlying contributor to the metabolic syndrome, the classification is summarised in table (2).

EGIR have recommended a number of changes to the WHO definition. It excluded patients with type 2 diabetes. Central obesity is defined as waist circumference ≥80 cm for women and ≥94 cm for men. Use of fasting insulin levels to estimate insulin resistance (instead of the euglycemic clamp) and impaired fasting glycemia as a substitute for impaired glucose tolerance in epidemiologic studies.

For a person to be diagnosed with the metabolic syndrome by the NCEP ATP III definition they must have three or more of the five risk factors summarised in table (3).

Because the metabolic syndrome comprises established cardiovascular disease risk factors, both the WHO and the NCEP ATP III definitions have been shown to be predictive of cardiovascular disease.

Type 2 diabetes can be regarded as a cardiovascular disease risk equivalent and hence the need to target these cardiovascular disease risk factors is fundamental when treating patients with type 2 diabetes. Higher proportions of the population meet the NCEP ATP III definition of the metabolic syndrome than the WHO definition because the NCEP ATP III definition has a lower diagnostic threshold for certain characteristics.

In 2004, the International Diabetes Federation (IDF) held an expert workshop to examine how the currently available definitions for the metabolic syndrome could be improved and developed with the aim of reaching a consensus for the introduction of a new and unifying definition. This is summarised in table (4).

Table 2: The WHO definition of the metabolic syndrome (1999)

For a person to be diagnosed with the metabolic syndrome they must have glucose intolerance, IGT or diabetes and/or insulin resistance together with two or more of the following components listed below:
1. Impaired glucose regulation or diabetes. 2. Insulin resistance (under hyperinsulinemic euglycemic conditions, glucose uptake below lowest quartile for background population under investigation). 3. Raised arterial pressure ≥140/90 mmHg. 4. Raised plasma triglycerides (≥1.7 mmol/L; 150 mg/dL) and/or low HDL cholesterol (<0.9 mmol/L, 35 mg/dL men; <1.0 mmol/L, 39 mg/dL women). 5. Central obesity (males: waist to hip ratio > 90 cm; females: waist to hip ratio > 85 cm) and/or BMI >30 kg/m2. 6. Microalbuminuria (urinary albumin excretion rate ≥20g/min or albumin: creatinine ratio ≥30 mg/g).

Table 3: The NCEP ATP III – The metabolic syndrome definition (2001)

For a person to be diagnosed with the metabolic syndrome they must have three or more of the following five risk factors:	
Risk Factor	**Defining Level**
(1) Abdominal obesity (Waist circumference) Men Women	 >102 cm (>40 in) >88 cm (>35 in)
(2) Triglycerides	≥150 mg/dL (1.7 mmol/L)
(3) HDL cholesterol Men Women	 <40 mg/dL (1.04 mmol/L) <50 mg/dL (1.29 mmol/L)
(4) Blood pressure	≥130/ ≥85 mmHg
(5) Fasting glucose	≥110 mg/dL (6.1 mmol/L)

Table 4: The International Diabetes Federation (IDF) consensus definition of the metabolic syndrome

Central Obesity	**Waist circumference** (ethnicity specific) for Europids: Male ≥ 94 cm Female ≥ 80 cm
Plus any two of the following:	
(1) Raised Triglycerides	≥150mg/dL (1.7mmol/L) Or specific treatment for this lipid abnormality
(2) Reduced HDL Cholesterol	<40mg/dL (0.9 mmol/L) in males <50mg/dL (1.1 mmol/L) in females or specific treatment for this lipid abnormality
(3) Raised blood pressure	Systolic: ≥130 mmHg or Diastolic: ≥85 mmHg or Treatment of previously diagnosed hypertension
(4) Raised fasting plasma glucose	Fasting plasma glucose ≥100 mg/dL (5.6 mmol/L) or Previously diagnosed type 2 diabetes If above 5.6 mmol/L or 100 mg/dL, OGTT is strongly recommended but is not necessary to define presence of the syndrome.

It is still unclear how insulin resistance per se could result in atherosclerosis. Some possible mechanisms by which insulin resistance could result in the observed increase in cardiovascular disease have been suggested.

Elevated plasma free fatty acids (FFA) concentrations are common in type 2 diabetes (47). It has been shown that early changes in FFA concentrations are predictive of the transition of patients from impaired glucose tolerance (IGT) to type 2 diabetes (48, 49). Some studies indicated that elevated circulating FFA might directly contribute to the development of both peripheral and hepatic insulin resistance (50, 51). High plasma FFA concentrations are associated with a number of cardiovascular risk factors linked to insulin resistance, including hypertension, dyslipidemia, hyperuricemia and abnormal fibrinolysis.

Although the exact relationship between elevated FFA, insulin resistance and beta-cell dysfunction requires further investigation, there is good evidence that FFA concentrations represent an important therapeutic target in obesity and type 2 diabetes.

It has been suggested that a number of potentially antiatherogenic properties of insulin may be impaired in the insulin resistant state. This includes impaired anti-aggregation of platelet effect, nitric oxide release from the endothelium, inhibition of fibrinogen synthesis and impaired inhibition of migration of vascular smooth muscle cells (52-55).

Insulin resistance is also associated with high levels of inflammatory factors, which may be one of the adverse consequences of obesity. It has become increasingly clear that visceral fat cells have an endocrine function. In particular, these cells produce a cytokine tumour necrosis factor (TNFα), which lowers insulin sensitivity and induces low-grade inflammatory activity. This leads to a thrombogenic tendency and may be involved in the mechanisms that lead to vascular dysfunction, hypertension and atherosclerosis (51). It is still not clear whether insulin resistance contribute to the development of some of the complications of type 2 diabetes through a pathway that is distinct from those related to the classical risk factors.

There is evidence linking vascular abnormalities with both insulin resistance and cardiovascular disease, supporting the fact that primary defects in insulin signalling pathway leads to reduce glucose uptake in skeletal muscle and adipose tissue as well as altered endothelial nitric oxide (NO) synthesis (56). It has been postulated that, it is the combination of these defects in insulin signalling pathway, which is manifested as metabolic abnormalities e.g.(Hyperglycaemia and dyslipideamia) in addition to vascular insulin resistance, which leads to cardiovascular disease (56). One can speculate that it is the "vascular insulin resistance" which will subsequently result in endothelial dysfunction and predispose to cardiovascular disease (56).

1.3 Evidence of the genetic basis of insulin sensitivity

1.3.1 Family studies

The role of genetic variation in the pathogenesis of type 2 diabetes and insulin resistance was seen in family studies. This includes studies of monozygotic and dizygotic twins and extended families and pedigrees (57).

It has been shown that the concordance rates for type 2 diabetes are higher in monozygotic twins compared to dizygotic twins (58, 59). Studies of different ethnic groups have demonstrated an early presence of insulin resistance in nondiabetic relatives of subjects with type 2 diabetes. It has been shown that in non diabetic first degree relatives of type 2 diabetes patients, there is threefold increased risk of developing type 2 diabetes compared to the general population (60, 61). Gulli *et al.*who used the euglycaemic hyperinsulinaemic clamp technique to measure insulin resistance, had reported impaired glucose disposal in the offspring of type 2 diabetic parents compared to the controls (62). Both first and second phase insulin secretion were impaied in the offspring, indicating compensatory hyperinsulinaemia in these subjects (63).

The evidence that insulin resistance has an underlying genetic predisposition was supported by the familial clustering both in Pima Indians and Caucasians using the euglycaemic hyperinsulinaemic clamp technique and the frequently sampled intravenous glucose tolerance (FSIVGTT) respectively (64, 65), to measure insulin resistance.

Furthermore the fact that insulin resistance is present as an early feature in non diabetic first degree relatives who are at higher risk for developing type 2 diabetes produce indirect evidence of the genetic basis of insulin resistance (63, 66).

It has been shown previously, that patients with type 2 diabetes with clinical evidence of underlying insulin resistance, have defects of insulin stimulated glucose uptake into skeletal muscles. Cultured myotubes from these subjects tend to retain these defects after prolonged culture, which appear to be specific to the insulin signalling pathway, indicating an underlying genetic basis of these defects of insulin action (67).

Other evidence for the genetic basis of inherited defects of insulin resistance comes from the finding of reduced insulin responsiveness of glucose uptake in culture myoblasts from insulin resistant non diabetic relatives of type 2 diabetic families (68). However both type 2 diabetes and insulin resistance are complex traits with multiple genetic and environmental factors combine to contribute to the pathogenesis of these conditions, and this makes it challenging to study the genetic basis of these conditions (69).

1.3.2 Heritability of insulin sensitivity

Heritability is defined as the proportion of variance in a trait that is attributable to the additive gene effect. Heritability of insulin resistance using fasting insulin as indirect measure was estimated to be 0.35 in a study of extended pedigree (70). Twin studies have demonstrated the heritability of glucose and insulin values and estimated the heritability of insulin resistance to range from 0.47-0.66 (58, 71-73).

In middle-aged twin pairs in a Dutch study, the heritability estimate for fasting insulin was 0.25. Another twin pairs from Finland, which used the euglycaemic hyperinsulinemic clamp technique to measure insulin resistance, estimated the heritability of insulin resistance to be 0.37 by variance in glucose uptake clamp and more than 0.55 heritability of insulin secretion, using the intravenous glucose tolerance test (74).

A recent Finnish study aimed to evaluate the genetic and environmental effects on plasma glucose, insulin secretion and insulin resistance. They studied 85 monozygotic and 85 dizygotic twins and showed the heritability estimates for fasting insulin to be 0.43 (75).

A recent Danish twin study used euglycaemic hyperinsulinemic clamp technique in 110 younger and 86 older twins and showed tnat the heritability estimates for peripheral insulin sensitivity to be 0.53 and 0.55 in the younger and older group respectively.

Another study used samples from the Diabetes UK Warren type 2 consortium. They included 811 nondiabetic relatives from 278 pedigrees of northern European extraction with at least 2 siblings with known diabetes. They described the heritability estimates for fasting glucose to be 0.72 and 0.29 for insulin sensitivity using the HOMA model. The heritability for features of insulin resistance was also significantly high in these families with increased susceptibility of type 2 diabetes (76). Freemen et al studied families from

the same background but with no increase in susceptibility to type 2 diabetes nor characterized by a high degree of insulin resistance. They reported relatively lower but still significantly high heritability estimates in fasting glucose and similar estimates for the features of insulin resistance as the previous study (77).

In summary there are consistent findings of significant heritability for insulin resistance, but values may vary and this may simply reflect differences between methods of measurement of insulin resistance and or differences in study populations.

1.4 Carotid artery intima-media thickness (c IMT):

1.4.1 c IMT as a marker of cardiovascular disease

Carotid artery intima –media thickness (cIMT), imaged by high resolution B-mode ultrasonography has been shown to be a reliable marker of sub-clinical atherosclerotic cardiovascular disease (78).

In addition, increased cIMT has been shown to be a predictor of myocardial infarction and stroke, independent of the classical risk factors in older subjects lacking a prior history of cardiovascular disease (79, 80). As expected, it has been confirmed that the classical cardiovascular disease risk factors such as hypertension, hyperlipidaemia, male sex, age, smoking and postmenopausal status all contribute to an increase in carotid IMT (80-82).

The carotid intima-media consists of endothelial cells, connective tissue, and smooth muscle and constitutes the site of lipid deposition in plaque formation (83). In healthy adults they range from 0.25 to 1.5 mm, (84) and values greater than1.0 mm are regarded as abnormal (83). Age is considered as a powerful determinant of c IMT with 0.01 to 0.02 mm increase each year (84, 85). Carotid IMT has been proposed as a surrogate marker of cardiovascular disease (86). It has been suggested that ultrasonographic defined carotid intima media thickness is a better model to study CVD than clinical manifestation of cardiovascular disease, such as myocardial infarction and stroke. The later were described to be distant phenotypes from their genes and gene products (87). As carotid intimal-medial thickness is predictive of clinical cardiovascular disease, and can be imaged and

then measured noninvasively using ultrasound, it can provide an alternative method to study early atheroscleoris. On the other hand genetic variants reported to be associated with clinical cardiovascular disease show weak or no relationship to carotid atherosclerosis. This could be explained by the inconsistency in the associations between clinical cardiovascular disease and the genetic variants or simply carotid atherosclerosis and clinical cardiovascular disease are two different phenotypes or represent different stages of the disease, e.g. IMT as a marker of vessel disease but myocardial infarction as a vessel disease marker in addition to other thrombotic factors leading to plaque instability and rupture (88). Recently linkage and association studies of carotid atherosclerosis, prove to be more encouraging (89).

1.5 Evidence of the genetic basis for cIMT

1.5.1 Heritability of cIMT

There is emerging evidence to support the role of familial, possibly genetic factors in determining the variability in cIMT. A number of studies have used family and twin based methods to estimate heritabilities for cIMT, with the hypothesis that a significant heritability estimate provides support for the involvement of gene effects. Duggirala et al studied sib-ships of mixed ethnicity in Mexico City and found a very high heritability estimate of 0.90 for the cIMT after adjustment for a number of established CVD risk factors, suggesting a major genetic component for the development of carotid artery atherosclerosis (90).

A second study used the same family based approach reported a lower (0.30) but still significant heritability estimate (91). A more recent study estimated cIMT heritability using the classical twin based approach (92). They studied 71 monozygotic and 61 dizygotic twin pairs and estimated the heritability to be 0.31. While this did not reach statistical significance, perhaps reflecting the difficulty in achieving adequate power with twin-based studies, the value nonetheless is almost identical to that of 0.30 estimated by Zannad et al (91) using the family based method.

The twin and family based studies described so far have been conducted in non-diabetic and otherwise healthy individuals. Lange et al have estimated the heritability of cIMT in the type 2 diabetic members of families in which there were at least two relatives with type 2 diabetes (93). Carotid IMT was measured in 252 type 2 diabetic patients from 122 families. The heritability estimate for cIMT was significantly increased at 0.32 after adjustment for age, sex and ethnicity. Interestingly, the heritability estimate increased (to 0.41) and remained significant after adjustment for the key classical cardiovascular risk factors (94) The increased heritability estimate of cIMT in type 2 diabetic families raises the intriguing question as to whether there are pleiotropic gene effects that increase susceptibility to both diabetes and CVD.

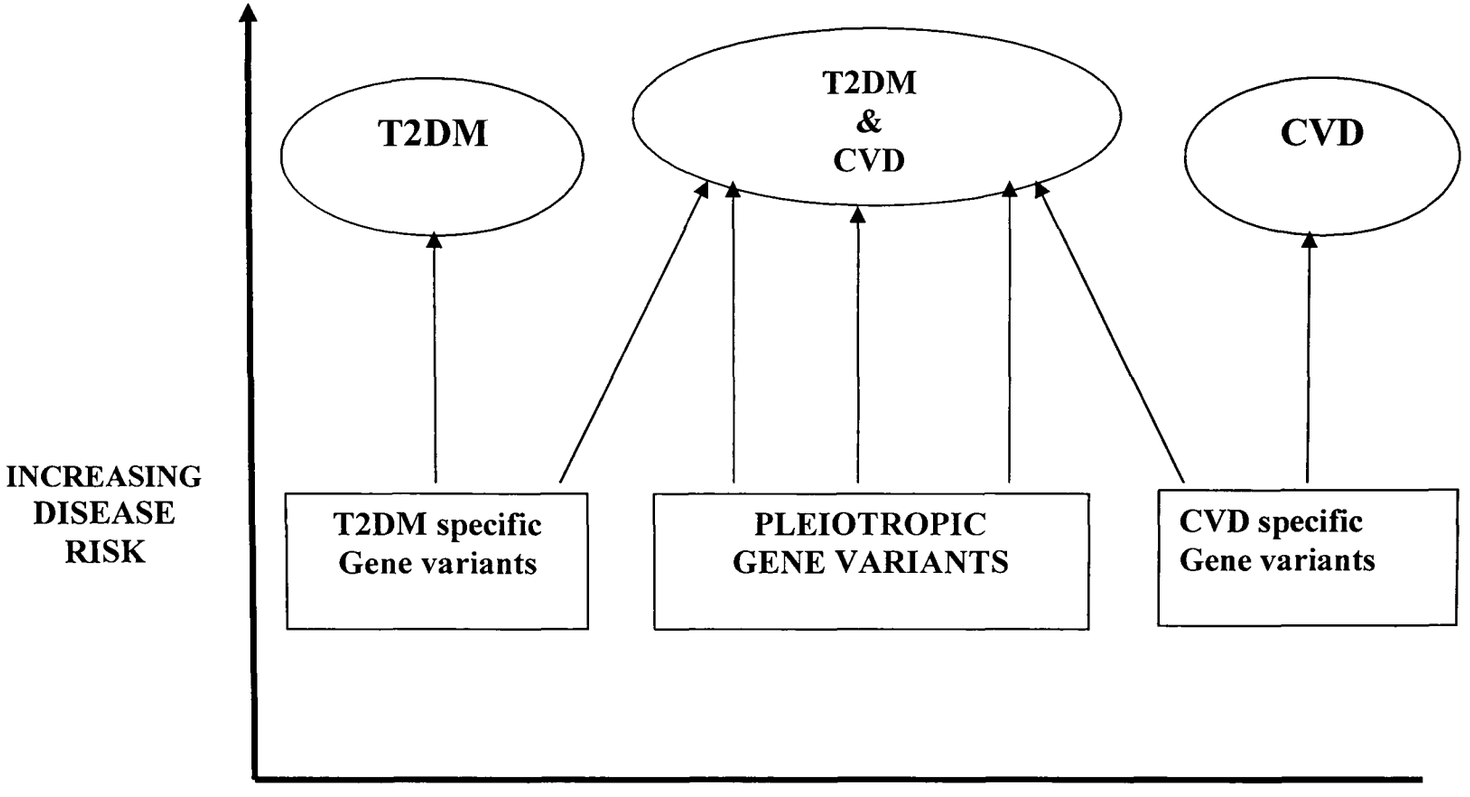


Figure 1 Common soil hypothesis

1.5.2 Known gene effects for cIMT

The schematic diagram in figure (1) aims to show how gene effects might increase the risk of type 2 diabetes and cardiovascular disease. It is postulated that there are pleiotropic gene effects that increase the risk of both type 2 diabetes and cardiovascular disease, in keeping with the "common soil" concept. These pleiotropic gene effects could then combine with disease specific gene effects to further increase susceptibility and lead to type 2 diabetes and cardiovascular disease, either alone or in combination.

Naturally, non-genetic factors will also impact upon the overall disease risk. However, an important point illustrated in the diagram is that a gene might at first be identified as type 2 diabetes susceptibility gene, but if pleiotropic, could also increase cardiovascular disease risk in the non-diabetic population. It is this concept that underlies the hypothesis to be investigated in this thesis. We will examine whether the common variants of type 2 diabetes susceptibility genes, are associated with carotid IMT as a marker of cardiovascular disease in non-diabetic population.

1.6 Type 2 Diabetes Susceptibility Genes

At present there is a great effort to define the genetic basis of type 2 diabetes. Several common gene variants have been identified that increase type 2 diabetes susceptibility. What is not clear so far is whether these are disease specific or pleiotropic variants.

1.6.1 PPARγ

The thiazolidinediones (TZDs) are insulin-sensitizing agents used in the treatment of type 2 diabetes, and mediate their effects through the nuclear transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ). This focused attention on the PPARγ gene as a potential susceptibility gene for insulin resistance and type 2 diabetes. Two loss of function mutations (P476L and V290M) in the ligand-binding domain of PPARγ were described which produced the clinical phenotype of severe insulin resistance, and early onset hypertension and type 2 diabetes (95).

A common variant has also been reported in exon B of the PPARγ2 gene, which leads to an amino acid substitution in codon 12 (Pro12Ala) (96). The Pro12 Pro allele was found to be associated with increased insulin resistance and an increased risk of type 2 diabetes (97, 98). Although the risk conferred to the individual was comparatively low, because the Pro allele is so common in the population (frequency 0.85) it generates a high population diabetes attributable risk of around 25% (98).

This observation lends support to the concept that genetic susceptibility to complex traits such as type 2 diabetes and insulin sensitivity is likely to result from the accumulation of common gene variants of weak functional effect.

Several genetic variants in PPARγ_1 gene have been reported but they are less frequent (99). The prevalence of the Ala allele varies from 4% to 28% in different ethnic populations

(100, 101). There is emerging evidence that the Ala allele variant is associated with increased insulin sensitivity in the non diabetic Caucasian population (97). Several Meta analyses confirmed that the Pro12Pro allele increases the susceptibility risk to type 2 diabetes (98, 102).

Because hyperglycaemia per se affects both insulin sensitivity and insulin secretion, it is necessary to examine the association of these gene variants in the nondiabetic or the prediabetic state.

A recent meta-analysis included 32000 non-diabetic individuals in 57 studies (103). It studied the effect of Pro12Ala polymorphism on the pre-diabetic phenotype. Across all studies, Pro12Ala polymorphism had no significant effect on the features of the insulin resistance syndrome. Subsequent subgroup analysis showed that in the obese subgroup, the Pro/Pro allele was associated with higher fasting glucose, and lower insulin sensitivity as measured by the HOMA model. On the other hand subjects homozygous for the Ala allele had lower fasting insulin compared to those homozyogous for the Pro allele.

One of the shortfalls of this meta-analysis is that it did not include data from studies that used the euglycaemic hyperinsulinaemic clamp technique to measure insulin sensitivity, as data were too heterogeneous to be included for analysis (103).

Recently attention has focused on the role of PPARγ in vascular function and atherosclerosis. Many of the principal vascular cells express PPARγ, including the endothelial and vascular smooth muscle cells (VSMCs) (104, 105). It has been shown that PPARγ activation inhibits gene expression and migration in VSMCs. In addition, PPARγ agonists (TZDs) were found to inhibit the formation of the early atheromatous lesions in a number of models of atherosclerosis (106, 107).

It has been shown that PPARγ is present in human differentiated macrophages in vitro, as well as in acetylated low-density lipoprotein (AcLDL)-loaded macrophages, suggesting that PPARγ could play an important role in atherosclerosis (108). The same study showed that subjects homozygous for the Ala allele had lower cIMT independent of other CVD risk factors, including insulin resistance, FFA, dyslipidaemia and inflammatory markers (108). Due to the low allele frequency very few studies have evaluated the effect of the Ala allele homozyotes separately. In these studies it was clear that carriers of Ala allele differ from the pro allele carriers with respect to its effect on BMI (98, 108), blood pressure and serum triglycerides (101).

In summary, therefore, there is emerging evidence to suggest that PPARγ might be a pleiotropic gene that mediates susceptibility to both type 2 diabetes and cardiovascular disease.

