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GENETICS
OF
TYPE 2 DIABETES
AND
THE METABOLIC SYNDROME

ACADEMIC DISSERTATION

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Abstract

Type 2 diabetes and the metabolic syndrome are highly prevalent disorders with severe complications such as cardiovascular disease. The aetiology of type 2 diabetes and the metabolic syndrome is not known, but the interaction between genetic factors and environmental triggers is important. The aim this thesis was to identify genetic factors that may increase susceptibility to these disorders by investigating candidate genes regulating lipolysis (hormone-sensitive lipase, HSL, lipoprotein lipase, LPL and phosphodiesterase 3B, PDE3B), thermogenesis (uncoupling protein 2, UCP2) and adipogenesis (peroxisome proliferator-activated receptor gamma, PPARG). Four of the genes were screened for mutations and identified variants were tested for association in large intra-familial and case-control association studies. Variability in the UCP2 gene was not associated with alterations in basal metabolic rate or with obesity. The gene encoding HSL was associated with type 2 diabetes in a case-control study, and the LIPE marker of the HSL gene showed distorted transmission to abdominally obese offspring. The PDE3B gene was associated with hyperinsulinaemia in genotype-discordant siblings. Haplotypes including several variants on chromosome 11 were unequally transmitted to offspring with abnormal glucose tolerance. The studies also provided evidence for an interaction between a variant in the LPL gene and insulin sensitivity. In a large, family-based multi-step study we could show that genetic variability in the gene encoding PPARG is associated with a reduced risk for diabetes, supported by the consistent results in a meta-analysis on the same variant. In conclusion, variability in genes regulating lipolysis and adipogenesis increase susceptibility to type 2 diabetes and the metabolic syndrome. Prospective studies will be helpful to establish the risk associated with the potential genetic riskfactors presented in this thesis.

Key words: diabetes, metabolic syndrome, UCP2, HSL, LPL, PPARG, PDE3B

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I want to know God’s thoughts.
The rest are details.

Albert Einstein

As far as the laws of mathematics refer to reality
they are not certain,
and as far as they are certain,
they do not refer to reality.

Albert Einstein

Scientific papers included in this thesis

Paper I	Klannemark M., Orho M. and Groop L.C.: **No relationship between variants in the uncoupling protein 2 gene and energy expenditure.** *Eur J Endocrinology* 139: 217-223, 1998.
Paper II	Klannemark M., Orho M., Langin D., Laurell H., Holm C., Reynisdottir S., Arner P. and Groop L.: **The putative role of the hormone-sensitive lipase gene in the pathogenesis of Type II diabetes mellitus and abdominal obesity.** *Diabetologia* 41: 1516-1522, 1998.
Paper III	Klannemark M., Suurinkeroinen L., Orho-Melander M., Groop L. and Taskinen M.R.: **Interaction between the Asn291Ser variant of the *HSL* gene and insulin resistance on dyslipidaemia in high risk individuals for Type 2 diabetes mellitus**. *Diabetic Medicine*, 17: 599-605, 2000.
Paper IV	Altshuler D., Hirschhorn J.H., Klannemark M., Lindgren C.M., Daly M., Vohl M.-C., Nemesh J., Lane C.R., Schaffner S.F., Bolk S., Brewer C., Tuomi T., Gaudet D., Hudson T.J., Groop L. and Lander E.S.: **The common *PPARγ* Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes.** *Nature Genetics*, 26: 76-80, 2000.
Paper V	Klannemark M., Orho-Melander M., Almgren P., Degerman E., Manganiello V. and Groop L**.: Discordant sibling analysis of the phosphodiesterase 3B gene and association to postprandial hyperinsulinemia.** *Preliminarily accepted in Diabetes*, 2001.

Contents

Abbreviations

%fat	percent body fat
ANOVA	analysis of variance
aP2	adipocyte lipid/ fatty acid binding protein
APO	apolipoprotein
ATP	adenosine triphosphate
BMI	body mass index
BMR	basal metabolic rate
bp	base pair
cAMP	cyclic adenosine monophosphate
CEBP	CAAT enhancer binding protein
CI $_{95\%}$	95% confidence interval
cM	centiMorgan
CV	coefficient of variation
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
FCHL	familial combined hyperlipidaemia
FFA (=NEFA)	free fatty acids (non-esterified fatty acids)
FP	fluorescence polarisation
FRET	fluorescence resonance energy transfer
GYS1	muscle glycogen synthase gene
HDL	high density lipoprotein
HOMA-IR	homeostasis model assessment insulin resistance index
HSL	hormone-sensitive lipase
IDDM (=T1DM)	insulin dependent diabetes mellitus (type 1 diabetes)
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IRC	insulin requiring for control
IRS	insulin requiring for survival
IVGTT	intravenous glucose tolerance test
kb	kilo base pairs
LADA	latent autoimmune diabetes in adults
LDL	low density lipoprotein
LPL	lipoprotein lipase
Mb	mega base pairs
MIDD	mitochondrial inherited diabetes and deafness
MIN	mixed IDDM/NIDDM
MODY	maturity onset diabetes of the young
NGT	normal glucose tolerance
NIDDM (=T2DM)	non-insulin dependent diabetes mellitus (type 2 diabetes)

NIR	non-insulin requiring
OGTT	oral glucose tolerance test
OR	odds ratio
OSD	observed sum of differences
PCR	polymerase chain reaction
PDE3B	phosphodiesterase 3B
PPARγ	peroxisome proliferator-activated receptor γ
QTDT	quantitative transmission disequilibrium test
RFLP	restriction fragment length polymorphism
SBE	single-base extension
SD	standard deviation
SEM	standard error of the mean
SSCP	single-strand conformational polymorphism
TDT	transmission disequilibrium test
TNFα	tumour necrosis factor α
TZD	thiazolidinedione
UCP2	uncoupling protein 2
UCP3	uncoupling protein 3
WHO	world health organization
WHR	waist-to-hip ratio
VLDL	very low density lipoprotein