1.6.2 Adiponectin

The adipose tissue has been established as an active endocrine organ secreting a variety of proteins that regulate glucose levels, lipid metabolism, and energy homeostasis A susceptibility locus for type 2 diabetes, abdominal obesity and the metabolic syndrome has been mapped to chromosome 3q27 (109). A particularly attractive candidate gene at this locus is ADIPOQ gene that encodes the protein adiponectin, which is released into the circulation from adipose tissue (110). In both animal (111) and human studies (112), an inverse relationship has been observed between circulating adiponectin levels and the degree of insulin resistance.

Decreased circulating adiponectin concentrations have also been reported in obese individuals with CAD and type 2 diabetes (113,114).

Investigation of the adiponectin gene has revealed several single nucleotide plymorphisms (SNPs) associated with type 2 diabetes. In a Japanese cohort, the G alleles at SNP 45 in exon 2 and the G allele at SNP 276 in intron 2 were both associated with an increased risk of type 2 diabetes (115). Conversely, these associations were not confirmed in a study of Caucasian type 2 diabetic patients (116). However, this group did find an association between type 2 diabetes risk and a haplotype (G-G) derived from variation at 2 SNPs within the gene promoter (-11391 and –11377). Furthermore, the G-G haplotype was strongly associated with circulating adiponectin levels. It has been suggested that this association might be explained by the presence of an enhancer sequence adjacent to –11377 (117). The same group reported that variation in the proximal promoter region of the adiponectin gene confers a 2 fold relative risk for T2DM in morbidly obese subjects.

The overall attributable risk for the development of type 2 diabetes, in subjects with the G-G haplotype, was estimated to be 12.7% in a French population (118). The difference between these two studies needs to be explained, but may simply reflect ethnic differences between the study populations.

Lower circulating adiponectin levels have been observed in cardiovascular disease, obesity and type 2 diabetes compared to healthy controls (115,118). Circulating adiponectin levels have been shown to be negatively correlated with carotid intima media thickness (cIMT) (119) and higher plasma levels have been associated with a lower risk of myocardial infarction (118). Furthermore adiponectin has been shown to suppress the transformation of macrophages into foam cells (120) and is detected in catheter-injured vessels but not in intact vessels (121).

A number of SNPs in the adiponectin gene have been described to be associated with cardiovascular disease (117,122). SNP T 45G allele has been associated with coronary artery disease (123) and abdominal obesity (124) in French Caucasians and type 2 diabetes in Japanese populations (116). A common haplotype of SNPs G-11391A and C-11377G in the promotor region of the adiponectin gene has been associated with circulating adiponectin levels and type 2 diabetes in French Caucasians (117).

Recent studies have shown the SNP A-11426G in the proximal promotor region to be associated with fasting plasma glucose levels and type 2 diabetes (122,125). While this is still a comparatively novel candidate gene, there is emerging evidence to suggest that variation in the adiponectin gene influence type 2 diabetes risks, and might also be a determinant of cardiovascular disease.

1.7 Aims and Hypothesis

The overall aim of the study is to investigate the hypothesis that common T2DM susceptibility gene variants exert pleotropic effects and influence CVD as assessed by cIMT.

Specific aims:

To use the RISC healthy subjects' cohort that has been carefully characterized for insulin sensitivity & cIMT to:

1. Investigate the relationship between the Pro12Ala variant of the PPARγ2 gene and insulin sensitivity & carotid intima media thickness (cIMT).
2. Investigate the relationship between the+45 T-G SNP of the adiponectin gene and insulin sensitivity & carotid intima media thickness (cIMT).
3. Investigate the relationship between the promototer SNPs (-11391, -11426 and –11377) of the adiponectin gene and insulin sensitivity & carotid intima media thickness (cIMT).

2. General methods:

2. The RISC Project

The project is entitled ''Relationship between Insulin Sensitivity and Cardiovascular disease" (RISC) (40). The study objective was to recruit 1500 unrelated healthy subjects from the background population and collect anthropometric, demographic, and lifestyle data. In addition, each subject at baseline assessment underwent a 75g oral glucose tolerance test to determine glucose tolerance status and to exclude those with the diagnosis of diabetes. Whole body insulin resistance was measured using hyperinsulinaemic-euglycaemic clamp technique. B mode ultrasound was used to measure carotid artery IMT.

The aim of the study is to repeat these measurements at intervals of 3, 5 and 10 years from baseline. The study has been powered to address whether insulin resistance predicts the development of cardiovascular disease as measured by change in cIMT. It was important to put in place training and data quality control assessment to ensure that the measurements were recorded in the exactly the same way. Specifically, an ultra-sonographer from each recruitment centre has been trained at the co-ordinating centre in Pisa. Moreover, the carotid artery ultra-sound recordings were sent to Pisa on sVHS videocassettes for quality control assessment and subsequent cIMT measurement performed by a single person blinded to the identity of each participant. As well as investigating the specific role of insulin resistance, a secondary aim of the project is to investigate the role of other metabolic, environmental and genetic determinants of cardiovascular disease. To this end, the Newcastle centre was responsible for generating

high quality DNA for the future investigation of candidate genotypes in the progression of cardiovascular disease and insulin resistance.

2.1 Study design

This is a multicentre prospective observational study, however baseline cross sectional data was used for this thesis.

2.1.1 Sampling and recruitment:

Healthy Caucasian volunteers were recruited from 19 European centres as part of the RISC study. Recruitment centres are listed in the appendix. Participants were recruited from each centre from the local population according to specific inclusion criteria. Recruitment and baseline examination began in June 2002 and finished in August 2005. Before starting the study, local ethics committee approval was obtained by each recruitment centre. Each participant was given detailed written information about the study as well as verbal explanation either over the telephone or in person. Written consent for the study in addition to separate consent for the genetic study was obtained (appendix).

The following recruitments methods were used in Newcastle centre:

2.1.1.1 Poster recruitments:

Posters were designed and displayed in the University of Newcastle upon Tyne, Inland Revenue, Blood Bank and Newcastle hospitals NHS trust.

2.1.1.2 Snow balling:

This involves approaching people who have already been recruited for the study and asked if they could identify a friend or a family member who is eligible for the study and

may be interested. Those who agreed to take part in the study were subsequently contacted and recruited if they fulfilled the eligibility criteria.

2.2 Study population

Newcastle is one of 19 European clinical research centres in 14 European countries that are collaborating to investigate the role of insulin resistance in the development of cardiovascular disease in a prospective cohort study.

2.2.1 Entry criteria:

The following were the entry criteria to the study:

1. Clinically healthy volunteers.
2. Age between 30 and 60 years.
3. The participant will be available for follow –up over the next 10 years.

2.2.2 Exclusion criteria:

The following were the exclusion criteria for the study:

1. Treatment for obesity, hypertension, lipid disorders or diabetes.
2. Pregnancy.
3. History of cardiovascular disease.
4. Weight change of 5 kg or more in last month.
5. Steroid treatment.
6. Chronic lung disease.
7. Cancer (in the last 5 years).
8. Renal failure or renal replacement therapy.
9. Recent major surgery
10. Seizure disorder or epilepsy.

11. Inability to give informed consent.

12. Blood pressure > 140/90 mmHg or treatment.

13. Fasting plasma glucose of ≥ 7mmol/l or 2h plasma glucose ≥ 11.1mmol or treatment.

14. Total cholesterol ≥ 7.8 mmol/l or treatment.

15. Triglycerides ≥ 4.6 mmol/l or treatment.

16. ECG abnormalities of acute myocardial ischaemic injury or pericarditis.

17. Poor ultrasound imaging of carotid artery.

2.3 History and medical history questionnaire:

Demographic data and socio-economic status of the volunteers were collected. Medical history and family history of CVD, stroke, hypertension and diabetes in addition to information on body shape of the participants and their family members, smoking and alcohol habits and physical activity as well as treatment history were recorded. The life style questionnaire was designed and prepared in English, translated to 11 different languages of the countries participated in the study and then back translated to English to ensure homogeneity of the data (Appendix).

2.4 Physical examination

Height was measured with a standard clinic stadiometer. Subject was standing on a flat, firm surface and backed up to a wall until heels, buttocks and/or shoulder blades touched the wall, with eyes straight ahead and head in the horizontal plane and feet/ankles together. Then headboard was placed over the crown of the head to form a right angle with the ruler. Subject was asked to take a deep breath. Height was recorded to nearest 1 cm.

Waist circumference was measured according to a standardized written protocol with a plastic one-sided tape measure. It was measured on bare skin at the smallest circumference between the costal margins and the iliac crests. The tape was checked to make sure it was horizontal (mirror was used to check if it was leveled at the back or with the help of second person). The subject was asked to breathe out gently looking straight ahead, with arms hanging loosely at the sides.Measurements were record to nearest 1 cm.

The hip circumference was measured by a tape measure using a standardized written protocol. The hip circumference was measured at the level of the greater trochanters and observer checked whether the tape was leveled. If trochanters were not palpable, the largest gluteal circumference was measured and recorded to nearest 1 cm.

Blood pressure was measured in each centre with the centrally provided OMRON 705cp blood pressure device (Omron Healthcare GmbH, Hamburg, and Germany). The outer garments of subjects were removed to expose the non-dominant arm. The subject was seated and had rested for at least 5 minutes before recording. The measurement was performed automatically and when finished, the systolic and diastolic blood pressures were displayed. The subject was allowed to rest for two minutes and then the blood pressure and heart rate measurement were repeated for three times in total.

Body weight, body mass index (BMI) and free fat mass were evaluated using TANITA bioimpedance balance (Tanita International Division, UK). The subject was fasting and dressed only in underclothes, and was asked to empty their bladder prior to the measurment. Results were printed on thermic paper, giving results on body weight, BMI, fat free mass, fat percent and total body water.

A resting 12 lead electrocardiogram (ECG) was obtained to exclude subjects with ECG changes of acute myocardial ischemic injury or pericarditis. Copy of the ECG was send to the co-ordinating centre for central coding.ECGs were coded according to the Minnesota Code (126).

2.5 Baseline Assessment

2.5.1 Oral glucose tolerance test (OGTT)

Subjects fasted from 20:00 hours the previous evening with only calorie-free, caffeine-free beverages (water) to be taken. Subjects were asked not to come to the examination by bicycle and to refrain from heavy or unusual physical exercise for 2 days before each test. The test started at around 9:00am. Subjects were seated throughout the test and refrained from smoking. A polythene cannula was inserted under local anaesthesia into an ante-cubital vein for venous blood sampling. All fasting blood samples were taken for local and central analysis, that include: glucose, insulin-peptides, haemoglobin, red cell count, platelets, total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL), triglycerides, urea and electrolytes, urate, creatinine, liver enzymes, γGT and albumin.

After fasting blood samples were drawn, a 75 gram glucose load was taken by subjects over a period of 5 minutes. Blood samples for the measurement of glucose, insulin and C peptides were then taken at 30, 60, 90 and 120 minutes. A snack was given at the end of the test, after the 120 minute blood sample.

2.5.2 Euglycaemic Hyperinsulinaemic clamp

This was conducted on a separate day to the OGGT. The study started at 09:00am, the subject fasted for 12 hours without caloric or caffeinated food. Subjects were asked not to come to the examination by bicycle or to undertake heavy or unusual physical activity for the 2 days preceding the study. Subjects were asked to pass urine before the start of the clamp, then to rest in the supine position on a bed for the duration of examination. A bedside glucose analyser was used to measure plasma glucose every 5-10 minutes; the analyser was calibrated before the start of the clamp and every 10 samples. A heat box was used to warm the canulated hand and thus arterialise venous blood. Two polythene cannulae were introduced under local anaesthesia. One cannula was placed retrogradely into a dorsal vein in the hand; this was warmed for arterialised sampling. The other cannula was placed in an ante-cubital vein for administration of infusates. The cannulae were kept patent with 0.9% NaCl flushes. Syringes were labelled for their contents. 47 ml 0.9 % NaCl was drawn into a 50 ml syringe for insulin infusion and 3 mls of the subject's blood was added to the 50 mls syringe as a carrier and mixed well. A 150 cm extension tube was then connected to 50 ml syringe containing insulin and placed in a pump. Blood was withdrawn from the heated hand cannula for baseline plasma glucose measurement to decide the clamp level. The target plasma glucose was clamped at a predetermined level (the average of 3 fasting plasma glucose levels). If the fasting plasma glucose level was between 4.5-5.5 mmol/l, then this was chosen as the clamp level (isoglycaemic clamp). If the fasting plasma glucose level was > 5.5 mmol/l, the target clamp glucose was 5.5 mmol/l. If the fasting plasma glucose level was < 4.5 mmol/l, the target clamp glucose was 4.5 mmol/l.

Glucose and insulin infusions were then attached to the antecubital vein by a 3- way stopcock, and the clock time set to clamp time 0 minute. The clamp was maintained by titration of a variable-rate of glucose infusion against a fixed-rate infusion of soluble insulin (priming dose followed by a constant maintenance dose). The priming dose consisted of 4 times constant infusion rate (60 ml/hour) for 0- 4 minutes followed by 2 times constant infusion rate (30 ml/hour) for time 5-7 minutes and this was followed by the constant infusion rate of 15 ml per hour from time 8-120 minutes.

The insulin infusion rates (240 pmol $.min^{-1}.m^{-2}$) were calculated as a dose per unit of surface area rather than by body weight, in order to avoid over-insulinization of overweight individuals. Insulin infusion was prepared according to body surface area ($40Mu.min^{-1}$ $.m^{-2}$). Body surface area (BSA) was calculated using the equation; $BSA=W^{0.425}$ X $H^{0.725}$ X71.84 X $10^{-}4$.(BSA= body surface in square cm, W= weight in kg, H= height in cm).The BSA was divided by volume infused per hour (15 ml) and multiplied by the syringe volume (50 ml) to give the units of insulin to be infused. This was typically around 10-15 units of insulin to be added to 50 mls of 0.9% NaCL.

The glucose infusion rate was computed on the basis of blood glucose concentrations measured at 5-10 minutes intervals, to ensure that blood glucose concentrations remain within 0.8 mmol/l (±15 %) of the target glucose concentration.

The starting glucose infusion rate, 20% glucose solution, was decided on the basis of the fasting blood glucose. If fasting glucose was ≤ 5.5 mmol/l (100 mg/dl) glucose infusion was commenced at time 4 minutes. If fasting glucose was > 5.5 mmol/l (100 mg/dl) glucose reduction to 5.5 mmol/l was awaited before starting glucose infusion.

At time 120 minutes the clock was stopped and the subject was given a snack and observed for a few hours before allowed to go home if no adverse reaction was noted. Blood pressure was measured and recorded at 0 and 120 minutes. The clamp procedure was standardised across centres with the use of a demonstration video. The data from each clamp study were transferred to the coordinator centre for a quality control check with pre-set criteria.

2.5.3 Study biological samples:

After overnight fast, fasting blood samples were collected during the 75 g OGTT and the euglycaemic insulin clamp. Samples were sent to local laboratories to evaluate study exclusion criteria and to central laboratories for central analysis for standardized central laboratory assays. Samples were separated into plasma and serum after centrifuging,they were aliquoted and stored at -20^0 C for glucose and glucagons and -80^0 C for insulin, c-peptides, insulin split products, lipids and non esterified free fatty acids. Urine samples were stored at -20^0 C. Samples were transferred on dry ice at a pre-arranged intervals to central laboratories.

2.5.4 Serum insulin measurements:

Serum insulin was measured by specific time-resolved fluoroimmunassay (Auto DELFIA^{TM} insulin kit, Wallac, Oy, Turku, Finland).The method uses two murine monoclonal antibodies that bind to two different epitopes on the insulin molecule.The assay is sensitive (detection limit 5 pmol/L), accurate (101% recovery with 50 pmol/L insulin added to samples, 95% with 100 pmol/L, and 89% with 300 pmol/L), and fast (results within 3 h), and has a high analytical capacity (done in microtiter plates). The working assay range selected is 5-600 pmol/L, corresponding to a clinically useful range.

Because of its specificity, this two-site immunoassay gives results that are lower than those obtained by using a competitive radioimmunoassay, both in normal individuals and in patients with type 2 diabetes (127).

2. 5.5 Plasma adiponectin measurements:

Plasma adiponectin was determined by a novel in-house time-resolved immunofluorometric assay (TR-IFMA) based on two antibodies and recombinant human adiponectin (R & D Systems, Abingdon, UK) (132). The adiponectin molecule is known to form a wide range of polymers, of which the predominant polymers include trimers, hexamers and highly congregated multimers (133). Previous experiments have demonstrated that both antibodies used are able to detect several adiponectin polymers in serum, including the major three molecular forms. All standards and unknown samples were analysed in duplicate, with the exception of non-specific binding (NSB), which was analyzed in quadruplicate. The intra-assay coefficient of variation (CV) was < 5 % and the inter-assay CV was < 10 %.

2.5.6 Calculation of insulin sensitivity:

The mean glucose infusion rate (GIR) was calculated between 80 and 120 minutes, in mg min^{-1} kg LBM^{-1}. The mean value is calculated from the integral of glucose infusion rate, using the time and values reported during the last 40 minutes of the clamp. A glucose space correction (GSC) was calculated using the following formula; GSC= V (G120-G80) = V ΔG where V is the glucose distribution volume, assumed to be 290 ml/ kg LBM, G120 and G80 are the glucose values at 80 and 120 minutes respectively. in

mg/ml. The value of 290 ml/ kgLBM obtained by dividing a typical distribution volume value found in the literature, GSC is expressed in mg min-1 kgLBM.

The M value was calculated as M = GIR - V ΔG. Insulin sensitivity was expressed as the ratio of the M/I value averaged over the final 40 min of the 2 hour clamp and normalised by the fat free mass to the mean insulin concentration measured during the same interval (M/I;in units of μmol min^{-1} kg_{ffm}^{-1} nM^{-1})(129).

2.5.7 Calculation of lean body mass:

Lean body mass (LBM) was calculated using Watson formula for LBM based on total body water provided by Tanita (Tanita International Division, UK). This provides an estimate of the total body water (TBW) from anthropometric measurements (Sex, weight, height and age). LBM is calculated from this estimate as LBM= TBW/0.732 (0.732 is the water space in the lean tissue). Watson's formula agrees reasonably well with the Amsterdam and Rome DEXA (128).

2.6 Ultrasound examination:

2.6.1 Measurement of (c IMT):

For subjects attending the Newcastle centre, this was performed by Yasmin McGrady, a certified sonographer from the department of radiology, Newcastle Hospital NHS trust and in the rest of the centres by a certified radiographer, trained and certified in Pisa, Italy.

Carotid artery intima media thickness (c IMT) was imaged following a validated protocol (130). The subject was asked to lie supine on a bed, without a pillow in order to relax the muscles of the neck, with the head turned to the opposite side of the examination with the sonographer seated behind the subject's head. The ECG was used to monitor the heart cycle and to select images for measurement off-line. The c IMT was measured during the diastolic phase of the cardiac cycle.

A randomised log-number was provided by the co-ordinating centre for each examination (scan number). This number was written on the screen as identification. No subject name or number, or centre name was written on the screen. This was to ensure blind reading of the carotid scans when measured by a single examiner at the co-ordinating centre at Pisa. The right side was identified as RT and the left side as LT. A preliminary scan was performed on both sides (before the recording) to explore the anatomy. The preliminary scan allowed identification of points of reference for the common carotid artery (CCA), carotid bifurcation (CB) and flow divider (FD), internal carotid artery (ICA) and external carotid artery (ECA) (Figure 2). The preliminary scan was also performed to identify subjects with poor imaging of the arterial walls. Subjects with plaque, heavy mineralisation, acoustic shadowing, vessel tortuosity, kinking, or very high bifurcation

were excluded. Also when the sonographer was unable to image the cIMT at the far wall of the CCA and CB or if there was a high degree of stenosis (>70%) vessel occlusion subjects were excluded.

The whole imaging procedure was recorded on S-VHS tapes and read in a centralized reading centre (Pisa) by a single reader blinded to clinical data, using a high resolution video recorder (Panasonic AG- MD830) coupled with a computer-driven image analysis system developed by the Institute of Clinical Physiology, CNR, Pisa Italy. For the purpose of this study, IMT of the near and far wall of the right and left CCA were measured in digitized end-diastolic frames, ~10 mm proximal to bifurcation. The measurements were performed at 5 points for each wall, and the mean near- and far-wall IMTs were calculated by averaging the measurement points. The Mean CCA IMT used in the statistical analyses was calculated as the overall mean of all available CCA walls (up to four). Both near- and far-wall IMT were measured in this study. It was previously demonstrated that far-wall IMT alone had significantly lower association with cardiovascular outcomes than the combined near- and far-wall measurements (78).