1. Identification of the disease(s)

1.1 The metabolic syndrome

The metabolic syndrome is a clustering of features associated with insulin resistance and risk factors for cardiovascular disease (CVD)[1]. The syndrome has been given many names such as the *insulin resistance syndrome*, *syndrome X* and the *dysmetabolic syndrome*[2-4]. The definition of the metabolic syndrome has been much discussed. In 1998 the World Health Organization (WHO) proposed a definition of the metabolic syndrome including diabetes, *impaired glucose tolerance* (IGT) and insulin resistance as invariable components and obesity, dyslipidaemia, hypertension and microalbuminuria as variable components (Figure 1)[1]. To have the metabolic syndrome the patient should have at least one of the invariable and two of the variable components. The prevalence of the syndrome increases with deterioration of glucose tolerance. In the Botnia study, 10% of subjects with normal glucose tolerance (NGT), 50% of subjects with IGT or impaired fasting glucose (IFG) and 80% of subjects with type 2 diabetes have the metabolic syndrome[5]. Subjects with IGT or IFG have a 3.6-fold increased risk for stroke and diabetic patients have a 2.2-fold increased risk for coronary heart disease if they have the metabolic syndrome[5]. The aetiology of the syndrome is not known. It has been suggested that *insulin resistance* is the common denominator and could be the cause of the other components[2,6]. The metabolic syndrome is per definition phenotypically heterogeneous. Whether it also has a heterogenous aetiology is not known. Many factors, including genetic factors, may increase or reduce the risk of developing the syndrome.

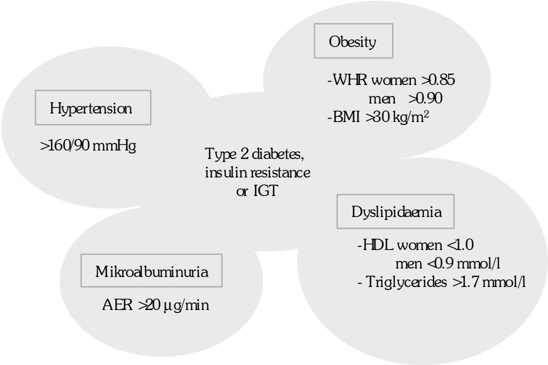


Figure 1. *The metabolic syndrome according to the WHO definition from 1998*[1]*. AER, albumin excretion rate; BMI, body mass index; HDL, high-density lipoprotein cholesterol; WHR, waist-to-hip ratio.*

1.2 Diabetes mellitus

Diabetes mellitus is a metabolic disorder defined by a disturbance in glucose metabolism leading to chronic hyperglycaemia. It is diagnosed by increased glucose levels in the fasting state or by a reduced glucose clearance after an *oral glucose tolerance test* (OGTT)[1,7]. Diabetes mellitus represents a spectrum of disorders with heterogenous aetiology but similar clinical signs (Figure 2). The worldwide prevalence of diabetes is about 4%, but varies between ethnic groups and degree of westernization[8]. About 25% of diabetic subjects have diabetes of *autoimmune* aetiology (type 1 diabetes, and latent autoimmune diabetes in adults, LADA).

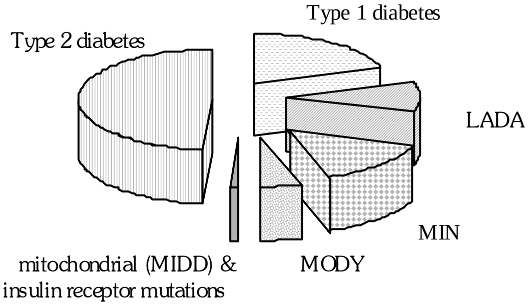


Figure 2. *Diabetes mellitus is a heterogenous disorder. Type 1 diabetes and LADA are caused by autoimmune destruction of the β-cells. Type 2 diabetes is associated with the metabolic syndrome. In some families both type 1 and type 2 diabetes occur and the diabetic phenotype of these subjects is intermediary between the two major forms of diabetes. We have called this form of diabetes MIN (mixed IDDM/NIDDM)[9,10]. Autosomal dominant forms of diabetes are caused by insulin secretory defects (MODY) or mutations of the insulin receptor. Mitochondrial inherited diabetes and deafness (MIDD) is highly penetrant and caused by mutations of mitochondrial DNA.*

Autoimmune forms of diabetes develop due to a destruction of pancreatic β-cells by self-directed T-cells. The patient usually loses endogenous insulin production rapidly, manifested by ketoacidosis at onset, and the patient requires insulin treatment for survival (IRS) (Figures 3 and 4)[1]. Type 2 diabetes develops over a long time period, probably decades (Figure 3). The onset is insidious, and the patients may not require insulin therapy to control glycaemia during the first years after diagnosis (*non-insulin requiring*, NIR, or *insulin requiring for control*, IRC) (Figure 4)[1]. Since symptoms can be lacking or sparse, many patients may remain undiagnosed for years until they start to suffer from macrovascular and microvascular complications. IGT is a prediabetic state (Figure 3) which leads to diabetes in 25-50% of the cases within 10 years[11] and the prevalence of IGT is higher among first degree relatives of type 2 diabetic subjects than among the general population[12]. The aetiology of type 2 diabetes is not fully understood. Most type 2 diabetic patients show impaired insulin action in target tissues (insulin resistance) as well as a reduced capability to compensate the insulin resistance with increased insulin production (relative insulin deficiency)[13,14]. Monogenic forms of diabetes can be caused by mutations of genes regulating insulin secretion and β-cell development[15].