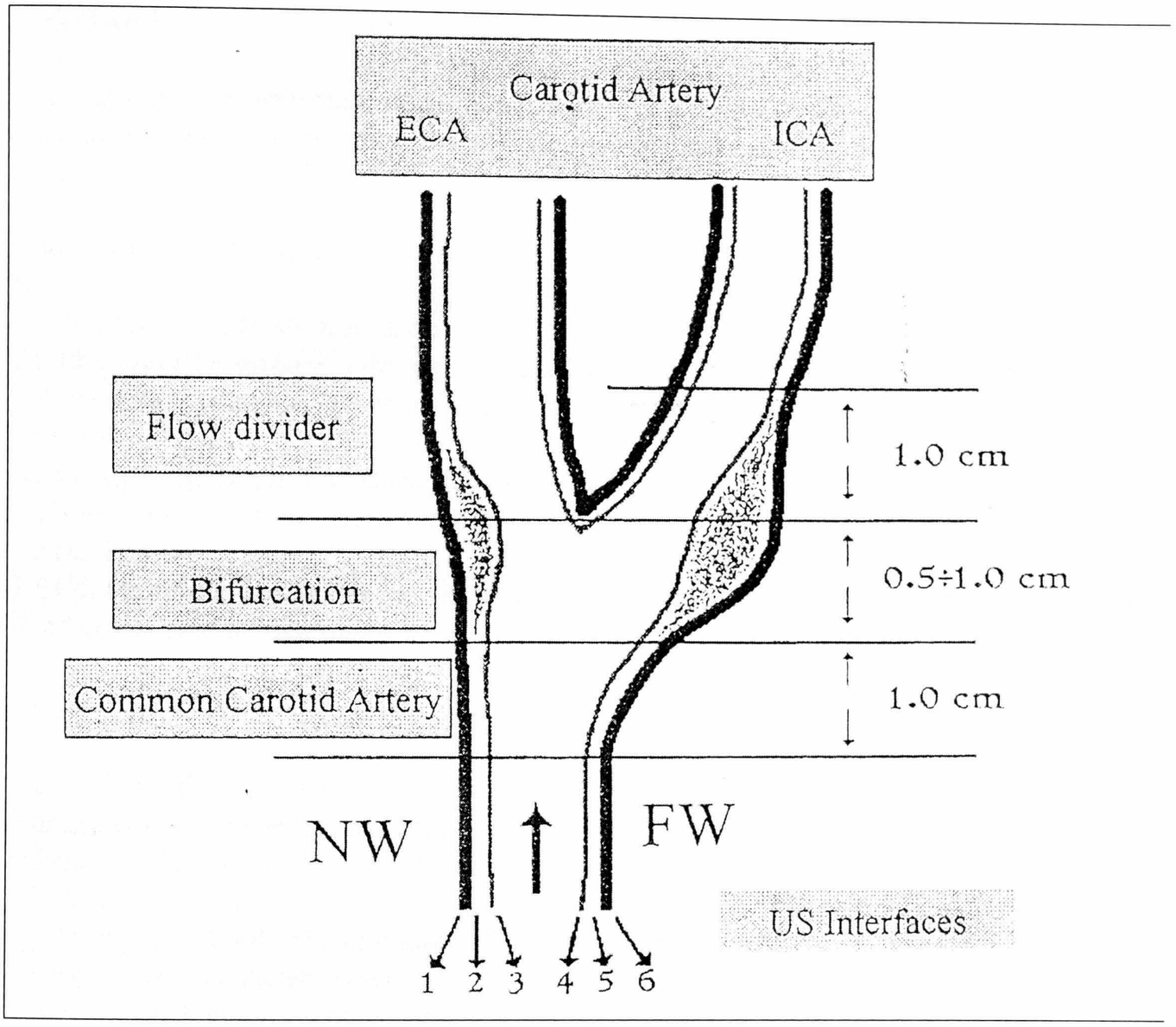


Figure 2: Carotid artery

ICA: Internal Carotid Artery

ECA: External Carotid Artery

NW: Near Wall

FW: Far Wall

2.7 DNA extraction

Samples were shipped from all other European centres to Newcastle upon Tyne, UK, where DNA extractions and genetic analysis took place. DNA samples were transferred on dry ice at pre-arranged intervals. Spare samples for each subject was kept at -80^0 c. Genomic DNA was extracted from lymphocytes using Nucleon DNA extraction kits (Tepnel Life Sciences, PLC, Manchester, UK).The procedure that was followed, was lysing of the blood cells with lysis buffer (Reagent A) and sodium dodecyl sulphate (SDS) (Reagent B), deproteinisation with sodium perchlorate, extraction of the DNA using chloroform and DNA precipitation with ethanol. A 5ml blood sample collected in potassium EDTA and stored at -80^0 C was thawed and transferred into a 50 ml sterile polypropylene tube to which 20 ml of cell lysis buffer (Reagent A) was added. The tube was mixed for 4 minutes then centrifuged at 3000 g for 4 minutes. The supernatant was discarded without disturbing the pellet and re-suspended in 2 ml of SDS (Reagent B).

The suspension was transferred into a 15 ml sterile polypropylene tube and 500 µl of sodium perchlorate was added and the tubes were inverted by hand 7 times. After proper mixing, 2 ml of chloroform was added and the tube was mixed again by inverting by hand until the phase had emulsified. 500 µl of Nucleon resin was carefully added by dropping slowly with a pipette tip.

The tubes were then centrifuged at 1300 g for 4 minutes. The upper aqueous phase above the nucleon resin layer was transferred to a fresh sterile 15 ml polypropylene tube and DNA was precipitated by adding 2 volumes (5 mls) of cold absolute ethanol.The tube was inverted several times to precipitate the DNA and then centrifuged for 5 minutes at 4000 g . The supernatant was removed and the DNA pellet was washed with 2 ml of 70%

ethanol then centrifuged for 5 min at 4000 g. The supernatant was removed and the DNA pellets were air dried and re-suspended in 500 µl of sterile DH_2O. The DNA pellet was left to re-hydrate overnight at room temperature then stored at 4^0 c.

2.7.1 DNA quantification:

The concentration and the quality of the DNA was checked on a lamda Bio 20 UV/VIS spectrophotometer (Perkin-Elmer, Warrington, UK). Absorbance values (A) at a wavelength of 260 and 280 nm were measured using a clean quartz cuvette. DNA quantity was calculated by the following formula:

DNA quantity =50xdilution factor xA260

Where 50= the optical density (OD) of DNA, 50 $\mu g.ml^{-1}$ of DNA gives an absorbance reading of 1.0 at a wavelength of 260 nm. DNA quality was checked by calculating the ratio of the two absorbance values at A260 and A280. A ratio above 1.8 indicates a low level of contamination.

2.8 PCR methods

PCR reactions were carried out on a Gene Amp PCR system 9700 thermal cycler (9600 emulation model AB, Warrington, UK). Two short primers (Forward and Reverse) were added to amplify specific regions of the genomic DNA using thermostable DNA polymerases. PCR reactions were set up using different volumes. PCR reaction was made from forward and reverse primers, d NTP, 1X PCR buffer, AmpliTag Gold DNA polymerase, m M $Mgcl_2$ and genomic DNA, the volume of each depending on the experiment . PCR reactions were set up on ice and reagents were stored at -20^0 c.

2.8.1 Agarose gel electrophoresis

Agarose gel of 1-3 % was used to resolve the PCR products for the restriction fragment length polymorphism (RFLP) assay. Agarose was dissolved in 1x TBE buffer and ethidium bromide was added and then boiled using a microwave oven. Agarose was then cooled and poured into a gel-casting tray. A comb was positioned in the casting tray prior to pouring the agarose gel so that wells could form in the gel. PCR products were mixed with DNA buffer and then samples were loaded into the wells. A DNA ladder was run alongside the samples to allow sizing of the PCR fragments. Gel electrophoresis was carried out between 60-130 V in 1x TBE buffer until the dye of the loading buffer migrated over two-thirds the length of the gel. PCR products were visualised using UV transilluminator (TMW-20, Flowgen Ltd, Lichfield, UK). Digital image was obtained using image acquisition apparatus (Alpha Imager 2000, Flowgen Ltd, Lichfield, UK).

2.8.2 Restriction enzyme digestion of PCR products

Restriction digests of PCR products were formed using 8µl of PCR product and 2µl of the appropriate enzyme and 10x reaction buffer. The digestion was then incubated at the recommended temperature for the specific enzyme. Finally the product of the digest was separated on agarose gel and visualised as described previously.This method was used to detect Pro12Ala polymorphism of the PPARγ_2 and described in detail in chapter 4.

2.9 SEQUENOM method:

Mass array homogenous mass extend (h M E) assay, through the application of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) to the analysis of primer extension products. The h M E assay is based upon the annealing of a Mass EXTEND primer adjacent to the polymorphic site of interest. The addition of a DNA polymerase, plus a cocktail mixture of nucleotides and terminators, allows extension of the primer through the polymorphic site (Fiqure3). The accuracy of the determined sequence is maintained through the application of four levels of stringency:

1. Correct hybridization of amplification primers and amplification of the target region.
2. Specific hybridization of the Mass EXTEND primer.
3. Extension through the polymorphic site with a high fidelity enzyme.
4. Correlation between mass of measured primer extension product and calculated values.

Step 1: Amplification:

Using standard methods, 2.5 ng of genomic DNA was amplified in a 5 µl volume using a 384-microtiter format.

Step2: Dephosphorylation:

Arctic Shrimp Alkaline Phosphatase (SAP) was added to samples, which are then incubated for 20 minutes at 37^0 C. This dephosphorylates any residual nucleotides, preventing their future incorporation and interference with the primer extension assay. Samples were then incubated at 85^0 C to inactivate the heat labile SAP.

Step 3: h M E Assay:

The MassEXTEND primer, DNA polymerase, and cocktail mixture of dNTPs and ddNTPs were added to initiate (h M E) primer extension reaction. This reaction generated allele-specific primer extension products that are generally 1-4 bases longer than the original MassEXTEND primer

Step 4: Sample conditioning:

Spectro CLEAN resin was added to the reaction to remove extraneous salts that interfere with MALDI-TOF analysis.

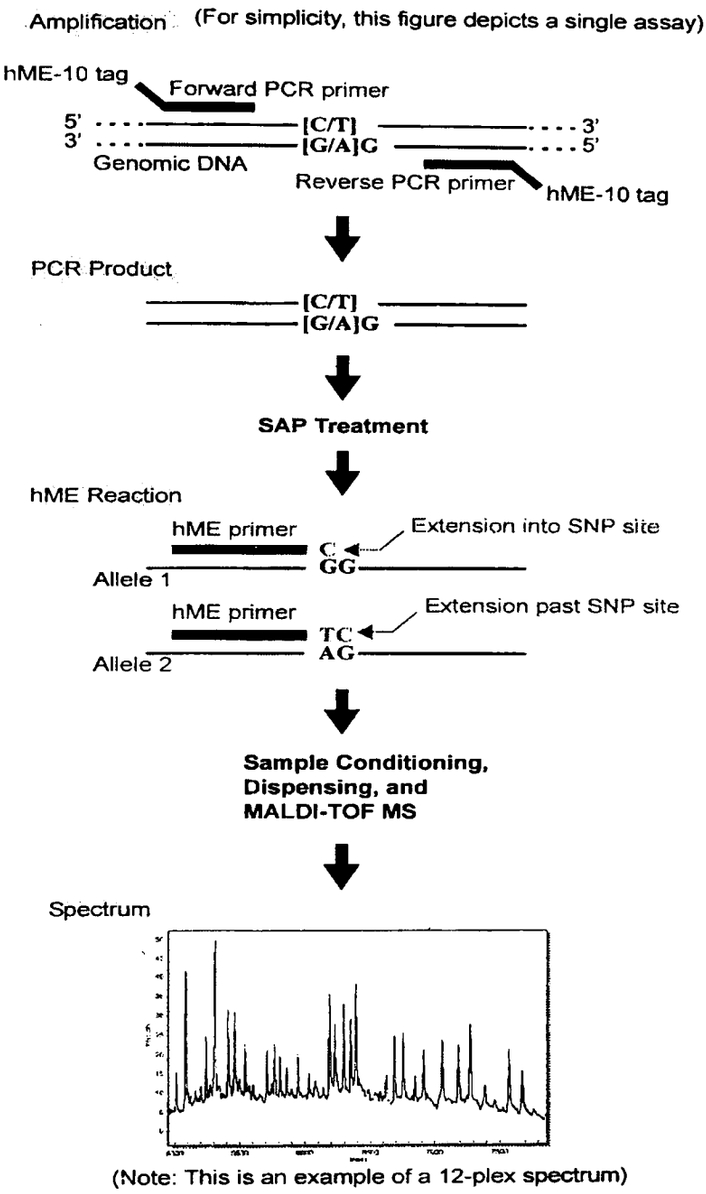
Step 5: Samples Transfer:

15 nl of sample was then transferred from the 384-microtiter plate and spotted onto the pad of the 384 SpectroCHIP microarray.

Step 6: Sample Analysis:

The Spectro CHIP was placed into the MALDI-TOF, which measures the mass of the extension products. Once determined the genotype was simultaneously called in real-time with SpectroTYPER RT software.

This method was used to detect SNP45 of the ADIPOQ gene and described in detail in chapter 5.3.1.



(Note: This is an example of a 12-plex spectrum)

Figure 3: Multiplexed Homogenous Mass EXTEND Sequenom assay

2.10 Quality control:

2.10.1 Quality control of genotying:

Of the genotyped samples, 5% were duplicates and there was at least 1 negative control per 96 well DNA plate. The accuracy of the genotyping was determined by the genotype concordance between duplicate samples. We obtained a 100% concordance between the genotyped duplicate samples for each of the SNPs. The genotyping success rate for each of the SNPs was >98 %.

2.10.2 Quality control of carotid IMT measurements:

In each recruiting centre, certified trained technicians performed ultrasound examination of extracranial carotid arteries following a standardized protocol. The ultrasound scanners, all with a 7.5 or 10.0 MHz linear array transducer, differed between centers (Acuson Aspen, Acuson Sequoia, Agilent Sonos 4500, Esaote Megas, Siemens Elegra, Toshiba Powervision). Whole imaging procedure was recorded on S-VHS tapes and read in a centralized reading center (Pisa) by a single reader blinded to clinical data, using a high resolution video recorder (Panasonic AG- MD830) coupled with a computer-driven image analysis system developed by the Institute of Clinical Physiology, CNR, Pisa Italy. Inter-individual variability of readings was tested in 167 subjects. The mean absolute difference and correlation coefficient between the two readers were 0.032 mm and 0.90 respectively. In 45 subjects, carotid B-mode imaging was performed twice in two different sessions. The mean absolute difference and correlation coefficient between the two acquisitions were 0.051 mm and 0.74 respectively.

2.10.3 Quality control of euglycaemic clamp:

To ensure consistency across study centres, the clamp procedure was standarised and each centre underwent pre-study training. Clamp data was then transferred and analysed at the RISC co-ordinating centres (Pisa, Italy), and quality assured against pre-set criteria. These were as follows: clamped glucose levels within 20% of target fasting glucose levels and coefficient of variation (CV) of < 15% and avoidance of hypoglycaemia (glucose of < 3.5 mmol/l).

2.11 Statistical Analysis

The observed genotype frequencies were checked using the Hardy-Weinberg equation for any differences from those expected.

$$P^2 + 2pq + q^2 = 1$$

Where p represents the frequency of the dominant allele and q represent the frequency of the recessive allele and 2pq represents the frequency of the heterozygote allele .The differences between the observed and expected frequencies were tested by the Chi square test.

Skewed variables were log transformed to normalise distributions for analysis and then back transformed and are presented as geometric means. Analysis of covariance was carried out using age, sex and body mass index as covariates. The adjusted means were presented as geometric means with 95% confidence intervals around the means.

Statistical analysis was carried out using Minitab version 15(Minitab Inc, USA).P values < 0.05 are highlighted in the Tables in line with conventional statistical significance.

Allowance for multiple testing was done in the interpretation of the significant results, such that p values <0.01 were considered of potential interest and relevance

The M//I value, as a measure of insulin sensitivity, was calculated and adjusted for free fat mass and mean insulin concentration measured during 2 hour euglycaemic clamp.

The ANOVA test was initially used to compare SNP genotypes with biological variables. Significant differences were further explored using independent samples t-Test. General linear Model (GLM) analysis was performed to test for associations between SNP genotypes and risk factors after adjusting for confounding metabolic and lifestyle variables (sex, age, BMI, waist smoking status, serum cholesterol and recruitment centre. While most subjects recruited to the RISC project were Caucasian, it was necessary to compare genotype frequencies between centres or groups of centres (based upon geographic and cultural proximity) to ensure that there were no significant differences. Where such centre effects are found, they will be taken into account by GLM.

3. Description of Data set:

3.1 Characteristics of Study Population

Table 5 shows the anthropometric and biochemical variables of the study population. Of the 1278 subjects studied, 579 were men and 699 were women. The mean age was 43.8 years and mean BMI was 25.6 kg/m^2. Means of anthropometric and metabolic variables including 2 hour glucose, 2 hour post glucose challenge insulin, and NEFAs are described in Table 5.

3.1.1 Male and female differences

Table 6 describes the anthropometric and biochemical characteristics in males and females. Although women were older than men, they had significantly lower BMI, waist circumference, fasting plasma glucose, fasting and 2 hours insulin and fasting and 2 hours NEFAs.

Women had lower total cholesterol, LDL, serum triglycerides, systolic and diastolic blood pressure. They also had higher HDL compared to men. However, there was no difference in 2 hour plasma glucose post OGTT challenge between males and females. Men had significantly higher carotid IMT compared to woman.

3.1.2 Northern and Southern centres

While all subjects recruited to the RISC project were Caucasians, it was necessary to compare between centres or groups of centres (based upon geographic and cultural proximity) to ensure that there were no significant differences. Centres were divided into northern and southern centres according to latitude.

Northern centres included; Amsterdam, Dublin, Frankfurt, Geneva, Glasgow, Kuopio, London, Malmö, Newcastle-upon-Tyne, Odense and Vienna.

Southern centres included; Athens, Belgrade, Madrid, Milan, Perugia Pisa, Rome and Lyon.

Table 7 describes the anthropometric and biochemical characteristics divided into northern and southern centers. Subjects from southern European centers were younger than those from northern European centers; they had significantly lower fasting plasma glucose, fasting and 2 hour insulin, 2 hour NEFAs, serum triglycerides, systolic and diastolic blood pressure. They also had higher HDL compared to those from north Europe. However, there was no difference in BMI, waist circumference, 2 hours plasma glucose, total cholesterol, LDL cholesterol or fasting NEFAs between the 2 groups.

3.2 Carotid intima media thickness (cIMT)

The mean of the carotid intima media thickness (cIMT) for the study population is described in Table 8. The means of common carotid artery (IMT_{CCA}), internal carotid artery (IMT_{ICA}) and the average of all these segments (IMT_{AVRG}) are described in table 8. Males had a higher c IMT compared to females. The mean of c IMT for males was [(617 [539-659] μm (Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]) and for females (581 [525-640] μm (Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]) and the difference was statistically significant with p value <0.00001.

3.2.1 Effect of centre on carotid intima media thickness (c IMT)

Table 9 shows the breakdown of the c IMT by center and data was presented as Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]. The name of the centre for the corresponding centre number is found in the appendix.

3.2.2 Northern versus Southern centres in relation to carotid intima media thickness (cIMT)

The number and the mean of the cIMT divided by northern and southern centres is illustrated in Table10. The southern centres had a lower c IMT compared to the northern centers. The mean of the IMT of the common carotid artery was 603 [543-665] μm Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]) vs. 591[553-654] (μm Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]) for the northern and the southern centres, respectively. The difference was statistically significant after adjustment for age and sex (p= 0.013).

3.3 Insulin sensitivity

Table 11 shows the mean and range of minimum and maximum value of the M/I value.

The mean of M/I value for the study population was 125.29 [92.0-178.6](μmol min^{-1} kg_{ffm}^{-1} nM^{-1}) [Geometric Mean interquartile range 25^{th} - 75^{th} percentile]. Males had a lower M/I value and hence were more insulin resistant compared to women and this was statistically significant (Geometric Mean interquartile range 25^{th} - 75^{th} percentile: 109.2 [80.0-151.1] vs. 139.9 [106.7-189.6](μmol min^{-1} kg_{ffm}^{-1} nM^{-1} : p=0.001).

3.3.1 Effect of centre on Insulin sensitivity

Table 12 shows the breakdown of the M/1 value by centre and data are presented as geometric mean [interquartile range 25^{th} - 75^{th} percentile]. The name of the centre for the corresponding centre number is found in the appendix.

3.3.2 Northern versus Southern centres in relation to insulin sensitivity

Table 13 shows the M/I values for the northern and southern centres. The mean of the M/1 value was 114.2 [97.2-186.6] vs.132.2 [83.6-155.7] μmol min^{-1} kg_{ffm}^{-1} nM^{-1} [Geometric Mean interquartile range 25^{th} - 75^{th} percentile]; for the northern and the southern centres, respectively. The difference between the 2 groups was statistically significant after adjustment for age and sex ($p=0.005$). This means that people from the southern part of Europe are more insulin sensitive compared to those from the northern part.

Table 5: The anthropometrics and biochemical variables of the study population

Data are presented as Mean ± (SD) and rang of minimum and maximum value.

Character	Mean ± (SD)	Range
Age (years)	43.8 ± 8.3	(29-63)
Sex (male/female)	(579/699)	Number 1278
BMI (kg/m^2)	25.6 ± 4.1	(16.8-43.9)
Waist circumference (cm)	86.7 ± 12.8	(49-147)
Fasting glucose (mmol/l)	5.1(0.5)	(2.9-7.0)
2 Hour glucose (mmol/l)	5.76 ± 1.48	(2.1-10.8)
***Fasting insulin** (pmol/l)	30.3 (21.0-44.0)	(3.0-118.0)
***2hour insulin** (pmol/l)	146 (88.7-244)	(10-332)
Total Cholestrol (mmol/l)	4.8 ± 0.85	(2.7-7.7)
***Triglycerides** (mmol/l)	0.95 (0.68-1.28)	(0.3-4.5)
HDL – cholesterol (mmol/l)	1.4 ± 0.4	(0.3-2.9)
LDL – cholesterol (mmol/l)	2.9 ± 0.8	(0.8-5.7)
Systolic BP (mmHg)	117.2 ± 12.1	(79-140)
Diastolic BP (mmHg)	74.3 ± 7.75	(50-90)
Fasting (NEFAs) (pmol/l)	0.54 ± 0.24	(0.03-3.23)
2 Hour (NEFAs) (pmol/l)	0.05 ± 0.11	(0.01-0.27)

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

Table 6: The characteristics of the study population separated into males and females. Data are presented as Mean ± (SD).

Character	Male Mean ± (SD)	Female Mean ± (SD)	P value
Number	579	699	Total (1278)
Age	43.1 ± 8.4	44.23± 8.1	0.005
BMI (kg/m^2)	26.5 ± 3.5	24.9± 4.4	0.000
Waist circumference (cm)	93.6± 10.3	81.3 ±12.0	0.000
Fasting Glucose (mmol/l)	5.3 ± 0.48	4.9 ± 0.56	0.000
2 Hour Glucose (mmol/l)	5.7 ± 1.4	5.7 ± 1.5	0.6
***Fasting Insulin** (pmol/l)	32.0 (22.0-46.0)	29.0 (20.0-40.0)	0.003
***2Hour Insulin**	157 (101-245)	132 (75-238)	0.000
Total Cholesterol (mmol/l)	4.9(0.85)	4.75(0.84)	0.01
***Triglycerides** (mmol/l)	1.10 (0.77-1.50)	0.85 (0.62-1.11)	0.000
HDL – cholesterol (mmol/l)	1.24 ± 0.30	1.58 ± 0.37	0.000
LDL – cholesterol (mmol/l)	3.06 ± 0.75	2.75 ± 0.78	0.000
Systolic BP (mmHg)	121.8 ± 10.1	113.6 ± 12.2	0.000
Diastolic BP (mmHg)	76.2 ± 7.1	72.8 ± 7.9	0.000
Fasting (NEFAs) (pmol/l)	0.59 ±0.25	0.47 ± 0.23	0.000
2 Hour (NEFAs) (pmol/l)	0.057 ±0.10	0.044±0.11	0.04
***Carotid IMT**(μM)	612(553-670)	581(525-640)	0.01

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P < 0.01$ is considered statistically significant

Table 7: The characteristics of the study population separated into northern and southern centres. Data are presented as Mean ± (SD).

Character	northern centers Mean ± (SD)	southern centers Mean ± (SD)	P value
Number	807	471	Total (1278)
Age	44.7 ± 8.2	42.3± 8.3	0.000
Male/Female	353/454	226/245	0.35
BMI (kg/m^2)	25.5 ± 3.9	25.8± 4.4	0.243
Waist circumference (cm)	86.8± 12.2	86.4 ±13.8	0.55
Fasting Glucose (mmol/l)	5.2 ± 0.52	4.95 ± 0.57	0.000
2 Hour Glucose (mmol/l)	5.7 ± 1.4	5.7 ± 1.5	0.5
***Fasting Insulin** (pmol/l)	32.6 (20-42)	29.3 (21-47)	0.003
***2Hour Insulin**	166.1(103-268)	137.7 (84.7-230)	0.001
Total Cholesterol (mmol/l)	4.83 ±0.83	4.75 ± 0.86	0.11
***Triglycerides** (mmol/l)	0.98 (0.70-1.31)	0.90 (0.63-1.25)	0.000
HDL – cholesterol (mmol/l)	1.35 ± 0.36	1.46 ± 0.38	0.000
LDL – cholesterol (mmol/l)	2.86 ± 0.78	2.92 ± 0.77	0.20
Systolic BP (mmHg)	118.3 ± 11.8	115.4 ± 12.3	0.000
Diastolic BP (mmHg)	74.7± 7.8	73.6 ± 7.7	0.007
Fasting (NEFAs) (pmol/l)	0.54 ±0.21	0.53 ± 0.27	0.32
2 Hour (NEFAs) (pmol/l)	0.07 ±0.05	0.03±0.16	0.000

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P < 0.01$ is considered statistically significant

Table 8: The value of c IMT in the study population and in males and females subjects: Data are presented as geometric mean [interquartile range 25th - 75th percentile].

Value	Number	Mean (μm)
IMT_{CCA} (μm)	1306	597 [539-659]
IMT_{ICA} (μm)	1306	620 [537-667]
IMT_{AVRG} (μm)	1306	663 [540-655]
IMT_{CCA} (μm)(**Male**)	579	612 [553-670]*
IMT_{CCA} (μm)(**Female**)	699	581 [525-640]

* Males vs Females: p=0.0001

Table 9 shows the distribution of the common carotid artery (CCA) by centre ID: Data are presented as geometric mean [interquartile range 25th - 75th percentile]

Centre Number	Number	Mean (μm)
1	80	605[547-669]
2	27	556[512-615]
4	78	571[523-641]
5	85	579[532-651]
7	66	580 [526-639
8	104	603 [544-667]
9	20	596 [540-655]
10	13	553 [506-609]
12	69	596 [542-655]
13	120	599 [541-661]
14	75	620 [562-671]
15	70	570 [522-641]
16	46	656 [596-691]
17	74	631[573-682]
18	74	570[520-643]
19	66	602[532-661]
20	19	592 [536-651]
21	67	625 [572-671]
22	41	599 [542-659]

Table 10: The distribution of the IMT value divided by Northern versus Southern centres. Data are presented as geometric mean [interquartile range 25^{th} - 75^{th} percentile].

Centre	Number	Male/Female	Mean (μm)
Northern centres	779	354/445	603 [543-665] *
Southern centres	499	240/259	591 [535-654]

* Northern vs Southern centres(age and sex adjusted): p =**0.013.**

Table 11: The mean [interquartile range 25th - 75th percentile] of the M/I value for the study population and in male and females subjects.

Value	Number	Geometric Mean ($\mu mol\ min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$)
M/I value ($\mu mol\ min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$)	1278	125.29 [92.0-178.6]
(M/I value **male**) ($\mu mol\ min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$)	570	109.2 [80.0-151.1] *
(M/I value **female**) ($\mu mol\ min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$)	708	139.9 [106.7-189.6]

* Males vs Females: p=0.001.

Table 12: The distribution of the M/I value by centre ID. Data presented as geometric mean [Interquartile ranges 25th - 75th percentile].

Centre	Number	Mean
1	84	96.0 (74.3-130)
2	32	134.0(102.5- 189.3)
4	86	141.7(110.0-195.0)
5	83	127.0(95.0-198.0)
7	70	156.3(124.4-198.0)
8	111	148.0(161.0-229.0)
9	85	98.4(73.0-141.3)
10	20	100.0(70.0-143.4)
12	76	126.7(101.3-156.6)
13	109	142.0(99.6-197.2)
14	79	164.7(124.0-219.4)
15	90	104.0(73.1-146.5)
16	41	137.7(78.4-137.7)
17	81	127.1(94.2-179.0)
18	76	120.5(92.4-180.2)
19	50	92.6(65.0-123.2)
20	19	112.5(80.2-145.7)
21	75	130.1(89.6-187.0)
22	52	140.0(97.3-210.4)

Geometic mean [interquartile range 25th - 75th percentile].

Table 13: The distribution of the M/I value divided by Northern versus Southern centers. Data is presented as geometric mean [interquartile range 25^{th} - 75^{th} percentile].

Centre	Number	Male/Female	Mean ($\mu mol\ min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$)
Northern Centers	814	377/437	114.2 ± (84.0-147.7) *
Southern Centers	464	205/259	132.2 ± (89.9-189.6)

* **Northern vs Southern centres (age and sex adjusted) p=0.005**

4. The role of the PPARγ Pro12Ala polymorphism in insulin sensitivity & CVD:

4.1 Introduction

PPARγ2 has been shown to influence insulin sensitivity and the risk of developing type 2 diabetes in various ethnic populations (97, 98). It plays a vital role in adipogenesis, lipid metabolism, insulin signalling and acts as a functional receptor for thiazolidinediones, insulin sensitizers used in the treatment of type 2 diabetes.

A common variant results from a cytosine to guanine nucleotide substitution and amino acid change, Proline to Alanine at codon 12 in exon 2 (Pro12Ala) polymorphism (96).The Pro 12 allele is very common in the general population with a frequency of 85% and this generates a diabetes population attributable risk of around 25% (98). Several Meta analyses confirmed that the Pro12Pro allele increases the susceptibility risk to type 2 diabetes (98,102). It was also previously shown that Pro12Ala variant of the PPARγ$_2$ gene influences insulin sensitivity, with evidence that this is mediated through altered body composition (97).

Frederiksen et al studied a cohort of non-diabetic Danish subjects who participated in the MONICA study, a population based study, and found that subjects homozygous for the Ala allele had decreased levels of serum triglycerides and diastolic blood pressure. However, there was no association with insulin sensitivity as assessed by HOMA- IR (101).

As the Ala/Ala genotype is very rare in the population with prevalence as low as 4% (103), there are very few studies, which included subjects homozygous for the Ala allele separately in the analysis, and hence it is difficult to assess whether the association with insulin sensitivity obesity and type 2 diabetes is a dominant or a recessive effect ie the effect is only seen in the Ala/Ala homozygous or is also seen in Pro/Ala heterozygote form.

There are very few data to date about the effect of the PPARγ gene on cardiovascular disease and early atherosclerosis. Recent data indicated that PPARγ is expressed in macrophages and suggest an effect on atherosclerosis (108). A more recent study investigated a total of 622 impaired glucose tolerant subjects, who participated in the RIAD study and they showed that carotid intima media thickness (cIMT) was significantly decreased in subjects homozygous for the Ala /Ala variant compared to the other two genotypes (108). It also demonstrated expression of the PPARγ gene in human atherosclerotic lesions and in cultured primary macrophages and foam cells. These studies together with the observation of the decreased (IMT) in diabetic patients treated with the PPARγ agonist troglitazone (106,107) support the hypothesis that PPARγ gene might be a pleiotropic gene and not only increase susceptibility to type 2 diabetes but to CVD as well.

4.2 Aims

The aims of this study are:

- To investigate the relationship between the Pro12Ala variant of the PPARγ2 gene and cardiovascular risk factors in a cohort of non diabetic healthy Caucasian population.
- To investigate the relationship between the Pro12Ala variant of the PPARγ2 gene and insulin sensitivity in a cohort of non diabetic healthy Caucasian population.
- To investigate the relationship between the Pro12Ala variant of the PPARγ2 gene and cIMT as a marker of CVD in a cohort of non diabetic healthy Caucasians population.

4.3 Detection of the Pro12Ala polymorphism by RFLP-PCR

A 270 base pair (bp) fragment of the PPARγ2 gene encompassing the pro12Ala polymorphism site was amplified by PCR using the primers described by Yen et al 1997(96).

Forward primer 5'-GCCAATTCAAGCCCAGTC- 3'.

Reverse primer5'-ATATGTTTGCAGACAGTGTATCAGTGAGGAATCGCTTTCCG3'.

A 20 µl volume PCR reaction was set up consisting of a final concentration of: 0.4 µM of each primer, 200µM of each dNTP, 0.75 U of Ampli Tag Gold, 1×PCR buffer, 1.5 Mm $Mgcl_2$ solution and 3 ng.ul^{1}of genomic DNA. PCR cycling condition was as follows: 5 min at 95^0 c for initial denaturing then 30 cycles consisting of 1 min at 95^0 c of denaturing followed by 1 min at 60^0c of annealing, I min at 72^0 c of extension, with final extension step at 72^0 c for 10 min .PCR products were then checked to confirm the correct size of 270 bp by taking 5µl of the PCR product and separating the products on 1% agrose gel.Restriction digests of PCR products were formed using 8µl of PCR product and 2µl of the appropriate enzyme (*Bst* UI restriction enzyme, New England BioLabs Ltd.(NEB),UK) and 10x recommended reaction buffer (supplied with the enzyme).

The primers used generated a Bst UI restriction site (5' ...CG‖CG... 3') only when the C to G substitution is present on nucleotide 34.

Products were digested for 2 hours at 60^0 c then they were separated on 3% agrose gel and visualised by ethiduim bromide staining. The expected products sizes after digestion were as follows.

Pro12Pro = 270 bp

Pro12Ala = 270, 227, 43 bp

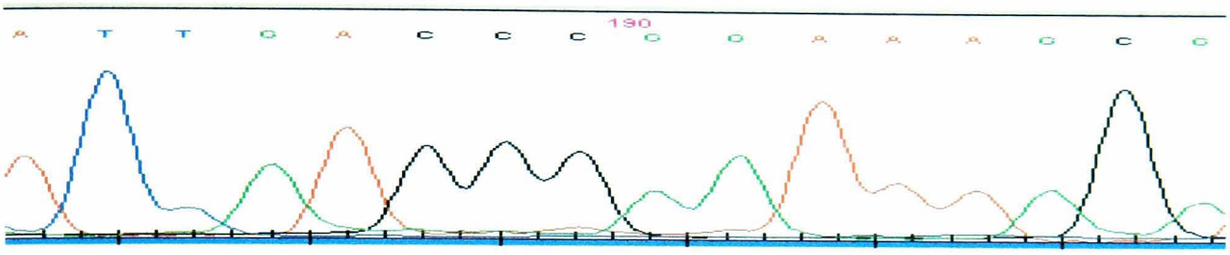
Ala12Ala = 227, 43 bp

To check for the correct sequence of the product, PCR products of Pro12Ala heterozygote, Pro12Pro and Ala12Ala homozygotes were sequenced and shown in figure (4).

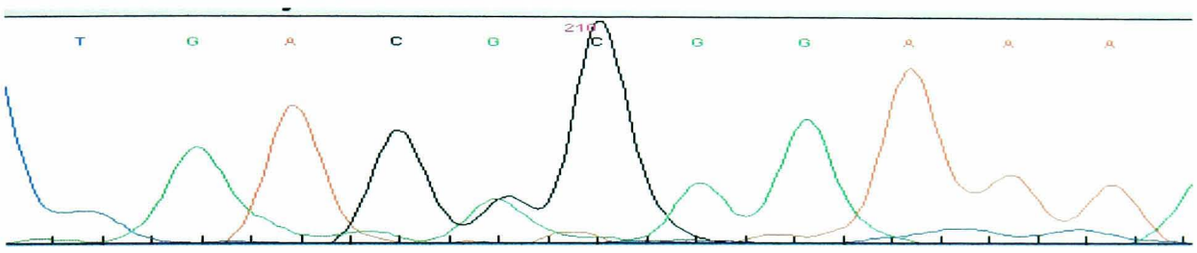
4.3.1 PCR product, purification and sequencing:

PCR was first purified using a Micron-PCR clean up kit (Millipore) following the specified kit protocol. The aim of the purification is to remove primers, nucleotides, salts and polymerase. Sequencing of PCR products were carried out by Dr. Sheila Patel. Oligonucleotides were used to prime PCR amplifications.

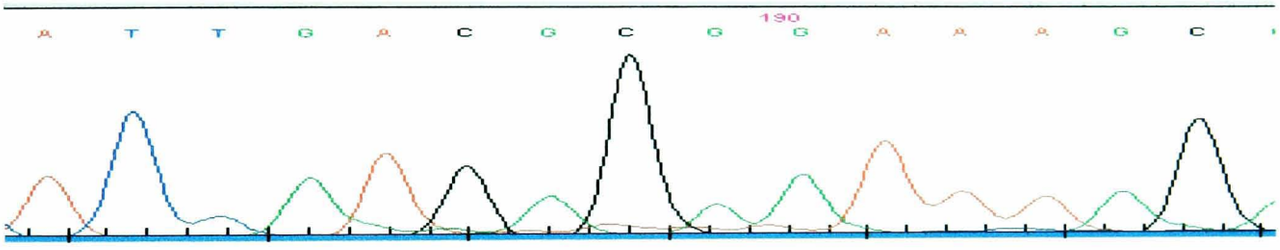
Sequencing was carried out using a ABI PRISM dye terminator cycle sequencing kit using the Gene Amp PCR system 9700 thermal cycler (Perkin-Elmer, Warrington,UK). Analysis of the sequence results were carried out using Navigator^{TM}version1.2.0 and Factura software^{TM} version 1.0.1 (ABI Perkin-Elmer, Warrington,UK).



Proline homozygote (Pro12Pro)



Proline/Alanine heterozygote (Pro12Ala)



Alanine homozygote (Ala12Ala)

Figure 4: An electropherogram of the immediate sequence surround the Pro12Ala polymorphism. A Pro allele homozygote, Pro12Ala heterozygote and Ala allele homozygote. There is a Bst UI restriction site at position 201pb ((5' ...CG‖CG... 3')

4.4 Statistical Analysis

Statistical analysis was carried out using Minitab version 15 (Minitab Inc, USA). Skewed variables were log transformed to normalize distributions and are presented as geometric means. P values < 0.01 were considered significant. The ANOVA test was used initially to compare SNP genotypes with biological variables. Significant differences were further explored using the independent samples t-Test. General linear Model (GLM) analysis was performed to test for associations between SNP genotypes and risk factors after adjusting for confounding metabolic and lifestyle variables (sex, age, BMI, waist smoking status, serum cholesterol and recruitment centre).

4.5 Results

4.5.1 Genotype and allele frequencies for the Pro12 Ala of the PPARγ:

The following allele frequencies were found for the Pro 12 Ala polymorphism:

Pro allele = 89%

Ala allele =11%

Observed genotype frequencies were not significantly different from those expected by Hardy-Weinberg equilibrium. Allele frequencies were similar to those reported in other European population (97-99).

4.5.2 Genotype / phenotype relationships for PPARγ

Table 14 summarises the metabolic and anthropometric data for the 3 genotypes of the Pro12 Ala variant, (Pro/Pro, Pro/Ala & Ala/Ala). Subjects homozygous for the Ala allele (Ala/Ala), had lower 2- hour insulin levels during OGTT, compared to other genotypes (Pro/Pro vs. Pro/Ala vs. Ala/Ala [146.2 (89-241) vs.134 (85-219) vs.72.6 (28.6-132.8)) geometric mean [interquartile range 25th - 75th percentile] pmol/l. p=0.007).

There were no significant differences between the 3 groups when analysed by ANOVA in relation to age, body mass index, waist circumference, blood pressure, total cholesterol, HDL cholesterol or fasting NEFAs.

Subjects homozygous for the Ala allele had higher body mass index and waist circumference, and lower circulating serum triglycerides when compared with carriers of the other genotypes, but none of these differences reached statistical significance.

When applying general linear model to correct for confounding factors (age, sex, BMI, waist and centre effect), this revealed significant difference between the 3 genotype groups (Table 15). In particular, Ala/Ala allele homozygotes had lower fasting insulin (p=0.01) and lower 2 hour insulin levels (p=0.007), together with lower circulating serum triglyceride levels (p=0.02)

As shown in Tables 14 and 15, subjects homozygous for the Ala allele (Ala/Ala) had a higher M/I value before (p=0.03) and after (p=0.007) correction for key covariates, indicating increased insulin sensitivity compared to subjects with the other 2 genotypes.

4.5.3 PPARγ and insulin sensitivity

As the main differences described above were between the Ala/Ala allele carriers and the other genotype groups, we then compared subjects homozygous for the Ala allele to the Pro allele carriers (Pro/Pro + Pro/Ala).

Table 16 shows that subjects homozygous for the Ala allele had a lower mean 2 hour insulin level compared to the Pro allele carriers which remained significant after correcting for age, sex, waist circumference, BMI and centre effects (Table 17). They also had lower fasting insulin levels after correcting for the same confounding factors (Table 17).

Subjects homozygous for the Ala allele were more insulin sensitive (M/I value; 120.8(93-175) vs. 176.2(133-214) [Geometric Mean [interquartile range 25th - 75th percentile] min^{-1} kg_{ffm}^{-1} nM^{-1}; p=0.002) compared to carriers of the Pro allele [Pro/Pro + Pro/Ala] after adjusting for age, sex, BMI, waist circumference and recruitment centre. Subjects homozygous for the Ala allele also had lower adjusted fasting triglyceride levels (0.70(0.68-1.2) vs. 0.94 (0.5-1.1) [Geometric Mean [interquartile range 25th - 75th percentile] mmol/l; p=0.01) (Table 17). However, this did not explain the greater insulin sensitivity, which remained statistically significant after including triglyceride levels as a covariate (Table 17).

Subgroup analysis was then performed to look into the effect of BMI and centre effect. For those with BMI $\leq$ 27 kg/m^2, subjects homozygous for the Ala allele showed greater insulin sensitivity compared to the other 2 alleles carriers after correcting for the same confounding factors. (Pro/Pro vs. Pro/Ala vs. Ala/Ala: 143.5 (111.3-191.5) vs.

144.0(104.1-195.1) vs. 188.4(146.9-224.0) [Geometric Mean [interquartile range 25th - 75th percentile] min^{-1} kg_{ffm}^{-1} nM^{-1}; p=0.027)(Table 18).

On the other hand there were no significant differences in subjects with BMI greater than 27 kg/m^2 in relation to insulin sensitivity when comparing the 3 different genotypes (Table19).

In subjects with a BMI $\leq$ 27 kg/m^2, homozygous Ala allele carriers were more insulin sensitive compared to carriers of the Pro allele [Pro/Pro + Pro/Ala] after correction for covariates (p=0.008) (Table 20) Although there was a similar trend towards higher insulin sensitivity in the Ala/Ala allele subjects in those with a BMI >27 kg/m^2, this didn't reach statistical significance (Table 21).

In subjects from the southern European centres, those homozygous for the Ala allele had a greater insulin sensitivity compared to other allele carriers (Pro/Pro vs. Pro/Ala vs. Ala/Ala: 129.8(96-159) vs.136.7 (104-183) vs. 181.9 (140-218) [Geometric Mean [interquartile range 25th - 75th percentile] min^{-1} kg_{ffm}^{-1} nM^{-1}; p=0.003) (Table 22). Again when comparing subjects homozygous for the Ala allele to carriers of the Pro allele [Pro/Pro + Pro/Ala] in subjects from the southern centres, subjects homozygous for the Ala allele were more insulin sensitive compared to the Pro allele carriers when correcting for the same confounding factors (131.4(98.2-162) vs. 181.9(140-218) [Geometric Mean [interquartile range 25th - 75th percentile] min^{-1} kg_{ffm}^{-1} nM^{-1}; p=0.002) (Table 24).

However there were no differences between the 3 alleles in relation to insulin sensitivity in subjects from the northern European centres (Tables 23 and 25).

4.5.4 PPARγ and carotid- intima media thickness (cIMT)

Table 26 summarises the mean of the carotid intima media thickness (cIMT) for the 3 genotypes of the Pro12 Ala variant,(Pro/Pro, Pro/Ala & Ala/Ala). There were no significant differences between the 3 genotypes for each IMT measurement

In subjects with BMI > 27 kg/m^2, there was a trend that subjects homozygous for the Ala allele had a lower c IMT compared to other genotypes; however this didn't reach statistical significane (Table 28).

Although subjects from the southern centres have significantly lower cIMT compared to subjects from northern centres (Table 10), there was no difference between the 3 genotypes groups for IMT measures when dividing the subjects into northern and southern centres. In the southern centres group Ala/Ala subjects had a lower c IMT compared to other genotypes group but this difference didn't reach statistical significance (Table 29 & 30).

Comparing subjects homozygous for the Ala allele to carriers for the Pro allele (Pro/Ala + Pro/Pro) showed no differences between IMT measures when analysed together and when subdivided by BMI and by latitude (data not shown).