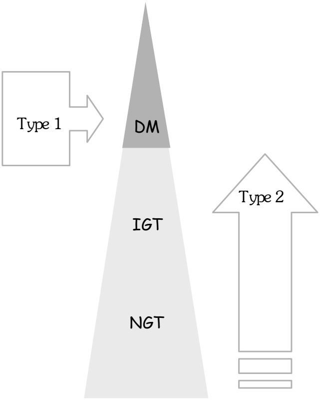


Figure 3. *Type 1 diabetes usually has a rather rapid onset whereas type 2 diabetes can develop over a long time-period, often preceded by stages like IFG or IGT.*

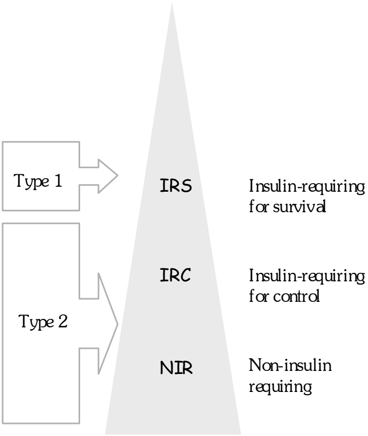


Figure 4. *Three subgroups of diabetes mellitus according to the need for insulin treatment*[1]*. Type 1 diabetes usually develops before the age of 35 years and requires insulin treatment for survival (IRS). Type 2 diabetes can be insulin requiring for control (IRC) or non-insulin requiring (NIR).*

1.3 Dyslipidaemia

Dyslipidaemia is defined by alterations in blood lipid levels. Lipids are transported in the blood in *lipoprotein particles* (Figure 5). The lipid levels can be very high in subjects affected by monogenic forms of dyslipidaemia[16] like familial hypercholesterolaemia or familial hypertriglyceridaemic disorders. In the latter conditions the plasma triglyceride levels are commonly above 6 mmol/l in affected subjects, and can be around 50 mmol/l if chylomicron metabolism is altered. The clinical appearance of these severe lipid disorders include acute pancreatitis, acanthosis nigrans, xanthomas and high incidence of cardiovascular disease[16].

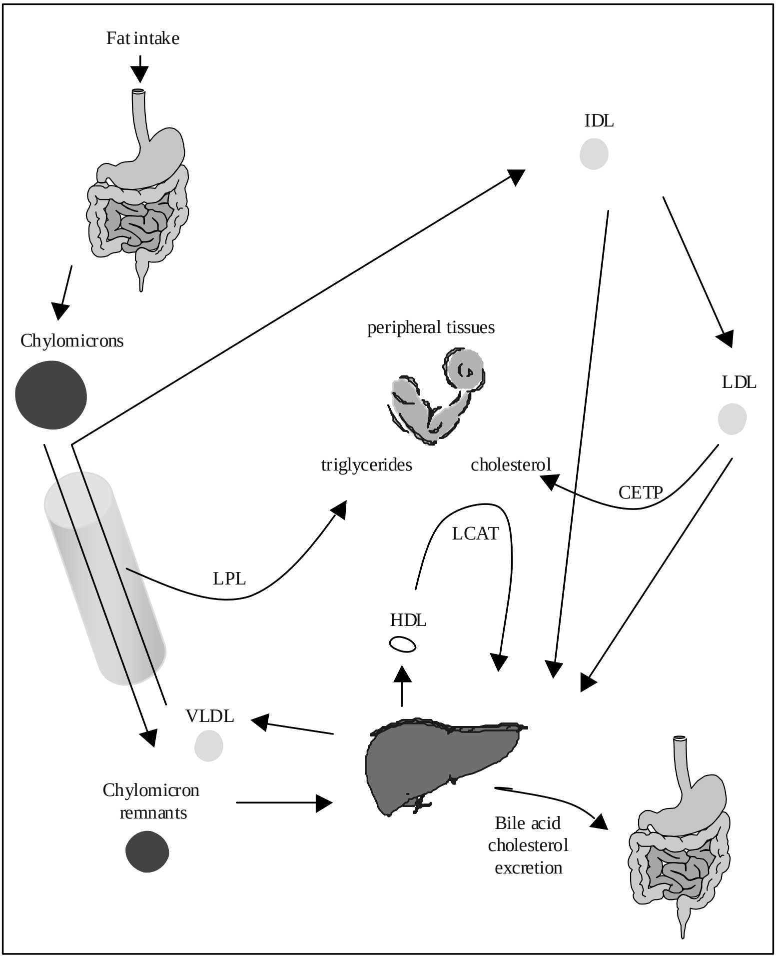


Figure 5. *A schematic description of lipid and lipoprotein metabolism. Lipids are transported in the blood in lipoprotein particles and delivered to peripheral tissues via hydrolysis/ esterification of triglycerides (lipoprotein lipase, LPL) and cholesterol (cholesterol ester transfer protein, CETP). Ingested fat is transported to the blood via the lymphatic system as chylomicrons. Remnant chylomicrons are metabolised by the liver. In between meals, the liver supplies peripheral tissues with triglycerides and cholesterol by secreting very-low-density lipoproteins (VLDL). VLDL increases in density when depleted of triglycerides, transforming into intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL). High-density lipoproteins (HDL) are produced in the liver to reverse cholesterol transport, a process requiring the enzyme lecithin-cholesterol acyl transferase (LCAT). Reverse cholesterol transport returns excess cholesterol from peripheral tissues for excretion as bile acids.*

The prevalence of milder forms of dyslipidaemia varies between populations and generally increases with degree of westernization of the society. Even the milder forms of dyslipidaemia are associated with an enhanced risk for *atherosclerosis*[17]. About 20-60% of patients with type 2 diabetes have some form of dyslipidaemia, mostly elevated plasma levels of triglycerides and reduced plasma levels of HDL cholesterol[18]. The excess plasma triglycerides reside in the VLDL subfraction and a large proportion of LDL is in the form of small and dense atherogenic particles.[18].