Table 14: ANOVA comparisons of means for Pro12Ala genotypes with anthropometric and metabolic variables (Data presented as means [SE])

Character	Pro/Pro N = 1001	Pro/Ala N = 264	Ala/Ala N = 13	P Value
Age (years)	43.5 (0.26)	44.4 (0.5)	45.6(2.9)	0.23
Male/Female	445/556	125/139	6/7	0.46
BMI kg/m2	25.5 (0.12)	25.6 (0.26)	26.6 (0.8)	0.54
Waist circumference (cm)	86.6 (0.4)	87.3 (0.9)	92.8 (2.7)	0.1
Fasting Glucose mmol/l	5.1(0.02)	5.1 (0.04)	5.2(0.15)	0.92
*Fasting Insulin pmol/l	30.2 (21-44)	28.5(20-40)	24.5(19-34)	0.16
Fasting NEFAs mmol/l	0.53(0.01)	0.55(0.01)	0.56(0.07)	0.54
2 hours Glucose mmol/l	5.7 (0.05)	5.7 (0.11)	5.4 (0.530)	0.73
*2 hours Insulin pmol/l	146.2 (89.0-241)	134(85.0-219.0)	72.6(28.6-32.8)	**0.007**
2 hours NEFAs mmol/l	0.05 (0.004)	0.05 (0.08)	0.02 (0.006)	0.69
Total- cholesterol mmol/l	4.8(0.03)	4.9(0.05)	4.5(0.22)	0.16
*Triglycerides mmol/l	0.95 (0.68-1.28)	0.97(0.69-1.10)	0.78(0.53-1.10)	0.27
HDL - cholesterol mmol/l	1.4(0.01)	1.4(0.02)	1.4(0.11)	0.93
LDL - cholesterol mmol/l	2.9(0.02)	2.9(0.05)	2.7(0.20)	0.27
Systolic BP mmHg	117.1(0.4)	116.8(0.8)	117.4(2.8)	0.94
Diastolic BP mmHg	74.1(0.2)	74.4(0.5)	74.1(1.8)	0.86
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	124.2(93-176.3)	126.8(89-182.1)	171.8(133.7-210.4)	**0.03**

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 15: Analysis of Covariance PPARγ (Pro12Ala) Age, Sex, BMI, Waist, Centre adjusted means [SE]) with anthropometric and metabolic variables

Character	**Pro/Pro** **N = 1001**	**Pro/Ala** **N= 264**	**Ala/Ala** **N = 13**	**P** **Value**
Fasting Glucose mmol/l	5.1(0.03)	5.0(0.05)	5.0(0.15)	0.79
*Fasting Insulin pmol/l	30.0 (20-44)	28.4 (21-42)	21.7 (19-35)	**0.01**
Fasting NEFA mmol/l	0.53(0.01)	0.53(0.01)	0.56(0.06)	0.85
2 hours Glucose mmol/l	5.7 (0.06)	5.9 (0.11)	5.5 (0.43)	0.48
*2 hours Insulin pmol/l	143.5(88-239)	133.6(84-218)	75.5 (29-136)	**0.007**
2 hours NEFA mmol/l	0.05 (0.00)	0.04(0.01)	0.03(0.02)	0.30
Total- cholesterol mmol/l	4.7(0.03)	4.8(0.05)	4.4(0.22)	0.15
*Triglycerides mmol/l	0.94 (0.68-1.28)	0.93(0.69-1.11)	0.69(0.53-1.11)	**0.02**
HDL - cholesterol mmol/l	1.4(0.01)	1.4(0.02)	1.5(0.08)	0.49
LDL - cholesterol mmol/l	2.9(0.02)	2.9(0.04)	2.5(0.2)	0.22
Systolic BP mmHg	117.7(0.4)	116.3(0.7)	114.4(2.9)	0.22
Diastolic BP mmHg	74.4(0.3)	74.7(0.5)	72.7(2.0)	0.63
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	120.5(92.7-172)	122.5(88.7-180)	176.2(133.4-214.3)	**0.007**

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 16: ANOVA comparisons of means for Pro allele carriers (Pro/Pro+ Pro/Ala) vs (Ala/Ala) genotype with anthropometric and metabolic variables (Data presented as means [SE])

Character	**P/P+P/A** **N = 1265**	**Ala/Ala** **N = 13**	**P** **Value**
Age (years)	43.7 (0.2)	45.6(2.9)	0.4
BMI kg/m2	25.5 (0.1)	26.6 (0.8)	0.33
Waist circumference (cm)	86.6 (0.4)	92.8 (2.7)	0.08
Fasting Glucose mmol/l	5.1(0.02)	5.2(0.15)	0.698
*Fasting Insulin pmol/l	29.8(20-43)	24.5(19.2-34)	0.19
Fasting NEFA mmol/l	0.53(0.01)	0.56(0.07)	0.7
2 hours Glucose mmol/l	5.7 (0.05)	5.4(0.52)	0.51
*2 hours Insulin pmol/l	143.5 (88-236)	72.6 (28.5-164.8)	**0.004**
2 hours NEFA mmol/l	0.05 (0.00)	0.03 (0.01)	0.43
Total- cholesterol mmol/l	4.8(0.02)	4.5(0.24)	0.18
*Triglycerides mmol/l	0.95 (0.68-1.28)	0.78(0.53-1.1)	0.13
HDL - cholesterol mmol/l	1.4(0.01)	1.4(0.1)	0.93
LDL - cholesterol mmol/l	2.9(0.02)	2.7(0.2)	0.32
Systolic BP mmHg	117.0(0.35)	117.4(2.8)	0.91
Diastolic BP mmHg	74.2(0.22)	74.1(1.8)	0.96
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	124.7(93-176)	171.8(133-210)	**0.03**

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 17: Analysis of Covariance PPARγ (Pro12Ala) for Pro allele carriers (Pro/Pro+ Pro/Ala) vs (Ala/Ala) genotype with anthropometric and metabolic variables (age, BMI, sex, centre adjusted means [SE])

Character	**P/P+P/A** **N = 1265**	**Ala/Ala** **N = 13**	**P** **Value**
Fasting Glucose mmol/l	5.1(0.02)	5.0(0.14)	0.63
*Fasting Insulin pmol/l	30.0(21-44)	22.3 (18-32)	**0.02**
Fasting NEFA mmol/l	0.53(0.01)	0.57(0.06)	0.576
2 hours Glucose mmol/l	5.8(0.06)	5.5(0.43)	0.589
*2 hours Insulin pmol/l	142.6 (88-233)	77.1(29-176)	**0.004**
2 hours NEFA mmol/l	0.05(0.00)	0.03 (0.02)	0.61
*Triglycerides mmol/l	0.94 (0.68-1.2)	0.70(0.5-1.1)	**0.01**
Total- cholesterol mmol/l	4.7(0.03)	4.3(0.2)	0.07
HDL - cholesterol mmol/l	1.4(0.01)	15(0.08)	0.39
LDL - cholesterol mmol/l	2.9(0.02)	2.6(0.2)	0.12
Systolic BP mmHg	117.2(0.3)	114.5(2.9)	0.36
Diastolic BP mmHg	74.4(0.2)	72.9(2.0)	0.45
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	120.8(93-175)	176.2(133-214)	**0.002****

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

** P= 0.004 after including Triglycerides levels as a coveriate

Table 18: ANOVA comparisons of means for Pro12Ala genotypes in relation to insulin sensitivity (M/I value) for those with BMI ≤27 kg/m^{2}Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	Pro/Pro Number=669	Pro/Ala Number=175	Ala/Ala Number=8	P Value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	143.5 (111.3-191.5)	144.0 (104.1-195.1)	188.4 (146.9-224.0)	0.19	**0.027**

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 19: ANOVA comparisons of means for Pro12Ala genotypes in relation to insulin sensitivity (M/I value) for those with BMI> 27 kg/m^{2}Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	Pro/Pro Number=332	Pro/Ala Number=89	Ala/Ala Number=5	P value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	91.0 (66-128.8)	95.9 (67.9-129.2)	143.0 (120.1-198)	0.167	0.10

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 20: ANOVA comparisons of means for Pro allele carriers (Pro/Pro+ Pro/Ala) vs. (Ala/Ala) genotype in relation to insulin sensitivity (M/I value) for those with BMI ≤27 kg/m^{2}Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	P/P+P/A Number=844	(A/A) Number=8	P Value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	143.5(111.3-191.5)	188.4(146-224)	0.07	**0.008**

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 21: ANOVA comparisons of means for Pro allele carriers (Pro/Pro+ Pro/Ala) vs. (Ala/Ala) genotype in relation to insulin sensitivity (M/I value) for those with BMI >27 kg/m^{2}Data presented as geometric mean [interquartile range 25th - 75th percentile]

Character	P/P+P/A Number=421	(A/A) Number=5	P Value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	92.0(68-120)	142.9(120-197)	0.08	0.08

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 22: ANOVA comparisons of means for Pro12Ala genotypes in relation to insulin sensitivity (M/I value) for those from the southern centres. Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	Pro/Pro Number=394	Pro/Ala Number=88	Ala/Ala Number=5	P value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	129.8 (96-159)	136.7 (104-183)	181.9 (140-218)	**0.049**	**0.003**

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 23: ANOVA comparisons of means for Pro12Ala genotypes in relation to insulin sensitivity (M/I value) for those from the northern centres. Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	Pro/Pro Number=607	Pro/Ala Number=176	Ala/Ala Number=8	P Value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	115.6 (84-148)	106.0 (68-128)	129.09 (102-162)	0.35	0.37

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 24: ANOVA comparisons of means for Pro allele carriers (Pro/Pro+ Pro/Ala) vs. (Ala/Ala) genotype in relation to insulin sensitivity (M/I value) for those from the southern centres. Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	P/P+P/A Number=482	(A/A) Number=5	P Value	P$^{\#}$ Value
M/1 min^{-1} kg_{ffm}^{-1} nM^{-1}	131.4(98.2-162)	181.9(140-218)	**0.038**	**0.002**

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 25: ANOVA comparisons of means for Pro allele carriers (Pro/Pro+ Pro/Ala) vs. (Ala/Ala) genotype in relation to insulin sensitivity (M/I value) for those from the northern_centres. Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	P/P+P/A Number=695	(A/A) Number=8	P Value	P$^{\#}$ Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	113.8(89-149.2)	129.0(102-63.2)	0.722	0.591

P$^{\#}$ value after analysis of covariance (age, sex, BMI, waist and centre adjusted means)

Table 26: Comparisons of means for Pro12Ala genotypes in relation to(c IMT) measures Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	Pro/Pro N = 934	Pro/Ala N= 247	Ala/Ala N = 13	P Value	P$^{\#}$ Value
IMT_{CCA} (μm)	599(540-653)	602(543-658)	580(538-643)	0.67	0.32
IMT_{BULB} (μm)	764(699-814)	773(703-830)	716(633-862)	0.39	0.31
IMT_{ICA} (μm)	615(556-671)	628(570-684)	570(528-633)	0.26	0.22
IMT_{AVRG} (μm)	662(603-728)	669(609-731)	636(571-685)	0.47	0.32

P$^{\#}$ value after analysis of covariance (age, BMI, waist, sex, centre, smoking, total cholesterol, systolic blood pressure, fasting glucose adjusted means)

Table 27: Comparisons of means for Pro12Ala genotypes in relation to(c IMT) measures for those with BMI ≤27 kg/m^2. Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	Pro/Pro N = 669	Pro/Ala N= 175	Ala/Ala N = 8	P Value	P$^{\#}$ Value
IMT_{CCA} (μm)	588(544-650)	589(530-653)	595(543-649)	0.97	0.98
IMT_{BULB} (μm)	751(680-799)	761(696-811)	748(676-796)	0.72	0.48
IMT_{ICA} (μm)	606(543-662)	613(551-669)	591(532-645)	0.79	0.79
IMT_{AVRG} (μm)	650(591-716)	656(598-723)	659(601-725)	0.79	0.69

P$^{\#}$ value after analysis of covariance (age, BMI, waist, sex, centre, smoking, total cholesterol, systolic blood pressure, fasting glucose adjusted means)

Table 28: Comparisons of means for Pro12Ala genotypes in relation to(c IMT) measures for those with BMI > 27 kg/m^{2}Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	Pro/Pro N = 265	Pro/Ala N= 72	Ala/Ala N = 5	P Value	P$^{\#}$ Value
MT_{CCA} (μm)	625(560-685)	634(575-691)	544(528-590)	0.08	0.22
IMT_{BULB} (μm)	797(726-851)	802(732-854)	640(567-713)	0.07	0.20
IMT_{ICA} (μm)	641(576-702)	665(602-714)	536(531-599)	0.09	0.20
IMT_{AVRG} (μm)	692(630-734)	701(639-740)	582(568-628)	0.07	0.17

P$^{\#}$ value after analysis of covariance (age, BMI, waist, sex, centre, smoking, total cholesterol, systolic blood pressure, fasting glucose adjusted means)

Table 29: ANOVA comparisons of means for Pro12Ala genotypes in relation to (c IMT) measures for those from the Northern Centres. Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	Pro/Pro N = 607	Pro/Ala N= 176	Ala/Ala N = 8	P Value	P# Value
IMT_{CCA} (μm)	604(542-655)	606(540-661)	591(536-650)	0.88	0.59
IMT_{BULB} (μm)	772(706-826)	783(712-837)	738(667-802)	0.57	0.41
IMT_{ICA} (μm)	620(560-680)	633 (575-690)	598(548-653)	0.40	0.55
IMT_{AVRG} (μm)	668(605-717)	675(610-724)	654(591-703)	0.72	0.45

$P^{\#}$ value after analysis of covariance (age, BMI, waist, sex, centre, smoking, total cholesterol, systolic blood pressure, fasting glucose adjusted means)

Table 30: ANOVA comparisons of means for Pro12Ala genotypes in relation to(c IMT) measures for those from the Southern Centres. Data presented as geometric mean [interquartile range 25th - 75th percentile]

Value	Pro/Pro N = 327	Pro/Ala N= 71	Ala/Ala N = 5	P Value	P# Value
IMT_{CCA} (μm)	590(544-649)	593(538-648)	534(519-579)	0.61	0.76
IMT_{BULB} (μm)	751(680-815)	748 (676-812)	626(566-696)	0.43	0.46
IMT_{ICA} (μm)	607(645-685)	615(655-675)	585(535-640)	0.40	0. 74
IMT_{AVRG} (μm)	652(590-702)	655(591-702)	563(601-712)	0.43	0.47

$P^{\#}$ value after analysis of covariance (age, BMI, waist, sex, centre, smoking, total cholesterol, systolic blood pressure, fasting glucose adjusted means)

4.6 Discussion:

The Pro 12 Ala polymorphism of the PPARγ gene has been found to be associated with obesity, insulin resistance and type 2 diabetes in different study populations. It has been previously shown that the homozygous variant genotype (Ala/Ala) confers a decreased risk of type 2 diabetes, and protects against insulin resistance syndrome (100).

The Ala allele variant of the Pro12Ala polymorphism has also been shown to influence insulin sensitivity, with evidence that this is mediated through altered body composition (97).

In this study we replicated the association of Pro12 Ala and insulin sensitivity in healthy individuals, with subjects homozygous for the Ala allele more insulin sensitive than the rest of the population. Intriguingly this was independent of measures of adiposity assessed by BMI and waist circumference. In our study, as in other studies (97,135), we found an association between the Ala allele and lower fasting insulin levels, lower total triglycerides levels and higher insulin sensitivity.

The strength of our study is that it used the euglycaemic hyperinsulinaemic clamp to measure insulin sensitivity. It also used data from healthy volunteers, ie studied a pre or non diabetic population. Although by studying a healthy cohort we would have excluded some subjects with decreased insulin sensitivity with worse metabolic profile, for example, diabetes, dyslipidemia and hypertension, the range of insulin sensitivity in this healthy cohort was broad, with a 10 fold spread across the M/I range. The advantage of using a healthy, disease-free cohort is that it avoids the secondary metabolic effects of the established diabetic state that can confound and obscure the relationship between the genetic variants and insulin resistance.

It is evident from the data of our study that the key differences are between the Ala/Ala carriers and the other genotype groups. For this reason, we compared subjects homozygous for the Ala allele to Pro allele carriers. This showed that the Ala/Ala homozygotes had greater insulin sensitivity and lower serum triglyceride levels independent of measures of adiposity and body composition.The association with lower serum triglyceride levels didn't explain the greater insulin sensitivity, which remained after including triglyceride levels as a covariate.

The finding from our study is that, although subjects homozygous for the Ala allele tended to have a marginally greater BMI and waist circumference, they had greater insulin sensitivity. This was hypothesised to be due to the fact that the Ala12Ala allele of the PPARγ gene promotes peripheral deposition of adipose tissue and increased insulin sensitivity previously described by Gonzalez Sanhez et al (136).

We undertook further subgroup analysis to look into the relationship between the BMI and the Pro12 Ala polymorphism as this was reported to be complex in previous studies with some contradicting findings (137).We found no interaction between BMI and Pro12 Ala polymorphism of the PPARγ gene.

In a population-based Spanish study by Gonzalez Sanhez et al (136) the frequency of the Ala12 allele carriers (Ala/Ala + Pro/Ala) was higher in obese men than in lean men and Ala12 allele carriers had a higher BMI compared to non-carriers, lower total triglycerides levels and lower fasting insulin levels and higher insulin sensitivity as assed by HOMA (136). In our study we found similar results, in which Ala12 alle homozygotes had a higher BMI, higher insulin sensitivity and lower triglycerides levels.

Interstingly in the same study, despite having high BMI, obese male carriers of the Ala12 allele had a lower sagittal abdominal diameter than Pro12 homozygous carriers (136).

In our study, we found that, in lean subjects with BMI less or equal to 27 kg/m^2, subjects homozygous for the Ala allele had greater insulin sensitivity compared to the other 2 genotypes after correcting for age, sex and recruiting centre. On the other hand there were no significant differences in subjects with BMI greater than 27 kg/m^2 in relation to insulin sensitivity when comparing the 3 different genotypes. It is very difficult to draw any conclusions from this finding as the numbers are very small and this needs pooling of all the study results to look specifically for the association of insulin resistance in relation to Pro 12 Ala polymorphism in subjects with BMI greater than 27 kg/m^2 and study the fat distribution using MRI in this group.It should be noted that,in the study by Gonzalez et al they used a cut off of 30 kg/m^2 for the BMI to divide subjects into lean and obese respectively, they also combined Pro 12 Ala and Ala 12 Ala individuals and compared them to Pro12 Pro and used HOMA-IR to assess insulin sensitivity.

On the other hand the association between lower BMI and greater insulin sensitivity in subjects homozygotes for the Ala allele of the PPARγ gene was previously described by Deeb et al (97).

The reasons for the discrepancies in association studies between Pro 12 Ala polymorphism of the PPARγ gene and BMI could be explained by ethnic differences, study design and or differences in PPARγ expression between subcutaneous and visceral fat (138).

Another large, UK based well-characterized case-control study with either positive family history and/ or early onset of Type 2 diabetes, studied 971 UK case samples and 1257 ethnically matched control subjects. This study found conflicting association between the Pro12 Ala variant and BMI. They found that subjects homozygous for the Pro 12 allele had increased BMI in the young onset Type 2 diabetes group and decreased BMI in the Warren 2 sib-pair probands group. This divergent BMI association in 2 different diabetic populations in this study suggest that the relationship between Pro12 Ala polymorphism of the PPARγ gene and BMI warrants further studies (137).

To examine the gene- environment interaction in this large European study with diverse countries difference such as Greece and Finland, we performed subgroup analysis, dividing centres into northern and southern centres according to latitude. In subjects from the southern European centres, those who were homozygous for the Ala allele had greater insulin sensitivity than other pro 12 genotypes. This could be explained by different environmental and dietary factors e.g the presence of the mediteranian type diet in the southern centres. The finding of increased insulin sensitivity in Ala allele homozyogtes in subjects from the southern European centres gives further evidence for gene-nutrient interaction at the PPAR γ gene. This was previously reported by Luan et al demonstrating that the effect of Pro12 Ala polymorphism of the PPAR γ gene may be affected by diet, with the role of PPAR γ as nutrient sensor regulating adipogenesis and insulin sensitivity (139).

They studied 592 nondiabetic Caucasian subjects from the Isle of Ely study. There was a significant inverse relationship between dietary polysaturated to saturated fat ratio (P:S) and fasting insulin after correction for age and sex. They found that as the P: S ratio

increases, both BMI and fasting insulin decreases in Ala allele carriers, but not in Pro allele homozygotes.Their data suggest that when the dietary P: S ratio is low, the mean BMI in the Ala allele carriers is greater than that in the Pro homozygotes which could explain our finding of higher BMI and insulin sensitivity in the Ala allele homozygotes.

Our study didn't show any significant association between Pro12 Ala polymorphism of the PPAR γ gene and c IMT which did not support the overall hypothesis of this study, that the Pro12 Ala polymorphism of the PPAR γ gene is T2DM susceptibility gene and exerts a pleotropic effect influencing CVD as assessed by c IMT.

The limitation of this study is that it used healthy volunteers and hence there would have been many exclusion criteria for those with unfavourable cardiovascular risk factors and the results would be an underestimate of the cardiovascular risk. It also used cross-sectional analysis of the RISC study. However, on the positive side, the RISC study is a longitudinal study of CVD and so we will, in time, be able to investigate the relationship between the Pro 12 Ala polymorphism of the PPAR γ gene and carotid IMT progression.

4.7 Conclusions:

We have confirmed that the Pro12Ala polymorphism of the PPAR γ gene influences insulin sensitivity in the healthy population. Specifically, subjects homozygous for the Ala allele are more insulin sensitive compared to the rest of the population, and this appears to be independent of differences in circulating triglyceride levels and measures of adiposity.

Subgroup analysis showed that, in lean subjects and those from the southern European centres, the Ala 12 Ala allele is associated with greater insulin sensitivity compared to the other allele genotypes.

In this cross sectional analysis of the RISC study, we found no significant association between carotid intima media thickness as a measure of cardiovascular disease and the Pro 12 Ala polymorphism in the PPAR γ gene.

5. The role of the T 45 G SNP of the ADIPOQ gene in insulin resistance and CVD

5.1 Introduction:

Several polymorphisms in the ADIPOQ gene have been reported to be associated with type 2 diabetes, obesity and coronary heart disease (115,118). In the Japanese population a T-G SNP in exon 2 (SNP+45) and a G-T SNP in intron 2 (SNP+276) were reported to be significantly associated with increase risk of type 2 diabetes (116). In a French population with type 2 diabetes Vasseur et al. found an association between the G allele of SN+45 of the ADIPOQ gene and coronary heart disease (117). The G allele of SNP+45 of the ADIPOQ gene was also found to be associated with CAD in patients with type 2 diabetes independent of other cardiovascular risk factors (123).

In a study by Hara et al. individuals with the G/G genotype of SNP+45 of the ADIPOQ gene had a higher risk of developing type 2 diabetes (116).In German individuals without type 2 diabetes, those who are G allele homozygotes of the SNP+45 of the ADIPOQ gene had higher BMI compared to T allele carriers (140). In the same study, the insulin sensitivity (measured by euglycaemic clamp) was significantly lower in the G allele carriers; however this didn't remain statistically significant after adjustment for BMI. Circulating adiponectin levels have been reported to be negatively correlated with c IMT (119) and higher plasma levels have been associated with a lower risk of myocardial infarction (118). In an animal model, Matsuda et al. found that adiponectin–deficient mice had severe neointamal thickening and increased proliferation of vascular smooth muscle cells in a mechanical injury model of re-stenotic change following balloon angioplasty (143).

Therefore, there is an emerging evidece that SNP+45 of the ADIPOQ gene may be associated with CVD and type 2 diabetes. However, there is limited work investigating the role of this SNP in healthy individuals.

5.2Aims:

The aims of this study were:

- To examine if the G allele of the +45 T-G SNP of the adiponectin gene is associated with cardiovascular risk factors in non diabetic population.
- To examine if the G allele of the +45 T-G SNP of the adiponectin gene is associated with insulin sensitivity in non diabetic Caucasian population.
- To examine if the G allele of the +45 T-G SNP of the adiponectin gene is associated witth cIMT as a marker of CVD in a cohort of the general non-diabetic population.

5.3 Methods:

5.3.1 Detection of T- 45- G SNP of the ADIPOQ gene using Sequenom Mass ARRAY assay

119 bp fragment of the adiponectin gene encompassing the site of the +45 T-G SNP was amplified by PCR. A matrix-assisted laser desorption/ ionisation time-of-flight (MALDI-TOF) mass spectrometry (Sequenom MassARRAY, San Diego, CA, USA) method was used to genotype SNP T45G, this was described in detail in chapter 2. The primers were designed using the Sequenom MassARRAY Assay design program version 2.0.4 (Sequenom, San Diego, CA, USA) and ordered from Metabion International AG, Martinsried, Deutschland, Germany.

The PCR primer sequences were as follows:

Forward primer 5'AGTGCACATGTGGATTCCAG 3',

Reverse primer 5' CCTTGAGTCGTGGTTTCCTG-3'.

A 5µl PCR reaction was set up using 2 ng DNA, 1.25 × HotStar Taq PCR buffer, total 3.5 mM $MgCl_2$ per reaction, 200 µM each dNTP, 100 nM each forward and reverse primers and 0.15 U HotStar Taq polymerase (Qiagen, UK). PCR amplification was performed in 384 well PCR plates and cycling conditions were as follows: 95°C for 15 min and 35 cycles of 95°C for 20 seconds, 58°C for 30 seconds, 72°C for 1 min, followed by a final extension step of 72°C for 3 min. Following this a primer extension reaction was performed using the mass extend primer 5-CTATTAGCTCTGCCCGG-3 and samples were then prepared according to the manufacturers methods (Sequenom, San Diego, CA, USA) for genotyping using MALDI-TOF.

5.3.2 Plasma adiponectin measurements

Plasma adiponectin was determined by a novel in-house time-resolved immunofluorometric assay (TR-IFMA) and described in detail in chapter 2.

5.3.3 Quality control:

Of the genotyped samples, 5% were duplicates and there was at least 1 negative control per 96 well DNA plate. The accuracy of the genotyping was determined by the genotype concordance between duplicate samples. We obtained a 100% concordance between the genotyped duplicate samples. The genotyping success rate was >98 %.

5.4 Statistical analysis:

All analysis was performed using SPSS version 13 (SPSS Inc., Chicago, IL, USA) and Minitab version 15 (Minitab Inc, USA). Skewed variables were log transformed for analysis and presented as geometric means and the interquartile range [25^{th} – 75^{th} percentile].The ANOVA test was initially used to compare SNP genotypes with biological variables.Significant differences were further explored using the independent samples t-Test.General linear Model (GLM) analysis was performed to test for associations between SNP genotypes and risk factors after adjusting for confounding variables.

5.5 Results:

5.5.1 Genotype and allele frequencies for the T-45-G SNP:

The following allele frequencies were found for the +45 T-G SNP:

T allele = 89%

G allele = 11%

Observed genotype frequencies were not significantly different from those expected by the Hardy-Weinberg equilibrium. Allele frequencies were similar to those reported in other European populations (117,140).

5.5.2 Genotype and metabolic features for the T-45-G SNP:

The study cohort consists of 1278 subjects (579 men and 699 women) aged 43.8 ± 8.4 yrs (mean ±SD), with a mean BMI of 25.6 ± 4.0 kg/m2.

Table 31 summarizes the metabolic and anthropometric data for the 3 genotypes of the SNP +45 of the ADIPQ gene. This revealed differences between the 3 genotype groups for fasting NEFA levels when analyzed by ANOVA. This remained significant after correcting for confounding factors (age, sex, BMI, circumference and recruitment centre) (T/T vs. T/G vs. G/G: 0.53(0.01) ± vs. 0.52(0.01) ± vs. 0.71(0.05) ± [geometric mean (SE)] mmol/l; p=0.03) (Table 33).

It appears from these data that the key differences are between the G/G homozygotes and the other genotype groups. For this reason, we then compared subjects homozygous for the G allele to the T allele carriers (T/T + T/G) as shown in Table 32. This showed that subjects homozygous for the G allele also had a higher waist circumference compared to T allele carriers; (G/G) vs. (T/T+T/G) 90.0[1.7] vs. 87.0[0.2] cm; p=0.02.

We found no association between serum adiponectin and the 3 genotypes of the SNP +45 of the ADIPQ gene.

5.5.3 SNP + 45 of the ADIPOQ gene and insulin sensitivity:

As shown in Table 34, subjects homozygous for the G allele had higher fasting NEFA levels (G/G) vs. (T/T+TG): 0.71[0.05] vs. 0.53 [0.00] mmol/l; p=0.001) after correction for the same covariates. They also had a lower M/I value; (G/G) vs. (T/T+T/G): 102.5[92.4-179.1] vs. 121.3[84.5-141.6] min^{-1} kg_{ffm}^{-1} nM^{-1} (geometric mean [interquartile range 25^{th} - 75^{th} percentile]); p=0.04 after correction for age, sex, BMI and centre. However this association didn't remain significant after correction for waist circumference and circulating fasting NEFA levels; p=0.25.

In subgroup analysis, lean subjects with BMI $\leq$ 27 kg/m^2, subjects homozygous for the G allele had greater insulin resistance compared to other allele genotypes after correcting for sex, age,BMI, plasma adiponectin level and recruitment centre((T/T+TG) vs. (G/G): 144.5[110-189] vs. 117.0[69-185] min^{-1} kg_{ffm}^{-1} nM^{-1}(geometric mean [interquartile range 25^{th} - 75^{th} percentile]); p=0.04, but didn't remain significant after correction for waist circumference and circulating fasting NEFA levels (Table 35). The same analysis could not be performed for the subjects with a BMI > than 27 kg/m^2 because of the small number of homozygous G allele carriers (n=4).

There were no significant differences between the genotypes and insulin sensitivity when the cohort was subdivided into northern and southern centres (Table 36 and 37).

5.5.4 SNP + 45 of the ADIPOQ gene and c IMT:

Table 38 summarises the relationships between carotid intima media thickness (c IMT) and the 3 genotypes of the SNP + 45 of the ADIPOQ gene. (T/T, T/G & G/G).There were no significant differences between the 3 genotyes and measures of c IMT before and after correction for age, sex, BMI, waist circumfrence , plasma adiponectin and the usual CVD risk factors.

There was no consistent relationship between the genotypes and measures of IMT when the cohort was subdivided by BMI (Table 39 and 40).

In subjects from the Southern European centres, subjects homozygous for the G allele tended to have a greater c IMT as measured by IMT_{ICA} (607(545-664) vs. 603(545-659) vs. 750(679-793) (μm); (Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]) p=0.04, but this didn't remain statistically significant after correcting for the same confounding factors (Table 42). However when we compared subjects homozygous for the G allele to the T allele carriers in subjects from the southern European centres, it was clear that subjects homozygous for the G had a greater $IMT_{ICA,}$ and a greater IMT_{BULB} compared to the T allele carriers (T/T + T/G) before and after correction for the usual covariates and after correcting for waist circumference, fasting NEFA and insulin sensitivity (Table 45). This was not seen in the subjects from the northern centres (Tables 41 and 44).

Table 31: ANOVA comparisons of means for SNP+45 genotypes with anthropometric and metabolic variables (Data presented as means [SE])

Character	T/T N=1003	T/G N=258	G/G N=17	P Value
Age (years)	43.7 (0.26)	43.5 (0.5)	45.0(2.2)	0.74
Male/Female	446/557	123/135	8/9	0.46
BMI kg/m2	25.5 (0.11)	25.5 (0.28)	24.8 (0.8)	0.73
Waist circumference (cm)	86.7 (0.4)	86.3 (0.84)	88.6 (1.7)	0.47
Fasting Glucose mmol/l	5.1(0.02)	5.0 (0.04)	5.1(0.14)	0.15
*Fasting Insulin pmol/l	29.8 (20-43)	30.6(21-44)	26.1(18.5-38.6)	0.48
Fasting NEFA mmol/l	0.53(0.01)	0.52(0.01)	0.68(0.15)	**0.03**
2 hours Glucose mmol/l	5.8 (0.06)	5.7 (0.10)	5.5(0.47)	0.84
*2 hours Insulin pmol/l	142.1 (86.3-261.5)	148 (86.3-261.5)	176.0 (96-238)	0.65
2 hours NEFA mmol/l	0.05 (0.00)	0.06 (0.01)	0.05 (0.02)	0.12
Total- cholesterol mmol/l	4.8(0.03)	4.8(0.05)	4.7(0.22)	0.97
*Triglycerides mmol/l	0.95 (0.67-1.27)	0.99(0.69-1.37)	0.89(0.64-1.20)	0.42
HDL - cholesterol mmol/l	1.43(0.01)	1.4(0.02)	1.4(0.11)	0.76
LDL - cholesterol mmol/l	2.9(0.02)	2.9(0.04)	2.8(0.19)	0.97
Systolic BP mmHg	117.3(0.38)	116.2(0.75)	118.5(2.8)	0.38
Diastolic BP mmHg	74.3(0.25)	73.5(0.48)	74.9(1.97)	0.32
*Serum Adiponectin mg/l	7.7 (5.6-10.3)	7.6 (5.4-10.5)	8.7 (5.6-10.3)	0.41
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	125.4(92.9-177.4)	124.3(91.5-185.1)	114.4(84.-141.)	0.74

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 32: ANOVA comparisons of means for SNP+45 genotypes (T allele carriers (T/T+T/G) vs G/G homozygotes) with anthropometric and metabolic variables (Data presented as means [SE])

Character	T/T+T/G N=1261	G/G N=17	P Value
Age (years)	43.7 (0.23)	45.00 (2.2)	0.52
Male/Female	569/692	8/9	0.25
BMI kg/m2	25.5 (0.11)	24.8 (0.84)	0.55
Waist circumference (cm)	87.0 (0.2)	90.0 (1.7)	**0.02**
Fasting Glucose mmol/l	5.1(0.02)	5.1 (0.14)	0.96
*Fasting Insulin pmol/l	30.0 (21-43.3)	26.1(18.4-38.6)	0.33
Fasting NEFA mmol/l	0.53(0.01)	0.68(0.16)	**0.01**
2 hours Glucose mmol/l	5.7 (0.05)	5.5 (0.47)	0.60
*2 hours Insulin pmol/l	143.3(88.0-238)	176.0(96-238)	0.37
2 hours NEFA mmol/l	0.05 (0.00)	0.05 (0.02)	0.99
Total- cholesterol mmol/l	4.8(0.02)	4.7(0.20)	0.72
*Triglycerides mmol/l	0.96 (0.68-1.28)	0.89(0.64-1.20)	0.52
HDL - cholesterol mmol/l	1.42(0.01)	1.43(0.1)	0.94
LDL - cholesterol mmol/l	2.9(0.02)	2.9(0.19)	0.89
Systolic BP mmHg	117.06(0.35)	118.5(2.86)	063
Diastolic BP mmHg	74.2(0.22)	74.9(1.97)	0.68
*Serum Adiponectin mg/l	7.6 (5.6-10.4)	8.7(6.4-11.05)	0.2
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	125.1(92-177)	114.3(84.5-141)	0.46

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 33: Analysis of Covariance SNP+45 of the ADIPOQ gene (age, sex, BMI and centre adjusted (Data presented as means [SE])

Character	T/T N=1003	T/G N=258	G/G N=17	P Value
Fasting Glucose mmol/l	5.08(0.02)	5.01 (0.03)	5.01(0.12)	0.22
*Fasting Insulin pmol/l	29.6(20-43)	30.3 (21-44)	26.7 (18.5-38.6)	0.52
Fasting NEFA mmol/l	0.53(0.01)	0.52(0.01)	0.71(0.05)	**0.03**
2 hours Glucose mmol/l	5.7 (0.06)	5.8 (0.10)	5.6(0.3)	0.83
*2 hours Insulin pmol/l	138(87-261)	147.2(86-261)	191.4(99-238)	0.17
2 hours NEFA mmol/l	0.05 (0.00)	0.05 (0.01)	0.05 (0.02)	0.96
Total- cholesterol mmol/l	47(0.03)	4.8(0.05)	4.7(0.2)	0.94
*Triglycerides mmol/l	0.93(0.67-1.2)	0.96(0.69-1.3)	0.91(0.64-1.20)	0.50
HDL - cholesterol mmol/l	1.4(0.01)	1.4(0.02)	1.4(0.07)	0.82
LDL - cholesterol mmol/l	2.85(0.03)	2.86(0.05)	2.9(0.17)	0.96
Systolic BP mmHg	117.2(0.37)	116.9(0.69)	118.8(2.5)	0.73
Diastolic BP mmHg	74.4(0.25)	74.0(0.46)	75.7(1.7)	0.53
*Serum Adiponectin mg/l	6.24(5.2-10.2)	6.18(5.1-10.3)	6.9(5.1-10.3)	0.84
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	120.7(92.9-177.4)	123.0(91.5-185.1)	102.5(84.5-141.6)	0.46

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 34: Analysis of Covariance for SNP+45 of the ADIPOQ gene (age, sex, BMI, and centre adjusted (Data presented as means [SE])

Character	(T/T+ T/G) N=1261	(G/G) N=17	P Value	* P Value
Fasting Glucose mmol/l	5.06(0.02)	5.01 (0.01)	0.7	0.7
*Fasting Insulin pmol/l	29.7(20-43)	26.7(18-38)	0.35	0.4
Fasting NEFA mmol/l	0.53(0.00)	0.71(0.05)	**0.001**	**0.006****
2 hours Glucose mmol/l	5.7 (0.06)	5.6 (0.4)	0.72	0.7
*2 hours Insulin pmol/l	139.6(87-264)	190.9(96-252)	0.13	0.3
2 hours NEFA mmol/l	0.04 (0.00)	0.05 (0.02)	0.8	0.7
Total- cholesterol mmol/l	4.7(0.02)	4.7(002)	0. 87	0.8
*Triglycerides mmol/l	0.94(0.67-1.2)	0.90(0.64-1.2)	0.68	0.7
HDL - cholesterol mmol/l	1.4(0.01)	1.38(0.07)	0.8	0.7
LDL - cholesterol mmol/l	2.8(0.02)	2.9(0.17)	0.88	0.8
Systolic BP mmHg	117.1(0.34)	118.9(2.5)	0.49	0.6
Diastolic BP mmHg	74.3(0.23)	75.8(1.7)	0.39	0.6
*Serum Adiponectin mg/l	7.4(5.6-10.1)	8.3(5.6-10.2)	0.19	0.3
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	121.3(92.4-179.1)	102.5(84.5-141.6)	**0.04**	0.25

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

P* (after adjustment for NEFA and waist circumference)

** (after adjustment for waist circumference)

Table 35: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to insulin sensitivity (M/I value) measures for those with BMI ≤27 kg/m^{2}Data presented as Geometric Mean [interquartile range 25th - 75th percentile])

Character	**T/T+G/G Number=858**	**(G/G) Number=13**	**P value**	**P* Value**	**P** Value**
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	144.5(110-189)	117.0(69-185)	**0.05**	**0.04**	0.34

P* (after correction for age, sex, BMI, plasma adiponectin and recruitment centre)

P* (after correction for fasting NEFA and waist circumference)

Table 36: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to insulin sensitivity (M/I value), for those from the northern centres. (Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	**T/T Number=624**	**T/G Number=151**	**G/G Number=9**	**P value**	**P* Value**
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	112.2(69-129)	121.8(66-230)	103.2(71-159)	0.3	0.15
Character	**(T/T +T/G) Number=775**	**G/G Number=9**		**P value**	**P* Value**
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	114.2(71-129)	103.3(71-159)		0.56	0.95

P* (after correction for age, sex, BMI, plasma adiponectin, waist, NEFA and recruitment centre)

Table 37: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to insulin sensitivity (M/I value) measures for those from the southern centres. (Data presented as geometric mean [interquartile range 25th - 75th percentile])

Character	**T/T** **Number=379**	**T/G** **Number=107**	**G/G** **Number=8**	**P**	**P***
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	133.5(96-180.9)	125.8(96-182)	125.4(89-184)	0.4	0.1
Character	**(T/T +T/G)** **Number=486**		**G/G** **Number=8**	**P**	**P***
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	131.9(89-179)		125 .4(96-182)	0.75	0.29

P* (after correction for age, sex, BMI, plasma adiponectin, waist, NEFA and recruitment centre)

Table 38: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to(c IMT) measures (Data presented as geometric mean [interquartile range 25^{th} - 75^{th} percentile])

Value	T/T N =1003	T/G N= 258	G/G N =17	P Value	P* Value
IMT_{CCA} (μm)	600(540-654)	601(542-658)	594(504-660)	0.96	0.84
IMT_{BULB} (μm)	767(696-813)	763(693-889)	780(710-837)	0.86	0.87
IMT_{ICA} (μm)	617(557-671)	616(558-672)	634(569-683)	0.88	0.66
IMT_{AVRG} (μm)	665(606-731)	660(601-725)	666(607-631)	0.87	0.94

P^* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 39: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to (c IMT) measures for those with BMI ≤ 27 kg/m^2(Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	T/T N = 689	T/G N= 169	G/G N = 13	P Value	P* Value
IMT_{CCA} (μm)	590(529-644)	579(513-643)	598(537-652)	0.27	0.87
IMT_{BULB} (μm)	758(687-806)	737(666-783)	800(733-857)	0.14	0.31
IMT_{ICA} (μm)	608(546-665)	600(542-656)	623(558-672)	0.72	0.85
IMT_{AVRG} (μm)	655(595-721)	637(574-6950	670(610-634)	0.10	0.63

P* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 40: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to(c IMT) measures for those with BMI > 27 kg/m^2 (Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	T/T N = 314	T/G N=89	G/G N = 4	P Value	P* Value
IMT_{CCA} (μm)	585(528-633)	621(558-669)	652(592-718)	**0.03**	0.55
IMT_{BULB} (μm)	731(660-886)	791(724-848)	826(859-883)	0.19	0.37
IMT_{ICA} (μm)	642(588-698)	658(600-714)	663(606-719)	0.75	0.73
IMT_{AVRG} (μm)	689(628-755)	714(746-763)	657(597-723)	0.20	0.37

P* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 41: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to(c IMT) measures for those from the northern Centres. (Data presented as geometric mean [interquartile range 25^{th} - 75^{th} percentile])

Value	T/T N = 624	T/G N= 151	G/G N = 9	P Value	P* Value
IMT_{CCA} (μm)	606± 3	605± 7	583 ±40	0.78	0.84
IMT_{BULB} (μm)	778± 6	770 ± 14	703 ± 33	0.32	0.91
IMT_{ICA} (μm)	623 ± 5	625 ± 12	548 ± 20	0.23	0.44
IMT_{AVRG} (μm)	672 ± 4	666 ± 10	609 ± 31	0.72	0.33

P^* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 42: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to (c IMT) measures for those from the southern Centres. (Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	T/T N =379	T/G N= 107	G/G N = 8	P Value	P* Value
IMT_{CCA} (μm)	589(529-643)	594(531-647)	610(549-664)	0.74	0.39
IMT_{BULB} (μm)	749(677-796)	752(682-800)	884(813-941)	0.06	0.09
IMT_{ICA} (μm)	607(545-664)	603 (545-659)	750(679-793)	**0. 04**	0. 08
IMT_{AVRG} (μm)	652(592-718)	650(588-718)	744(673-781)	0.08	0.08

P* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 43: ANOVA comparisons of means for T allele carriers (T/T+ T/G) Vs (G/G) genotypes in relation to(c IMT) measures (Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	T/T+ T/G N = 1261	G/G N= 17	P Value	P* Value
IMT_{CCA} (μm)	600±2	594 ± 24	0.73	0.63
IMT_{BULB} (μm)	766 ± 4	780 ± 42	0. 72	0.73
IMT_{ICA} (μm)	617 ± 4	634 ± 44	0.63	0.26
IMT_{AVRG} (μm)	664 ± 3	666 ± 31	0.92	0.93

P* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 44: ANOVA comparisons of means for T allele carriers (T/T+ T/G) Vs. (G/G)homozygotes of the ADIPOQ gene in relation to(c IMT) measures for those from the northern centres(Data presented as geometric mean [interquartile range 25^{th} - 75^{th} percentile])

Value	T/T+ T/G N =775	G/G N=9	P Value	P* Value
IMT_{CCA} (μm)	583±3	605±40	0.48	0.58
IMT_{BULB} (μm)	703 ±5	776±33	0.16	0.38
IMT_{ICA} (μm)	547±5	623± 24	0.08	0.56
IMT_{AVRG} (μm)	609 ± 4	671± 29	0.09	0.89

P^* (after adjustment for age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

Table 45: ANOVA comparisons of means for T allele carriers (T/T+ T/G) Vs. (G/G) homozygotes of the ADIPOQ gene in relation to (c IMT) measures for those from the southern Centres. (Data presented as geometric mean [interquartile range 25th - 75th percentile])

Value	T/T+ T/G N =486	G/G N=8	P Value	P* Value	P** Value
IMT_{CCA} (μm)	591(529-643)	610(549-664)	0.58	0.86	0.69
IMT_{BULB} (μm)	750(680-996)	883(812-941)	**0.02**	**0.04**	**0.049**
IMT_{ICA} (μm)	606(544-663)	750(679-793)	**0.01**	**0.03**	**0.036**
IMT_{AVRG} (μm)	651(591-714)	744(673-781)	**0.03**	0.08	0.41

P* value after analysis of Covariance (Age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure)

P** (after adjustment for fasting NEFA and M/I value)

5.6 Discussion:

This is a cohort of 1278 healthy non diabetic Caucasian subjects recruited from the RISC study. We found that G/G allele homozygote subjects for the SNP+45 of the ADIPOQ gene had increased insulin resistance, waist circumference and fasting NEFA levels compared to carriers of the T allele. These findings support the previous published data from the French and Japanese populations. (116,124).

A recently published study by Daimon et al (144) identified a lower serum adiponectin level as an independent risk factor for progression to T2DM and those with a serum adiponectin concentration in the lowest tertile had a 10-fold higher risk for development of type 2 diabetes during a 5-year follow-up period.The G allele of SNP+ 45 of the ADIPOQ gene was found to be associated with lower serum adiponectin and markers of insulin sensitivity in different study populations (116,124,140). However, these associations were not confirmed in another study of Caucasian type 2 diabetic patients (117).

Interstingly in our study, adiponectin levels were not different between the 3 genotypes and the association of the G/G allele and insulin sensitivity was independent of circulating adiponectin levels.The association between the G allele homozygotes of SNP+45 of the ADIPOQ gene and insulin sensitivity was not significant after correcting for waist circumference and fasting NEFA. This finding gives evidence that SNP+45 of the ADIPOQ gene influences insulin sensitivity through measures of adiposity and fasting NEFA levels.

There is emerging evidence that, it is the plasma fraction of high molecular-weight polymers rather than the total concentration of adiponectin, that is associated with changes in insulin sensitivity in db/db mice and in type 2 diabetic patients treated with thiazolidinedione (160).Thefore, future studies determining the different circulating adiponectin isoforms, including high molecular –weight adiponectin, may help to examine whether the association between SNP+45 of the ADIPOQ gene and insulin resistance is independent of serum adiponectin.

To date there are four large published studies on the effect of ADIPOQ gene variants and CVD disease , all consisting of type 2 diabetes subjects (123,151-153). In the French-Swiss population, the G/G allele of SNP+45 of the ADIPOQ gene was found to be a significant predictor of CAD, in cross-sectional sample of 162 cases with CAD and 315 control subjects without significant CAD, with odd ratio of 1.9(95% CI 1.2-2.9) (123). However, this wasn't confirmed in the other 3 studies (South Italy and USA). Meta analysis of all published data, including 827 CAD positive cases and 1887 CAD negative control subjects showed no association between SNP + 45 and CAD (152).