1.4 Obesity and abdominal obesity

Obesity is a major health problem associated with increased mortality[19]. Obesity is defined by an increase in body weight due to accumulation of excess body fat. Quantitative measures of obesity include the *body mass index* (BMI; height/weight^2), percent body fat (%fat) and the *waist-to-hip ratio* (WHR). Obesity (BMI above 30 kg/m^2) is very common, affecting 10-30% of populations in Europe and the US[20]. ”*Morbid obesity*” (BMI above 40 kg/m^2) is less common but associated with severe medical complications. Abdominal obesity is defined by accumulation of fat in the abdominal region (WHR above 0.85 for women and 0.9 for men). Abdominal obesity is characterised by fat deposition in the viscera, and is strongly associated with insulin resistance[21]. Abdominal obesity is more common among men[22] and the prevalence increases in women as they pass the menopause, suggesting that sex hormones may influence body fat distribution. Obesity can be caused by factors that influnce appetite or energy expenditure. Certain pharmacological agents, such as cortisol, can cause abdominal obesity as seen in Cushing's syndrome. Adult-onset obesity is often localised in the central regions and associated with increased adipocyte size, ”*hypertrophic obesity*”[23]. Subjects with generalised, early-onset obesity often have an increased number of adipocytes, so called *hyperplastic obesity*.

1.5 Hypertension

Hypertension is defined as a chronic elevation of blood pressure, above 160 mmHg systolic and/ or 90 mmHg diastolic pressure[1]. Hypertension is considered a major risk factor for cardiovascular disease[24,25]. The prevalence is 10-20% in most populations[26,27] and increases with age[28]. Hypertension can be caused by factors that influence blood volume, vasodilation, peripheral arterial resistance and heart rate. Hypertension is influenced by salt intake, but sensitivity to salt varies between individuals[29].

1.6 Microalbuminuria

Microalbuminuria is the presence of 20-200 μg/min albumin in overnight urine[1]. Microalbuminuria is an early sign of diabetic kidney disease[30], but also a predictor of cardiovascular disease[31]. About 30-40% of type 2 diabetic patients develop microalbuminuria after 25 years of diabetes duration[32]. In diabetic subjects microalbuminuria is strongly associated with hypertension [33]. Only about 7% of non-diabetic hypertensive subjects develop microalbuminuria[34]. Hyperglycaemia per se might increase albumin excretion in the urine[35].

1.7 Treatment

There are several options for the treatment of *type 2 diabetes* (sulphonylureas, metformin, thiazolidinediones (TZDs) and insulin), none of which has been able to change the inevitable deterioration of glucose tolerance characteristic of type 2 diabetes[36]. Therapeutic intervention can prevent microvascular complications if improved glycaemic control is achieved[37]. In contrast, most therapies have thus far had little or no effect on macrovascular complications[37]. This emphasises the need to treat not only hyperglycaemia in diabetic subjects, but also other risk factors like high blood pressure, obesity and dyslipidaemia.

Diet and exercise are important instruments to control most features of the metabolic syndrome. Diet-and-exercise intervention over a four-year period halved the number of IGT subjects that progressed to type 2 diabetes[38]. Unfortunately, compliance is not always good and it may be difficult to maintain weight reduction.

Pharmaceutical compounds used for the treatment of *obesity* include drugs that suppress appetite (sibutramin) or fat-absorption (orlistat). In general, the effect of these agents is restricted to a weight reduction of about 10%[39]. Although such small weight reduction has a beneficial influence on insulin sensitivity[40] it is not enough to reduce mortality in morbidly obese subjects. For these patients, surgery to reduce the size of the stomach is often considered (bariatric surgery). Once the excess weight has been lost many patients have reduced their energy expenditure[41] and easily gain weight again (the so called reduced obese state). WHO has proposed that obesity should be considered a chronic disease.

For the management of *dyslipidaemia* mainly statins and fibrates are used. Statins are HMG-CoA reductase inhibitors and reduce the levels of LDL-cholesterol and to some extent also plasma triglyceride levels[42]. Fibrates are particularly beneficial in patients with the metabolic syndrome, since they reduce the levels of plasma triglycerides, increase the levels of HDL cholesterol and reduce the density of LPL-particles[42]. These agents have been shown to reduce coronary artery disease (CAD) or coronary events by 25-60%[42]. Treatment of *hypertension* include among others β-blockers, diuretics, calcium channel blockers and ACE inhibitors.

Understanding the mechanism(s) that cause the metabolic syndrome may help to develop better pharmacological agents targeted at the underlying pathogenic processes.

2. Heritability of diabetes and the metabolic syndrome

2.1 How do we know if a disease has genetic components?

The factors that influence a trait can be divided into familial genetic, familial non-genetic and non-familial (Figure 6). The relative contribution of these components varies from trait to trait, and in different environments.