While there was no significant association between the 3 genotypes and measures of IMT in the whole cohort, we did find that in subjects homozygotes for the G allele from the southern centres had an increased IMT_{ICA} and IMT_{BULB} compared to the T allele carriers and this association appears to be independent of insulin sensitivity, serum adiponectin, waist circumference and circulating NEFA levels. However, this association needs to be

replicated in view of the small number of G/G homozygotes (n=8), particularly as it may reflect a gene-lifestyle interaction.

A previous study reported an association of the G/G homozyogous allele of SNP+ 45 of the ADIPOQ gene with CAD in patients with type 2 diabetes independent of other CVD risk factors (123). In the current study we were able to replicate this association in the healthy subjects from southern Europe; with subjects homozyotes for the G allele having greater c IMT independent of other CVD risk factors and adiponectin level. Nevertheless, the findings from our study provide very little evidence supporting the original hypothesis that genetic variation within the ADIPOQ gene exhibits pleiotropy and increase the risk of cardiovascular disease.

Recent data have demonstrated that the ADIPOQ gene product is expressed in tissues other than adipose tissue, including vascular tissue (144). ADIPOQ gene expression and its relation to enothelial function is an area for future research. As the RISC project is a longitudinal study of CVD risk, we will, in time, be able to measure the change in carotid IMT progression and investigate its relation to variation in the ADIPOQ gene.

5.7 Conclusions:

We have confirmed that the SNP +45 of the ADIPOQ gene influences insulin sensitivity in the healthy population. Specifically, subjects homozygous for the G allele have increased fasting NEFA levels and waist circumference and are less insulin sensitive compared to the rest of the population. The association of G/G allele of SNP +45 of the ADIPOQ gene with insulin resistance appears to be mediated through measures of adiposity and NEFA levels but independent of circulating adiponectin levels.

Subgroup analysis showed that in subjects from the southern European centres, variation in the adiponectin gene was associated with carotid IMT in a cohort of healthy subjects, and this was independent of circulating adiponectin levels and classical CVD risk factors. This observation needs to be replicated in other cohorts.

6. The role of the ADIPOQ gene promoter variants (SNPs A-11426G, G-11391A and C-11377G) in insulin resistance and CVD

6.1 Introduction

A recent, systemic analysis of the ADIPOQ gene locus in Europeans, suggested that the gene is organized in 2 linkage disequilibrium (LD) blocks separated by a region of a looser LD in the middle of the first intron (155). A common haplotype of SNPs G-11391A and C-11377G in the proximal promotor region of the ADIPOQ gene has been associated with circulating adiponectin levels and type 2 diabetes in a large cohort of type 2 diabetic French Caucasians (117). Furthermore a significant association with adiponectin levels was observed for SNPs 11391 & 276 indicating independent genetic effects, since these 2 SNPs belong to 2 different LD blocks (147).

Recent studies have shown that SNP A-11426G in the proximal promotor region is associated with fasting plasma glucose levels and type 2 diabete and SNP C-11377G with coronary stenosis and vascular events (122,144). Therefore it is evident that SNPs in the ADIPOQ gene may be associated with CVD and type 2 diabetes. However, there is limited work investigating the role of these SNPs in healthy individuals.

6.2 Aims:

- To examine the relationships between ADIPOQ promoter gene variants (SNPs C-11377G, G-11391A and A-11426G) and cardiovascular risk factors in non diabetic population.
- To study the relationships between ADIPOQ promoter gene variants (SNPs C-11377G, G- 11391A and A-11426G) and insulin sensitivity in non diabetic Caucasian population.
- To study the relationships between ADIPOQ promoter gene variants (SNPs C-11377G, G-11391A and A-11426G) and carotid IMT and plasma adiponectin levels in healthy subjects participating in the RISC study.

6.3 Methods:

6.3.1 Plasma adiponectin measurements

Plasma adiponectin was determined by a novel in-house time-resolved immunofluorometric assay (TR-IFMA) and described in detail in chapter 2. In this study we measured total adiponectin levels using two antibodies and recombinant human adiponectin. Adiponectin circulates as various polymers, which may differ in receptor affinities and metabolic effects (132,133). To date, virtually all studies have assayed the total concentration of plasma or serum adiponectin without differentiating between the various isoforms. The two antibodies used in this method are able to detect several adiponectin polymers in serum, including the major three molecular forms (133).

6.3.2 ADIPOQ promoter SNPs (11426, 11391 & 11377) genotyping

I initially genotyped SNPs G-11391A and C-11377G using the seqenom mass ARRAY assay method, however, the genotype frequencies were not in Hardy-Weinberg equilibriuim and so this was repeated by Dr. Sheila Patel who also genotyped SNP A-11426G using a TaqMan allelic discrimination assay (Applied Biosystems, Warrington, UK). Primers for the allelic discrimination assays were designed using the Assay by design service (Applied Biosciences, Warrington, UK). For each SNP, a 25 µl PCR reaction was set up with 10 ng of genomic DNA, 1x TaqMan® universal PCR MasterMix with AmpErase® UNG and 1 x primer and probes assay mix. Samples were amplified on a GeneAmp 9700 PCR machine (using the 9600 emulation mode; ABI, Warrington, UK). Cycling conditions were as follows: 50°C for 2 min followed by 95°C for 10 min, then 40

cycles of 92°C for 0.15 sec, 60°C for 1 min. Following PCR, an allelic discrimination assay was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Warrington, UK).

6.3.3 Quality control:

Of the genotyped samples, 5% were duplicates and there was at least 1 negative control per 96 well DNA plate. The accuracy of the genotyping was determined by the genotype concordance between duplicate samples. We obtained a 100% concordance between the genotyped duplicate samples for each of the SNPs. The genotyping success rate for each of the SNPs was >98 %.

6.4 Statistical analysis:

All analyses were performed using SPSS version 13 (SPSS Inc., Chicago, IL, USA) and Minitab version 15 (Minitab Inc, USA). Skewed variables were log transformed for analysis and are presented as geometric means and the interquartile range [25^{th} – 75^{th} percentile]. The ANOVA test was initially used to compare SNP genotypes with biological variables. Significant differences were further explored using the independent samples t-Test. General linear Model (GLM) analysis was performed to test for associations between SNP genotypes and risk factors after adjusting for confounding variables.

6.5 Results:

The study cohort consists of 1278 subjects (579 men and 699 women) aged 43.8 ± 0.2 yrs (mean ± SD), with a mean BMI of 25.5 ± 0.1 kg/m^2. The clinical and metabolic characteristic of the study population was described in detail in chapter 3. Table 46 shows the baseline characteristics of the RISC study subjects by tertiles of plasma adiponectin levels. All listed variables were significantly different between the three circulating adiponectin tertiles.

Study subjects in the lower adiponectin tertile (≤ 6.24 mg/l) had higher BMI's, increased waist circumference, higher fasting triglycerides and LDL-cholesterol and BP compared to the highest adiponectin tertile (>9.27 mg/l). The carotid IMT value was also significantly different between the adiponectin tertiles, (Geometric Mean [interquartile range) 616 (524-664), 597 (540-652) and 590(530-643) μm (lowest, middle and highest tertile of adiponectin).

All genotyped SNPs were in Hardy-Weinberg equilibrium.Table 47 shows detailed information for each SNP and genotype frequencies.genotype frequencies were similar to those previously reported in French and Swedish Caucasians (121,130).

6.5.1 Relationship between ADIPOQ gene promoter variants (SNPs 11377, 11391 & 11426) and cardiovascular risk factors:

Tables 48 to 50 summarise the metabolic and anthropometric data for the 3 genotypes of the SNPs C11377G, G11391A and the A11426G variants of the ADIPOQ gene promotor. There were no significant differences between the 3 genotypes of SNP C-11377G and G11391A of the ADIPOQ gene promotor and the metabolic and anthropometric measures. On the other hand subjects homozygous for the G allele of the SNP A11426 G of theADIPOQ promotor gene tended to have higher total cholesterol and LDL levels compared to other 2 genotypes alleles.

6.5.2 Relationship between ADIPOQ gene promoter variants (SNPs 11377, 11391 & 11426) and insulin sensitivity:

Tables 51 to 53 summarize the relationships between SNPs (C11377G, G11391A and A11426G) and insulin sensitivity (M/I). There was no significant association between the adiponectin gene promoter variants and insulin sensitivity in this healthy non diabetic cohort when correcting for known confounding factors. Further subgroup analysis dividing groups by sex, BMI (Lean and overwieght subjects) and northern and southern centres showed no association with insulin sensitivity.

6.5.2 Relationship between ADIPOQ gene promoter variants (SNP C11377G, SNP G11391A and SNP A11426G) and serum adiponectin and cIMT:

Of the 3 genotyped SNPs, A-11426G and the G-11391A SNPs of the ADIPOQ gene promoter were significantly associated with adiponectin levels (Tables 49 and 50).As shown in Table 54, homozygous carriers of the -11391G allele had significantly lower plasma adiponectin levels compared to A allele carriers (geometric mean [interquartile range]) 7.4 [5.4 - 10.0] vs. 9.1 [6.9 - 12.2] mg/l, P<0.0001). Plasma adiponectin levels remained significantly lower after adjusting for age, sex, BMI and recruitment centre (P<0.0001). Similarly, for SNP A-11426G, carriers of the G allele had significantly lower plasma adiponectin levels compared to the A allele homozygotes before (P=0.015) and after adjustment (P =0.005) for the same covariates.

On the other hand, only SNP C-11377G was associated with carotid IMT (Table 54). Carriers of the G allele had significantly greater carotid IMT values compared to C allele homozygotes (Geometric Mean [interquartile range]) 608 (543-643) vs. 596(540-654) μm, p=0.017). This difference remained after adjusting for age, sex, recruitment centre and BMI (p=0.02), and after adjustment of fasting adiponectin levels and other CVD risk factors (HDL and LDL cholesterol, triglycerides, systolic and diastolic BP and smoking status (p=0.029).

Table 46: Baseline characteristics of the study population according to their adiponectin tertiles (means ± SE)

	*Tertiles of plasma adiponectin (mg/l)			
Character	**≤ 6.25**	**6.25 to 9.28**	**>9.28**	**P values**
Number	426	426	426	
Age	42.8 ± 0.4	43.8 ± 0.4	44.9 ± 0.4	**0.001**
Sex (male/female)	314/112	199/227	69/357	**<0.0001**
BMI (kg/m^2)	26.7 ± 0.2	25.7 ± 0.2	24.1 ± 0.2	**<0.0001**
Waist circumference (cm)	92.6 ± 0.5	87.4 ± 0.6	79.9 ± 0.6	**<0.0001**
***Fasting Insulin** (pmol/l)	37.3 [26.0 - 54.0]	31.1 [22.0 – 44.0]	24.8 [17.7 - 34.0]	**<0.0001**
Fasting Glucose (mmol/l)	5.3 ± 0.04	5.1 ± 0.03	4.9 ± 0.03	**<0.0001**
***Triglycerides** (mmol/l)	1.16 [0.83 - 1.57]	0.97 [0.69 - 1.30]	0.78 [0.61 - 1.01]	**<0.0001**
HDL – cholesterol (mmol/l)	1.2 ± 0.01	1.4 ± 0.02	1.7 ± 0.02	**<0.0001**
LDL – cholesterol (mmol/l)	3.1 ± 0.04	2.9 ± 0.04	2.7 ± 0.03	**<0.0001**
Systolic BP (mmHg)	120 ± 0.5	118 ± 0.6	114 ± 0.6	**<0.0001**
Diastolic BP (mmHg)	76 ± 0.3	74 ± 0.4	73 ± 0.4	**<0.0001**
***Carotid IMT** (μm)	616 (524-664)	597 (540-652)	590 (530-643)	**<0.0001**
***M/1** (μm)	116.3(72.6-140)	138.3(94-167.8)	168.5(117.4-204)	**<0.0001**

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

P≤ 0.01 is statistically significant

Table 47 – ADIPOQ SNPS, genotypes and allele frequencies in the RISC study population

SNP (dbSNP ID number)	n	Genotypes n (%)			Allele 1 (%)	Allele 2 (%)
A-11426G (rs16861194)	1278	AA 1053 (82.6)	AG 208 (16.1)	GG 17 (1.3)	A (0.91)	G (0.09)
G-11391A (rs17300539)	1278	GG 1100 (85.3)	GA 170 (14.1)	AA 8 (0.6)	G (0.92)	A (0.08)
C-11377G (rs266729)	1278	CC 718 (56.2)	CG 495 (38.8)	GG 65 (5.0)	C (0.76)	G (0.24)

Table 48: ANOVA comparisons of means for SNP C-11377G of the ADIPOQ gene promoter with anthropometric and metabolic variables (Data presented as means [SE])

Character	C/C N = 718	C/G N = 495	G/G N = 65	P Value
Age (years)	43.6 (0.30)	43.8 (0.38)	45.3(1.09)	0.3
BMI kg/m2	25.6 (0.15)	25.4 (0.17)	25.0 (0.40)	0.4
Waist circumference (cm)	87.1 (0.49)	86.1 (0.53)	85.0 (1.6)	0.2
Fasting Glucose mmol/l	5.1(0.02)	5.1 (0.04)	5.1(0.07)	0.9
*Fasting Insulin pmol/l	31.5 (21.8-45.7)	29.4(19.9-41.6)	29.4(20.9-42.7)	0.07
Fasting NEFAs mmol/l	0.53(0.01)	0.54(0.01)	0.54(0.02)	0.7
2 hours Glucose mmol/l	5.8 (0.06)	5.8 (0.07)	5.5 (0.17)	0.25
*2 hours Insulin pmol/l	150.5 (93.3-251.2)	138.2 (81.3-223.8)	135.2(72-229)	0.16
2 hours NEFAs mmol/l	0.05 (0.004)	0.04 (0.08)	0.05 (0.01)	0.62
Total- cholesterol mmol/l	4.8(0.03)	4.9(0.03)	4.5(0.17)	0.16
*Triglycerides mmol/l	0.97 (0.69-1.33)	0.95(0.67-1.25)	0.90(0.63-1.30)	0.27
HDL - cholesterol mmol/l	1.41(0.014)	1.43(0.02)	1.44(0.11)	0.46
LDL - cholesterol mmol/l	2.92(0.03)	2.88(0.03)	2.83(0.08)	0.27
Systolic BP mmHg	117.1(0.4)	118.0(0.6)	116.6(1.6)	0.42
Diastolic BP mmHg	74.2(0.3)	74.6(0.4)	74.0(1.1)	0.86
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	123.9(89.1-173.8)	126.3(93.3-182.0)	130.0(93-177)	0.61
*Adiponecin(mg/l)	7.7(5.7-10.2)	7.6(5.7-10.4)	7.6(5.4-10.5)	0.90
*c IMT(μm)	596(540-654)	608(543-643)	597(538-665)	**0.01**

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 49: ANOVA comparisons of means for SNP G-11391A of the ADIPOQ gene with anthropometric and metabolic variables (Data presented as means [SE])

Character	G/G N = 1100	G/A N = 170	A/A N = 8	P Value
Age (years)	43.8 (0.3)	44.0 (0.6)	42.8 (3.2)	0.86
BMI kg/m2	25.6 (0.12)	25.2 (0.31)	25.4 (1.2)	0.56
Waist circumference (cm)	86.9 (0.4)	85.0 (1.0)	86.2 (3.5)	0.18
Fasting Glucose mmol/l	5.1(0.02)	5.1 (0.04)	4.9(0.17)	0.6
*Fasting Insulin pmol/l	30.5 (18-41)	31.2(18-37)	30.5(13.5-35.7)	0.86
Fasting NEFAs mmol/l	0.53(0.01)	0.55(0.02)	0.49(0.07)	0.48
2 hours Glucose mmol/l	5.7 (0.05)	5.8 (0.11)	5.4 (0.36)	0.56
*2 hours Insulin pmol/l	144.8 (88.0-244)	147.9 (84.0-244.5)	99.0 (23.7-274)	0.40
2 hours NEFAs mmol/l	0.05 (0.002)	0.06 (0.01)	0.11 (0.06)	0.06
Total- cholesterol mmol/l	4.8(0.03)	4.8(0.06)	4.6(0.30)	0.76
*Triglycerides mmol/l	0.96 (0.68-1.27)	0.98(0.68-1.39)	0.96(0.92-1.27)	0.76
HDL-cholesterol mmol/l	1.42(0.01)	1.44(0.03)	1.36(0.10)	0.66
LDL-cholesterol mmol/l	2.9(0.02)	2.9(0.05)	2.8(0.30)	0.89
Systolic BP mmHg	117.4(0.37)	117.4(0.96)	120.5(3.8)	0.78
Diastolic BP mmHg	74.3(0.24)	74.9(0.57)	75.8(2.39)	0.56
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	125.5(93.0-177.7)	125.7(89.5-179.3)	116.0(69-205.8)	0.95
*Adiponecin(mg/l)	7.4(5.4-10.0)	9.2(6.9-12.2)	8.2(6.3-12.0)	**0.001**
*c IMT(μm)	602(540-654)	597(538-665)	603(534-653)	0.61

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 50: ANOVA comparisons of means for SNP A-11426G of the ADIPOQ gene with anthropometric and metabolic variables (Data presented as means [SE])

Character	A/A N = 1053	A/G N = 208	G/G N = 17	P Value
Age (years)	43.8 (0.25)	43.6 (0.57)	45.2(2.10)	0.7
BMI kg/m2	25.5 (0.12)	25.7 (0.28)	26.9 (0.85)	0.2
Waist circumference (cm)	86.4 (0.4)	87.3 (0.9)	89.4 (3.0)	0.4
Fasting Glucose mmol/l	5.1(0.02)	5.1 (0.04)	5.4(0.17)	0.15
*Fasting Insulin pmol/l	30.6 (21-44)	29.9(20.2-43.5)	32.9(22.5-42.2)	0.72
Fasting NEFAs mmol/l	0.54(0.01)	0.50(0.01)	0.51(0.04)	0.18
2 hours Glucose mmol/l	5.7 (0.05)	5.8 (0.12)	6.2 (0.46)	0.36
*2 hours Insulin pmol/l	143.8 (88-243.2)	149.8 (85.8-255.3)	165.2 (105-318)	0.6
2 hours NEFAs mmol/l	0.05 (0.003)	0.03 (0.004)	0.03 (0.005)	0.75
Total- cholesterol mmol/l	4.8(0.03)	4.7(0.06)	5.3(0.27)	**0.03**
*Triglycerides mmol/l	0.95 (0.68-1.28)	0.98(0.70-1.29)	1.14(0.80-1.37)	0.28
HDL - cholesterol mmol/l	1.42(0.01)	1.40(0.02)	1.40(0.08)	0.84
LDL - cholesterol mmol/l	2.9(0.02)	2.9(0.05)	3.3(0.25)	**0.05**
Systolic BP mmHg	117.2(0.4)	118.6(0.8)	121.0(2.6)	0.14
Diastolic BP mmHg	74.2(0.2)	75.3(0.5)	77.2(2.3)	0.75
*M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	125.3(92.1-180.6)	127.2(94.4-175.5)	139.2(89-201.4)	0.65
*Adiponecin(mg/l)	7.7(5.6-10.5)	7.1(5.6-10.0)	7.2(5.4-9.6)	**0.01**
*c IMT(μm)	602(540-654)	597(538-655)	604(548-652)	0.45

*Geometric Mean [interquartile range 25^{th} - 75^{th} percentile]

$P \leq 0.01$ is statistically significant

Table 51: Analysis of Covariance of SNP C-11377G of the ADIPOQ gene promoter in relation insulin sensitivity (M/I) with subgroup analysis including; sex, BMI and centre (Data presented as geometric means [range])

Character	C/C	C/G	G/G	P Value
*M/I **(GLM)** $min^{-1}\ kg_{ffm}^{-1}\ Nm^{-1}$	121.0 (86-176) **N = 718**	121.3 (87-169) **N = 495**	124.4(98-174) **N = 65**	0.81
*M/I **(Male)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	109.0 (68-151) **N = 323**	108.9 (71-149) **N = 216**	114.4 (132.9-196.8) **N = 33**	0.87
*M/I **(Female)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	138.0 (105.1-186.) **N = 395**	142.5 (110.4-196) **N = 279**	149.6 (101-212) **N = 32**	0.46
*M/I **(BMI ≤ 27kg/m^2)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	142.2 (110-194) **N = 483**	146.2 (98-215) **N = 350**	148.9 (103-209) **N = 48**	0.54
*M/I **(BMI > 27kg/m^2)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	94.8 (61-174) **N = 235**	90.1 (69-168) **N = 145**	90.1 (69-169) **N = 17**	0.61
*M/I **(Northern centres)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	131.7 (97.2-186.2) **N = 436**	132.7(99.1-188.8) **N = 325**	135.0(92.8-191.0) **N =43**	0.94
*M/I **(Southern centres)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	112.7(81.0-154.4) **N = 282**	115.8 (87-162.7) **N = 170**	122.4(100.0-172.9) **N = 22**	0.67

*Geometric Mean [interquartile range 25th - 75th percentile]

$P \leq 0.01$ is statistically significant

(GML) (General linear model; after correcting for age, sex, BMI, adiponectin levels and recruitment centre).

Table 52: ANOVA comparisons of means for SNP G-11391A of the ADIPOQ gene promoter with insulin sensitivity (M/I) (Data presented as geometric means [range])

Character	G/G	G/A	A/A	P Value
*M/I **(GLM)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	121.6 (87.2-176.3) **N = 1100**	120.2 (87.1-171.5) **N = 170**	123.6(102.4-176.5) **N = 8**	0.95
*M/I **(Male)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	110.0 (71.2-153.5) **N = 499**	103.7 (67.5-149) **N = 73**	115.1 (132-196) **N = 6**	0.64
*M/I **(Female)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	138.2 (104-199) **N = 601**	142.5 (91-208) **N = 97**	118.8 (134-201) **N = 2**	0.82
*M/I **(BMI $\leq$ 27kg/m^2)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	143.5 (115-193.4) **N = 751**	147.8 (102-215) **N = 122**	142.5 (111-195) **N = 6**	0.6
*M/I **(BMI > 27kg/m^2)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	93.3 (62-171) **N = 349**	82.8 (61-158) **N = 48**	62.6 (68-170) **N = 2**	0.12
*M/I **(Northern centres)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	132.5(93.0-186.7) **N = 710**	130.9 (93.0-190.9) **N = 105**	141.4(90.1-211.0) **N = 4**	0.94
*M/I **(Southern centres)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	113.6(84.6-153.7) **N = 390**	118.8(85.3-179.2) **N =65**	95.3(61.5-174.6) **N = 4**	0.58

*Geometric Mean [interquartile range 25th - 75th percentile]

$P \leq 0.01$ is statistically significant

(GML) (General linear model; after correcting for age, sex, BMI, adiponectin levels and recruitment centre).

Table 53: ANOVA comparisons of means for SNP A-11426G of the ADIPOQ gene promoter with insulin sensitivity (M/I) (Data presented as geometric means [range])

Character	A/A	A/G	G/G	P
*M/I **(GLM)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	121.0 (86.5-176) **N = 1053**	120.2 (86-171) **N = 208**	134.6(105-180) **N = 17**	0.6
*M/I **(Male)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	109.4 (68.5-151) **N = 480**	110.1 (71.2-153) **N = 92**	113.2 (74-196) **N = 7**	0.5
*M/I **(Female)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	140.0 (107-186) **N = 573**	142.2 (102-224) **N = 116**	154.8 (101-219) **N = 10**	0.68
*M/I **(BMI ≤ 27kg/m^2)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	143.1 (112-207) **N = 738**	149.6 (102-224) **N = 135**	169.5 (123-230) **N = 10**	0.29
*M/I **(BMI > 27kg/m^2)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	92.5 (61-168) **N = 315**	94.4 (61-174) **N = 73**	108.0 (68-149) **N = 7**	0.70
*M/I **(Northern centres)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	133.3(99.6-189.1) **N = 658**	130.2(93.9-177.6) **N = 142**	138.7(82.2-212.7) **N = 11**	0.83
*M/I **(Southern centres)** $min^{-1}\ kg_{ffm}^{-1}\ nM^{-1}$	112.9(83.0-154.7) **N = 395**	120.9(95.4-157.2) **N = 66**	140(99.5-213.8) **N = 6**	0.38

*Geometric Mean [interquartile range 25th - 75th percentile]

$P \leq 0.01$ is statistically significant

(GML) (General linear model; after correcting for age, sex, BMI, adiponectin levels and recruitment centre).

Table 54: Relationship between ADIPOQ promoter gene, serum adiponectin & c IMT.

SNPs/ genotypes	*Plasma adiponectin (mg/l)	*carotid IMT (μm)
A-11426G		
AA	7.7 [5.6 - 10.5]	602(540-655)
AG + GG	7.2 [5.4 - 9.6]	598(548-652)
P value	**0.015**	0.676
†GLM Model 1 P value	**0.005**	0.428
G-11391A		
GG	7.4 [5.4 - 10.0]	602(540-654)
GA + AA	9.1 [6.9 -12.2]	598 (534-653)
P value	**<0.0001**	0.473
†GLM Model 1 P value	**<0.0001**	0.917
C-11377G		
CC	7.7 [5.7 - 10.2]	596(540-654)
CG + GG	7.6 [5.4 - 10.5]	608(543-643)
P value	0.669	**0.017**
†GLM Model 1 P value	0.273	**0.02**
‡GLM model 2 P value	-	**0.029**

*Geometric mean [interquartile range, 25th – 75th percentile].
P value after adjustment for age, sex, BMI and recruitment centre. ‡P value after adjustment for other CVD risk actors (HDL-, LDL- and total cholesterol, serum triglycerides, systolic and diastolic BP, smoking status) as well as age, sex, BMI, recruitment centre and adiponectin levels.

6.6 Discussion:

The novel observation of this analysis is that variation in the ADIPOQ gene promoter is directly associated with common carotid artery IMT in healthy subjects. Specifically, individuals with the G allele of the C-11377G SNP had significantly higher carotid IMT values compared to C allele homozygotes. This relationship remained significant after adjustment for key covariates (age, sex, BMI and recruitment centre) and after accounting for classical CVD risk factors (systolic and diastolic blood pressure, smoking status and serum cholesterol). Furthermore, this relationship was independent of circulating adiponectin levels. Our findings are supported by Hoefle *et al.* (2007) (150), who recently reported an association between SNP -11377 and coronary angiography determined CAD in a prospective study of 402 men. The SNP -11377 G allele was significantly associated with increased coronary stenosis and future vascular events independently of serum adiponectin levels and traditional CVD risk factors (150).

How might variation in the ADIPOQ gene directly influence carotid IMT? Clearly, our study does not allow us to address this directly, but it is interesting to note that a recent study reported that the ADIPQ gene is expressed in vascular tissue (148). Moreover, ACE inhibitor therapy given to type 2 diabetic patients resulted in a 2-fold increase in the vascular expression of the ADIPOQ gene and was associated with an improvement in endothelial function (148). This led the authors to speculate that adiponectin generated locally within the vasculature might directly influence endothelial function. This is supported by the observation that globular adiponectin applied to cultured endothelial cells increased both the expression and the activity of eNOS (149). The circulating serum/ plasma levels of adiponectin may not reflect the actual amount of adiponectin present at

the tissue level, for example the concentration in the sub-endothelial space where the anti-atherogenic targets for adiponectin is located (146,147). Therefore further *in vitro* studies are needed to explore whether variation in the ADIPOQ gene in vascular tissue affects local adiponectin expression, endothelial function and ultimately CVD risk.

Of the promoter SNPs, the A-11426G and G-11391A were significantly associated with plasma adiponectin levels. This association was independent of the effects of age, sex, BMI and recruitment centre. The SNP -11391A allele has been previously shown to be associated with higher adiponectin levels in European population (117,124). Putative binding sites for transcriptional factors have not been found in the promoter region of the ADIPOQ gene where SNPs -11391 and -11377 lie, however between these two SNPs and adjacent to the position of the -11377 SNP a nucleotide sequence which is similar to an enhancer element sequence in the epidermal growth factor receptor gene has been reported (119).

Although previous studies have shown that variants in the promoter region of the ADIPOQ gene was associated with insulin resistance (117,124). In this study there was no association between ADIPOQ promoter gene SNPs and insulin sensitivity. Examination of the genetic variation in the promoter region has not conclusively identified a functional variant and these previously reported associations may represent associations of these variants with adverse metabolic features in the study populations (154). Prospective gene association studies are more powerful to clarify these inconsistencies with the ability to estimate the risk of type 2 diabetes and CVD in the same cohort.

These differences could also be explained by gene-environment interaction. It has been previously hypothesized that certain environments predispose to CVD through interaction with the promoter block and others predispose to insulin resistance and type 2 diabetes, through interaction with other region in the ADIPOQ gene (154).

While there was a strong association between the A-11426G and G-11391A SNPs and circulating adiponectin levels, there was however, no association between these SNPs and carotid IMT. Furthermore, plasma adiponectin levels were not an independent predictor of carotid IMT after adjustment for the usual covariates and classical CVD risk factors. This suggests that the trend for decreasing carotid IMT across the adiponectin tertiles (Table 46) is due to other confounding differences between the adiponectin sub-groups. The majority of studies investigating ADIPOQ gene SNPs and adiponectin levels have been reported in disease states, especially in subjects with type 2 diabetes and CVD. Our study is a large healthy cohort and therefore we are able to dissect out relationships independent of the potentially confounding secondary effects of the disease. As mentioned before, the analyses have been conducted using cross-sectional data and it remains to be seen wether these relationships hold in the longitudinal analysis.

6.7 Conclusion:

Variation in the ADIPOQ gene promoter was associated with carotid IMT in a large cohort of healthy subjects, and this was independent of circulating adiponectin levels and classical CVD risk factors. However we found no association between ADIPOQ gene promoter and insulin sensitivity in this healthy European population.

This observation needs to be replicated in other cohorts, and further work is needed to explore potential mechanisms by which ADIPOQ gene variation might directly influence carotid IMT and CVD.

7. Summary/General Discussion:

The RISC study is a prospective, multicenter, observational study that has enrolled 1566 (Data presented for 1278) volunters at 19 centers across Europe. Subjects are mainly middle-aged healthy volunteers, with no preexisting diabetes, hypertension or known CVD. All subjects underwent testing for insulin sensitivity using the euglycemic clamp technique-the "gold standard" for measuring insulin resistance. Subjects also had their carotid intima media thickness measured by ultrasound, as a surrogate measure of cardiovascular disease.

The main strengths of our study were that, the RISC project was a large well characterized cohort, had a common methodology for measurements of carotid intima media thickness and had central laboratories. Furthermore it used the gold standard euglycaemic clamp to measure insulin resistance.

Clearly this study excluded subjects with unfavourable cardiovascular and metabolic profile and this may lead to a restricted cohort with low prevelance of disease risk allele. However, by studing healthy, disease–free population, we are able to avoid the secondary metabolic effects of established diabetes and or cardiovascular disease and to dissect out relationships independent of the potentially confounding secondary effect.

One of the findings of our study is the association of Pro12 Ala polymorphism of the PPARγ gene with insulin sensitivity independent of body composition (97). The relationship of this common gene variant and body mass index needs further investigation and it would be interesting to investigate visceral and peripheral deposition of adipose tissue using MRI scans in subjects homozygous for the Ala allele. This will help in further understanding some the effects of thiazolidinediones on adiposity and insulin resistance.

Furthermore, we found that in subjects from the southern European centres, subjects homozygous for the Ala allele had greater insulin sensitivity compared to those from the northern centres. This could be explained by difference in diet, for example, the presence of the Mediterranean type diet in the southern centres. This will give further evidence for the role of PPAR γ as nutrient sensor regulating adipogenesis and insulin sensitivity, but will need further evaluation (139).

The overall hypoythesis to be investigated was that variation within type 2 diabetes suceptibility genes (Pro12Ala and ADIPOQ) exhibit pleiotropy and increase the risk of CVD. We found no supporting evidence for this pleiotropy from this cross-sectional data and it remains to be seen whether we will find any association with both CVD and insulin resistance in the longitudinal analysis.

It would seem from the investigation into the genetic basis of insulin resistance and CVD, that variation in PPARγ predisposes to diabetes (possibly through increased insulin resistance) but we found no relationship with CVD. Conversley, variation in the ADIPOQ gene is associated with both insulin sensitivity (SNP+45) and c IMT (promoter region) . These findings could have great potential for future strategies of both insulin resistance and cardiovascular disease management. The interesting question is how to translate genetic association studies into clinical practice. The advantage of making the diagnosis well in advance of the appearance of clinical disease is attractive, however studying complex diseases like T2DM , insulin resistance and CVD remains difficult as there are multiple factors involved including: gene-gene and gene-environment interactions and these diseases don't exhibit simple modes of inheritance where we can make a molecular diagnosis by a simple blood test (156).

With the advances in genome wide scans in identifying emerging novel pleotropic genes for both T2DM and CVD and with the advances in our understanding of the underlying mechanisms for these complex diseases. It will be possible to identify gene-linked mutations by molecular genetics and screen people at risk and patients early in the course of the disease (156) .

A good example of applying this approach is a recent study by Kang et al. which found that patients with T2DM carrying the G allele at SNP+45 of the ADIPOQ had less response to PPARγ- agonist (Rosiglitazone) in reducing fasting glucose and increasing circulating adiponectin (157). This finding, although it needs to be replicated in a larger population, if translated into clinical practice, can identify patients response to treatment with more cost-effective approach to treatment of both T ype 2 diabetes and CVD (157).

The same approach could be applied if we manage to identify pleiotropic gene variants in patients presenting with impaired glucose tolerance or at a risk of developing 2 diabetes to tackle the cardiovascular disease risks if they are found to have these gene variants.

In conclusion the findings from our study do not support the original hypothesis, that variations in the PPAR γ and ADIPOQ genes, exhibit pleiotropy and increase the risk of cardiovascular disease. As the RISC study is a longitudinal study of CVD risk, we will, in time, be able to measure the change in carotid IMT progression and investigate its relation to ADIPOQ and PPAR γ gene variants .

7.1 Future work:

Although this study is cross-sectional; the RISC project is longitudinal study of CVD risk so it would have the advantage to investigate the relationship between these common type 2 diabetes gene variants and the carotid IMT progression. Furthermore with the genome wide association scans are now available to identify T2D susceptibility variants, we will be able to investigate other emerging novel type 2 diabetes genes in relation to cardiovascular disease, using c IMT as a marker in a non-diabeteic healthy individuals to test for the common soil hypothesis. Longitudinal RISC study cohort will help to define the role of the susceptibility alleles in the early pathogensis of type 2 diabetes and CVD.

Our study doesn't answer how variation in the ADIPOQ gene might directly influence carotid IMT. There is recent evidence that the ADIPQ gene is expressed in vascular tissue and adiponectin generated locally within the vasculature might directly influence endothelial function (148). Therefore further studies are needed to explore whether variation in the ADIPOQ gene in vascular tissue affects local adiponectin expression, endothelial function and ultimately CVD risk.

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Table1: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to insulin sensitivity (M/I value) measures for those with BMI ≤27 kg/m^{2}Data presented as Geometric Mean [interquartile range 25th - 75th percentile])

Character	T/T Number=689	T/G Number=169	G/G Number=13	P Value	P* Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	133.5 (109-189.6)	125.8 (112.5-203.)	125.4 (102.5-141.6)	0.38	0.27

P* GLM after correction for age, sex, BMI, waist, plasma adiponectin and recruitment centre

Table 2: ANOVA comparisons of means for SNP+45 of the ADIPOQgene in relation to insulin sensitivity (M/I value) measures for those with BMI l > 27 kg/m^{2}Data presented as Geometric Mean [interquartile range 25th - 75th percentile])

Character	T/T Number=314	T/G Number=89	G/G Number=4	P Value	P* Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	92.4. (66.7-127.4)	91.8 (66.3-134)	103.7 (71.6-159.8)	0.89	0.64

P* GLM after correction for age, sex, BMI, waist, plasma adiponectin and recruitment centre

Table 3: ANOVA comparisons of means for SNP+45 of the ADIPOQ gene in relation to insulin sensitivity (M/I value) measures for those with BMI l > 27 kg/m^{2}Data presented as (Geometric Mean [interquartile range 25th - 75th percentile])

Character	T/T+ T/G Number=403	(G/G) Number=4	P Value	P* Value
M/I min^{-1} kg_{ffm}^{-1} nM^{-1}	92.3(66-127)	103.7(71-160)	0.65	0.85

P* GLM after correction for age, sex, BMI, waist, plasma adiponectin and recruitment centre

Table 4: ANOVA comparisons of means for G allele carriers (G/G) Vs (T/T +T/G) genotypes in relation to(c IMT) measures for those with BMI ≤ 27 kg/m^2 (Data presented as Geometric Mean [interquartile range 25th - 75th percentile])

Value	T/T+ T/G N =858	G/G N=13	P Value	P* Value
IMT_{CCA} (μm)	591±3	581±6	0.16	0.6
IMT_{BULB} (μm)	758 ±5	741±11	0.44	0.78
IMT_{ICA} (μm)	608 ±5	602± 10	0.56	0.84
IMT_{AVRG} (μm)	655 ± 4	639 ± 7	0.07	051

$P^{\#}$ value after analysis of Covariance (Age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure adjusted means)

Table 5: ANOVA comparisons of means for T allele carriers (T/T+ T/G) Vs. G/G genotypes of the adiponectin in relation to(c IMT) measures for those with BMI ≤27 kg/m^2 (Data presented as Geometric Mean [interquartile range 25th - 75th percentile])

Value	T/T+ T/G N =858	G/G N=13	P Value	P* Value
IMT_{CCA} (μm)	589±3	599±6	0.69	0.88
IMT_{BULB} (μm)	753 ±5	800±11	0.29	0.16
IMT_{ICA} (μm)	607 ±5	623± 10	0.68	0.48
IMT_{AVRG} (μm)	651 ± 4	670 ± 7	0.54	0.49

P$^{\#}$ value after analysis of Covariance (Age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure adjusted means)

Table 6: ANOVA comparisons of means for T allele carriers (T/T+ T/G) Vs. G/G genotypes of the adiponectin in relation to(c IMT) measures for those with BMI greater than 27 kg/m^2 (Data presented as Geometric Mean [interquartile range 25th - 75th percentile])

Value	T/T+ T/G N = 403	G/G N=4	P Value	P* Value
IMT_{CCA} (μm)	627±5	585±28	0.35	0.73
IMT_{BULB} (μm)	798±8	731± 67	0.38	0.16
IMT_{ICA} (μm)	646 ±8	663± 57	0.80	0.93
IMT_{AVRG} (μm)	694± 6	658 ±45	0.51	0.28

P$^{\#}$ value after analysis of Covariance (Age, sex, BMI, waist, plasma adiponectin, recruitment centre, smoking, fasting glucose, total cholesterol, systolic blood pressure adjusted means [SE])

LOCAL Form 3: Consent Form - RISC Genetic Studies

Date ☐☐/☐☐/☐☐☐☐ Subject ID ☐☐/☐☐☐ ☐☐☐☐

To be printed on headed stationery of the hospital recruiting and examining the subject

Relationship between insulin sensitivity and cardiovascular disease risk (RISC)

Consent Form: Genetic Studies

Name of Volunteer (capitals) _____

		Please initial each box
1	I have read the participant information sheet on the above project and have been given a copy to keep. I have had the opportunity to ask questions about the project and I am satisfied with the information I have been given. I have discussed the study with ..... (name of physician)	
2	I agree to give blood samples for DNA analysis as part of this project. I understand that giving a sample for this research is voluntary and that I am free to withdraw at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that all data will be made anonymous prior to its circulation to other scientists and that all samples used in the genetic analysis will be anonymous.	
3	I understand that I am donating the DNA sample to the **European Group for the study of Insulin Resistance**, as outlined in the participant information sheet and that I will not derive any financial benefit from my participation in the study.	
4	I understand that information derived from the DNA sample - specific to me will not be passed back to me or to anyone else outside the project.	
5	I agree to take part in the study and know how to contact the research team if I need to.	

Volunteer's Signature_____ Date_____

I confirm that I have fully explained the nature of this study to the above named volunteer.

Signature of physician _____ Date_____

LOCAL Form 2: Consent Form - RISC Study

Date ☐☐/☐☐/☐☐☐☐ Subject ID ☐☐/☐☐☐ ☐☐☐☐

To be printed on headed stationery of the hospital recruiting and examining the subject

Relationship between Insulin Sensitivity and Cardiovascular Disease Risk (RISC)

Consent Form - General

Name of Volunteer (capitals)

_____

Please initial each box after reading text.

1	I have read the participant information sheet on the RISC project and have been given a copy to keep. I have had the opportunity to ask questions about the project and I am satisfied with the information I have been given. I have discussed the study with (name of physician)	
2	I agree to give blood samples and undergo the other medical examinations as part of this project and the information collected to be stored on file for use in the study. I understand that taking part in this research is voluntary and that I am free to withdraw at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that all data will be made anonymous prior to its circulation to other scientists.	
3	I agree to allow personal medical information to be collected about me from my GP, hospital, town hall, civic registry - on the understanding that it will be kept private and kept on file only for as long as necessary.	
4	I agree to being contacted by telephone at yearly intervals after the first examination and being asked questions about my state of health in the preceding year.	
5	I agree that I shall be contacted in approximately 3 years to make an appointment to have another hospital visit and undergo further tests.	
6	I understand that information derived from the tests specific to me will not be passed back to me or to anyone else outside the project - unless I specifically request it (below)	
7	**Women only** – I agree to undergo a pregnancy test if necessary, on the understanding that I will be informed of the result	
8	**If a disease or medical condition is found during the examinations**	**Circle as appropriate**
A	I would like to be informed	Yes / No
B	I would like my doctor to be informed of the results of the tests	Yes / No
9	I agree to take part in the study and I know how to contact the research team if I need to.	

Volunteer's Signature_____ Date _____

I confirm that I have fully explained the nature of this study to the above named volunteer.

Signature of physician_____ Date_____

Acknowledgements:

Participants

EGIR-RISC Study Group

RISC recruiting centres

Amsterdam, The Netherlands: R.J. Heine, J Dekker, G Nijpels, W Boorsma. Athens, Greece: A Mitrakou, S Tournis, K Kyriakopoulou. Belgrade, Serbia and Montenegro: N Lalic, K Lalic, A Jotic, L Lukic, M Civcic. Dublin, Ireland: J Nolan, TP Yeow, M Murphy, C DeLong, G Neary, MP Colgan. Frankfurt, Germany: T Konrad, H Böhles, S Fuellert, F Baer, H Zuchhold. Geneva, Switzerland: A Golay, V. Barthassat, V. Makoundou, TNO Lehmann, E. Harsch Bobbioni, T Merminod. Glasgow, Scotland: J Petrie, C Perry, F Neary, C MacDougall, K Shields, L Malcolm. Kuopio, Finland: M Laakso, U Salmenniemi, A Aura, R Raisanen, U Ruotsalainen, T Sistonen, M Laitinen. London, England: SW Coppack, N McIntosh, P Khadobaksh. Lyon, France: M Laville, F. Bonnet, A Brac de la Perriere, C Louche-Pelissier, C Maitrepierre, J Peyrat, A Serusclat. Madrid, Spain: R. Gabriel, EM Sánchez, R. Carraro, A Friera, B. Novella. Malmö, Sweden (1): P Nilsson, M Persson, G Östling, (2): O Melander, P Burri. Milan, Italy: PM Piatti, LD Monti, E Setola, F Minicucci, A Colleluori. Newcastle-upon-Tyne, England: M Walker, IM Ibrahim, M Jayapaul, D Carman, Y McGrady, D Richardson. Odense, Denmark: H Beck-Nielsen, P Staehr, K Hojlund, V Jensen, C Olsen. Perugia, Italy: GB Bolli, F Porcellati, C Fanelli, M Romolini, F Calcinaro, A Saturni. Pisa, Italy: E Ferrannini, A Natali, E Muscelli, S Pinnola, M Kozakova, L Landucci. Rome, Italy: G Mingrone, P Di Rocco, C Guidone, A Favuzzi. Vienna, Austria: W Waldhäusl, M Roden, C Anderwald, A Hofer.

Core laboratories and reading centres

Lipids Dublin, Ireland: P Gaffney, J Nolan, G Boran. Hormones Odense, Denmark: C Olsen, L Hansen, H Beck-Nielsen. Urine Albumin:creatinine Amsterdam, The Netherlands: A Kok, J Dekker; Genetics Newcastle-upon-Tyne, England: S Patel, M Walker. Stable isotope analysis Pisa, Italy: A Gastaldelli, D Ciociaro. Ultrasound reading centre Pisa, Italy: M Kozakova, E Ferrannini.

Data Management Villejuif, France: B Balkau, L Mhamdi. Mathematical modelling and website management Padova, Italy: A Mari, G Pacini, C Cavaggion. Coordinating office: Pisa, Italy: SA Hills, L Mota, L Landucci.

Further information on the RISC project and participating centres can be found on www.egir.org.

1. Pisa
2. London
4. Amsterdam
5. Newcastle
7. Lyon
8. Odense
9. Dublin
10. Perugia
12. Geneva
13. Frankfurt
14. Malmö
15. Rome
16. Glasgow
17. Vienna
18. Madrid
19. Athens
20. Milan
21. Belgrade
22. Kuopio