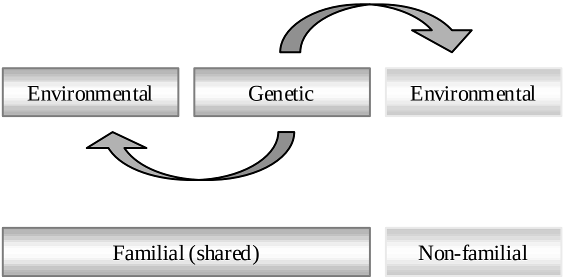


Figure 6. *Both genetic and environmental factors contribute to complex genetic diseases. Environmental factors can be familial and non-familial.*

Two variables that describe the size of the familial genetic component can be calculated from family data: heritability (h^2) and the lambda (λ) value. *Heritability* is the variability of a trait that can be accounted for by heritable (genetic) factors. Heritability can be calculated from the difference of concordance rate among monozygotic and dizygotic twins. Since monozygotic twins share 100% genetic material and dizygotic twins share 50% genetic material, the concordance rate should be larger among monozygotic twins if the trait/ disease has considerable genetic components. Twin studies may give variable results especially for late-onset diseases, and they are also very sensitive to selection bias. The *λ value* (relative risk) gives an estimate of the risk of disease in subjects related to affected individuals *relative* to the risk in the general population. The λ value cannot distinguish familial genetic from non-genetic components. Naturally, λ will be low for common diseases, such as type 2 diabetes.

Segregation analyses investigate the mode by which a (genetic) disease is transmitted through families. Mendelian genetic disease can be dominant/ codominant or recessive, autosomal or X-/Y-linked and maternally transmitted. When the mode of inheritance cannot be determined this usually means that the disease is not inherited in a Mendelian fashion. Non-Mendelian genetic diseases may need two or more genetic factors to precipitate (oligo- or *polygenic*), may be influenced by the sex of the parent that transmitted the disease (*imprinted*) or have other as yet unknown modes of transmission. Mendelian as well as non-Mendelian genetic diseases may have variable *penetrance*, which means that the phenotype can be variably expressed or not expressed at all in some individuals. Reduced penetrance can be the result of strong interaction with environmental factors.

2.2 Complex genetic disease

Diseases (or traits) that can be caused by a combination of genetic and environmental factors are complex or multifactorial. Many of our most common diseases are complex, such as cancer, asthma, diabetes, obesity and even infectious diseases. These diseases are heritable, but what is inherited is not the disease itself but rather the *susceptibility* to it. Many complex diseases are polygenic, but there are also examples of monogenic complex diseases (*conditional* mutations). Phenylketonuria has a high penetrance –all subjects that carry the mutation will develop the clinical manifestations if subjected to the environmental trigger (phenylalanine). The environmental influence on a disease may vary with the degree to which the studied population has been exposed to environmental factors.

A person who develops a complex disease may carry several genetic factors that predispose to the disease, and the number of prediposing genes will most likely affect the severity as well as the age at onset of the disease. Individuals who develop the disease without carrying any of the genetic factors are called *phenocopies*. Presumably, phenocopies will be more common among subjects without any family history of the disease. Complex genetic diseases that can be precipitated by environmental factors alone have a high rate of phenocopies and are particularly difficult to study genetically.

2.3 Quantitative traits and polygenic disease

All the traits of the metabolic syndrome have a continuous scale and are thus defined as *quantitative*. Quantitative genetic traits are generally believed to be polygenic, although monogenic traits can also be quantitative if the trait is influenced by environmental factors. The risk for cardiovascular disease increases as the associated traits become more pronounced, and defining when the disturbance becomes pathologic can be difficult.

2.4 Thrifty genes

Thrifty genes are genes that promote survival under a certain *environmental stress*. A classical example of a survival gene is the mutated form of the sickle-cell anaemia gene that in its heterozygous form protects against malaria. Another interesting example is the apolipoprotein (APO) E gene. The APOE ε4 allele is associated with increased risk for Alzheimer's disease and has a high frequency in Aborigines and low frequency in individuals from societies with a long-established agricultural economy[43]. Due to the risk of developing Alzheimer in old age, it has been suggested that the ancestral ε4 allele has been removed from populations that live longer.

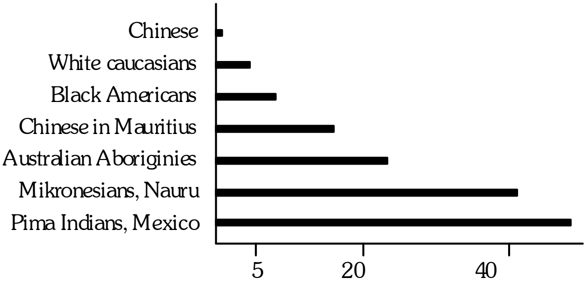


Figure 7. *The prevalence of diabetes increases with degree of westernization*[44]. *Diabetes is uncommon in the Chinese population, affecting less than 1% of the population. The frequency is higher in Chinese individuals born in China and living in Mauritius (15%). Aborigines (Australia, Nauru, Mexico) that have adopted the habits of a western society have much higher prevalence (>40%) than Caucasians (5-10%) and rural Aborigines (<1%).*

The hypothesis that thrifty genes could be important for the pathogenesis of type 2 diabetes and the metabolic syndrome was first introduced in 1962[45]. During a long time of human (and mammalian) evolution we have been subjected to long periods of famine and unpredictable food supplies. In such environments, genetic selection would favour energy conserving genotypes. For example, storing energy as fat rather than glycogen would protect the individual against fatal consequences of starvation. An animal that presumably has been naturally selected for thrifty genes is the desert-based Israeli sand rat (Psammomus obesus). This rat is well adapted for its natural niche in the desert, probably with high survival during periods of food shortages. In a laboratory environment, however, it develops obesity, insulin resistance and diabetes (i.e. the metabolic syndrome)[46]. Other examples of how energy conserving alleles can be advantageous are seen in the ob and db mice heterozygous for mutations of the leptin and leptin receptor genes, respectively. Although of similar weight, these mice survive starvation better than their wildtype littermates[47], a phenomenon that might be attributable to insulin resistance.

The thrifty genotype selection mechanism most likely also has been active in the evolution of human metabolism. The prevalence of most components of the metabolic syndrome is well known to vary between populations of different ethnic background[22,44,48]. After Australian aboriginies adjusted to the habits of a western society, the prevalence of type 2 diabetes increased to 25%[49]. In China the prevalence of diabetes has been 0.5% but currently the diabetes prevalence is escalating[50]. Micronesians of Nauru and Pima Indians in Texas have the highest prevalence of type 2 diabetes in the world, affecting more than 40% of the populations (Figure 7)[44]. The highest prevalence of obesity has been reported in Western Samoa, with 60% of the men and 80% of the women affected[51]. In populations with a high prevalence of diabetes and obesity, thrifty alleles may have accumulated due to genetic isolation.

2.5 Thrifty phenotypes

The development of type 2 diabetes, hypertension and other features related to the metabolic syndrome in adult life are associated with *low birthweight*[52]. The reason for this could be that poor intrauterine nutrition permanently programs the body to a constant starvation-state, which would lead to the accumulation of excess energy. However, genetic predisposition can influence both birthweight and the development of diabetes: Mutations in the glucokinase gene lead to the development of diabetes (MODY2) in heterozygous individuals (autosomal dominant inheritance)[53]. Carriers of MODY2 mutations have a mild insulin deficient/ insulin sensitive form of diabetes and they seldom become obese. Glukokinase mutations have also been associated with low birthweight. Recently it was shown that subjects who carry glukokinase mutations are small at birth only if they inherit the mutation from their father[54]. Carriers of glucokinase mutations can secrete insulin, but their β-cells have a higher glucose threshold for glucose-stimulated insulin secretion. If the mutation is transmitted from a diabetic mother the fetus will be subjected to high glucose levels (as glucose crosses the placenta) and the islets of the fetus will secrete normal insulin levels. This demonstrates that a mild insulin deficiency during growth may influence birthweight.

2.6 Evidence that type 2 diabetes is inherited

The risk of developing type 2 diabetes is approximately 3-4 times higher among first degree relatives of diabetic subjects compared to subjects without family history of diabetes (λ value around 3.5)[55]. Similar numbers have been calculated from offspring of diabetic subjects[56]. If one parent has diabetes the risk that the offspring will develop the disease is about 40%, and if both parents have diabetes the risk is approximately 70%[57,58]. This supports the hypothesis that there are familial factors that contribute to the disease and suggests that these factors to some extent are additive. Very high concordance rates of type 2 diabetes have been reported in monozygotic twins[59,60]. These studies most likely have overestimated the concordance by ascertaining twins based upon affection status[61], and do not distinguish familial genetic from non-genetic components. One population-based twin study suggested concordance rates of 34% among monozygotic and 16% among

dizygotic twin pairs[62]. Thus, approximately 40% of the variability of the diabetic phenotype may be heritable (familial genetic). In one recent study, the heritability seemed to be higher for IGT and diabetes (0.60), than for diabetes alone (0.26)[63].
There are also some monogenic forms of diabetes with some similarities to classical type 2 diabetes but generally developing at earlier ages. Maturity onset diabetes of the young (MODY) represents insulin deficient/ insulin sensitive forms of type 2 diabetes and make up about 5% of all diabetic cases[15,64]. MODY is caused by defects in the β-cell eventually leading to insulin deficiency. MODY1 is caused by mutations in the hepatocyte nuclear factor 4α gene (chromosome 20q12-q13.1), MODY2 by mutations in the glucokinase gene (chromosome 7p15-p13), MODY3 by mutations in the hepatocyte nuclear factor 1α gene (chromosome 12q24.2), MODY4 by mutations in the insulin promoter factor 1 (chromosome 13q12.1) and MODY5 is associated with mutations in hepatocyte nuclear factor 1β gene (chromosome 17cen-q21.3). Diabetes can also develop as a consequence of mutations in the insulin receptor gene (chromosome 19p13.2)[65] or in mitochondrial DNA ($tRNA^{Leu}$)[66], and familial forms of adipose tissue deficiency (partial and congenital lipodystrophy) are associated with diabetes[67].

2.7 Evidence that dyslipidaemia is inherited

Heritability estimates for plasma triglyceride and HDL cholesterol levels have ranged from 0.2-0.8[68]. In a recent study including twins reared apart, genetic factors contributed to one third of the variability of plasma triglycerides and nearly half of the variability of HDL cholesterol levels[69]. In particular, triglyceride levels seem to be highly influenced by individual-specific environmental factors. Severe lipid disorders are often due to mutations in genes regulating lipid metabolism, like familial hypercholesterolaemia (mutations in the LDL-receptor gene on chromosome 19p13.2), familial HDL deficiency (mutations in the gene encoding the ATP-binding cassette 1 on chromosome 9q22-q31) and type I hyperlipoproteinaemia (mutations in the *LPL* and *APOCII* genes on chromosomes 8p22 and 19q13.2)[16]. Monogenic forms of dyslipidaemia can be extremely rare (*APOCII* deficiency) to rare (familial combined hyperlipidaemia (FCHL)). FCHL is a relatively common form of familial dyslipidaemia, characterised by high incidence of cardiovascular disease representing 5-15% of subjects with coronary artery disease[70]. FCHL has been linked to several chromosomal regions[71] including 1q21-q23[71-73], and may be mono- or oligogenic. The clinical appearance of FCHL typically varies between the members of an affected family (high triglyceride or high cholesterol levels or both) [74,75].

2.8 Evidence that obesity is inherited

Heritability estimates have varied between 0.2-0.9 for obesity and between 0.3-0.5 for abdominal obesity[76-78]. Many of the available estimates include non-genetic familial factors, thus reflecting ”familiality”. In Pima Indians the familiality was 0.8

for body fat and waist circumference and 0.5 for BMI. Most studies agree on a heritability of BMI around 0.5, and the remaining variability of BMI seems to be largely attributed to shared environmental factors[78]. Studies of twins have shown that the propensity to gain weight in response to overfeeding is largely heritable.

Monogenic obesity often develops in childhood and progresses with time. It can be caused by mutations in genes related to the regulation of appetite (the melanocortin receptor 4, pro-opiomelanocortin and prohormone convertase 1 genes on chromosomes 18q22, 2p21 and 5q15-q21) or metabolism (the leptin, leptin receptor and peroxisome proliferator-activated receptor γ genes on chromosomes 7q31.3, 1p31 and 3p25)[79]. Mutations in most of these genes require two mutated alleles to cause disease. However, 3-5% of subjects with BMI above 40 kg/m^2 have mutations in the melanocortin receptor 4 in these families obesity is inherited in a dominant fashion[80]. Several chromosomal aberrations cause syndromes where obesity is one component, such as the fragile X, Prader-Willi/ Angelman, Bardet-Biedl and Turner syndromes. Familial partial lipodystrophy (adipose tissue deficiency) is an autosomal dominant disorder of fat redistribution that develops after puberty and is caused by mutations in the lamin A/C gene (chromosome 1q21.2)[79]. The body fat is redistributed from the periphery to central regions of the upper body, such as the abdomen, face and neck. There are also forms of generalised lipodystrophy, for example the Berardinelli-Seip syndrome (BSCL), which is congenital and seems to be a heterogenous autosomal recessive disorder caused by mutations in either *BSCL1* (chromosome 9q34)[79] or *BSCL2* (chromosome 11q13) [81].

2.9 Is the metabolic syndrome inherited?

All the components of the metabolic syndrome seem to have a complex polygenic background. Monogenic forms of each feature alone show that these disorders can be caused by single-gene mutations. In the general population it is likely that the risk alleles will be common and that protective alleles as well as risk alleles may be operating in the same individual. Although the features of the metabolic syndrome are common in most parts of the world, the prevalence varies a lot between rural and westernized populations, suggesting that genetic background and habitual factors play an important role.

Reports in the literature have suggested that 10 to 90% of the variability of metabolic cardiovascular risk factors is genetically determined. This emphasises the instability of heritability estimates. Inconsistent reports can be explained by differences in methodology, selection bias and data acquisition, and age of the studied population.

All features of the metabolic syndrome are associated with insulin resistance, which could be a common denominator. Most studies have shown that around 50% of the variability of insulin resistance may be genetically determined, and that the remaining variability is explained by individual-specific factors[78]. The familiality of insulin resistance is also illustrated by a higher prevalence among first-degree relatives of diabetic subjects (45%) compared to subjects with no family history of diabetes (20%)[12]. Hypertension and microalbuminuria are also highly heritable. It has been estimated that 10-80% of the variability of blood pressure is attributable to genetic factors. The heritability of systolic blood pressure may be highly age-dependent since the concordance is much higher in younger twins[82]. Intrauterine factors could also be important for the metabolic syndrome. Such factors may in particular be important for hypertension, as blood pressure variability seems to be partly explained by environmental factors shared by twins reared apart[78]. Most diabetic subjects with microalbuminuria also have hypertension, which makes it difficult to distinguish between factors that contribute to these traits separately. About 30% of the variability of albumin excretion seems to be heritable[83], but whether this is due to concomitant hypertension needs to be clarified. Several studies have shown that the heritability of coronary heart disease (CHD) exceeds 0.5[78]. It is important to remember that also behavioural risk-factors for CVD can be genetically related. Heritability estimates of smoking for example have suggested that a substantial proportion is genetic (28-84%)[78].

The metabolic syndrome may not always be inherited, but it seems heritable.

2.10 Are there one or several heritable traits of the metabolic syndrome?

The metabolic syndrome clusters predominantly in families with diabetes. About 80% of type 2 diabetic subjects have the metabolic syndrome[5] whereas only 30% of obese individuals are insulin resistant. First degree relatives of diabetic subjects often develop the metabolic syndrome already before the onset of diabetes[84], emphasising that hyperglycaemia develops as a consequence of the underlying metabolic defects. First-degree relatives of subjects with type 2 diabetes have increased abdominal deposition of fat, and are more insulin resistant than subjects without a family history of type 2 diabetes[12]. In these subjects, abdominal obesity usually precedes insulin resistance and the two variables are highly correlated. This does not *per se* mean that insulin resistance is caused by abdominal obesity; syndromes of severe insulin resistance are not always associated with obesity.

The metabolic syndrome may have a single central aetiologic component or it may be caused by different factors that increase the risk for CVD. Few studies have been performed to elucidate this question. In one study, twins reared either together or apart, BMI and insulin resistance were largely co-inherited[69]. Systolic blood pressure, plasma triglycerides and HDL cholesterol levels were to some extent influenced by the same genetic component, but also by independent genetic factors. Taken together this suggests that a single factor could initiate the syndrome by influencing fat accumulation or insulin sensitivity. Additional environmental factors and genetic factors influencing lipaemia and blood pressure may aggravate the associated symptoms.

3. Adipose tissue, lipolysis and energy expenditure

3.1 Adipogenesis

Differentiation of adipocytes into functional insulin sensitive cells of fat storage is called adipogenesis. Glucocorticoids and growth hormone promote adipogenesis in pre-adipocytes[85]. Certain internal factors are needed to initiate the transcriptional cascade that makes the cell to develop into an adipocyte. CCAAT/enhancer binding proteins (C/EBPα and C/EBPδ) and peroxisome proliferator-activated receptor gamma (PPARγ) are transcription factors that play important roles in the regulation of adipocyte differentiation[85]. Adult-onset obesity is associated with increased fat cell size[23], and large fat cells seem to be less insulin sensitive than smaller cells[86]. Whether this is a cause or a consequence of obesity is not known. Defective deposition of fat in adipose tissue seems to promote storage of fat in other organs, such as the liver, skeletal muscle and pancreas, and could be one important factor contributing to insulin resistance[87].

3.2 Adipose tissue and metabolism

The adipose tissue is an *endocrine organ* secreting several hormones/ cytokines into the blood stream. Factors secreted from adipose tissue may convey signals to the the brain, the β–cells and skeletal muscle to adapt to changes in fat stores by modulating feeding, insulin secretion and insulin sensitivity. The discovery of leptin has demonstrated a new role for adipose tissue in the regulation of appetite and energy expenditure by signalling the body's state of adiposity to the brain[88]. Resistin[89] and adiponectin[90] are considered to modulate insulin sensitivity in mice, although the role of resistin in human metabolism has been questioned[91]. The cytokine tumour necrosis factor alpha (TNFα) inhibits insulin signalling in adipose tissue[92], and can thereby stimulate lipolysis.

Disturbed adipose tissue development and metabolism may lead to insulin resistance, which may precipitate diabetes if insulin secretory capacity fails to compensate. Evidence to support this comes from studies of *lipodystrophies* associated with diabetes. The A-ZIP/F-1 mice lacks subcutaneous adipose tissue and is characterised by fat deposits in the liver, muscle and β-cells, as well as by insulin resistance and hyperglycaemia[93]. Insulin resistance and hyperglycemia can be reverted by implantation of adipose tissue[94]. Mice lacking the glucose-transporter 4 (GLUT4) in adipose tissue have normal adipocyte mass but develop insulin resistance in the liver and skeletal muscle[95]. This suggests that insulin sensitivity of the adipocyte may be important for the maintenance of whole-body insulin sensitivity.

An increased amount of adipose tissue in the visceral region is associated with insulin resistance, diabetes and CVD[96]. The reason for this is not known, but visceral fat is metabolically highly active, with a large output of FFA[97]. FFA from visceral depots is carried with the portal vein directly to the liver, and could interfere with gluconeogenesis and VLDL production. The OLETF (Otsuka Long-Evans Tokushima Fatty) rat represents another model of the metabolic syndrome with increased visceral fat accumulation and a high incidence of atherosclerosis[98].

3.3 Free fatty acids (FFAs)

FFA levels are increased in diabetic and obese individuals[99], and associated with an increased risk for type 2 diabetes[100] and cardiovascular disease[101]. There is evidence to suggest that increased serum FFA could be a familial trait predisposing to CVD. For example, the prevalence of CVD in the parental generation is increased in subjects with elevated serum levels of FFA[102]. FFA *per se* could have deleterious effects on insulin sensitivity and insulin secretion[103]. FFAs compete with glucose metabolism in skeletal muscle (*the Randle cycle*)[104], leading to insulin resistance and reduced glycogen synthesis[105]. In fact, forcing FFA to the skeletal muscle by overexpressing the *LPL* gene in muscle of transgenic mice leads to insulin[106]. *Intramuscular triglyceride levels* are increased in insulin resistant non-diabetic individuals and correlate with the rate of insulin stimulated glucose utilisation[107]. Long-term exposure of the β-cell to high serum FFA levels reduces insulin secretion (*lipotoxicity*)[108]. Recent studies have shown that depletion of such excess triglycerides from pancreas, liver and skeletal muscle using TZDs results in improved insulin sensitivity. Intracellular breakdown of triglycerides could in some tissues provide substrates for intracellular signalling molecules such as diacylglycerol and phospholipids.

3.4 Lipolysis

Fat storage is regulated by the hydrolysis of triglycerides to FFAs, a process called *lipolysis* (Figure 8). The rate-limiting step of intracellular lipolysis is catalyzed by hormone-sensitive lipase (HSL)[109]. The main function of HSL in normal physiology is to release stored fat during starvation or endurance training, providing energy as FFA to the heart and skeletal muscle. Catecholamines and glucagon stimulate and insulin inhibits intracellular lipolysis. After a meal, lipolysis of triglycerides stored in chylomicrons or VLDL particles produces FFA that can be transported into the cell for storage. This step is catalyzed by LPL, which is produced by adipose tissue and skeletal muscle and adheres onto the endothelial cell wall. For efficient transfer of fat from plasma into tissues, insulin activates LPL and inactivates intracellular lipolysis. The anti-lipolytic effect of insulin is mediated via the insulin receptor leading to the activation of phosphodiesterases (PDEs), predominantly PDE3B[110,111]. PDE3B hydrolyses the activator of HSL, cAMP, and thereby reduces serum FFA levels by 80% after a normal meal[112] [113].

HSL mRNA and protein levels are decreased in obese subjects[114]. Subjects with polycystic ovary syndrome (PCOS)[115] or FCHL[116] as well as obese subjects[117,118] and their first-degree relatives[119]show reduced response to catecholamine-induced lipolysis. Also, in many studies the suppression of FFA in response to insulin was impaired in diabetic[120-122] and obese prediabetic[121,123] subjects and in patients with FCHL[124] subjects. The sensitivity to the anti-lipolytic action of insulin was improved with TZD treatment[125].

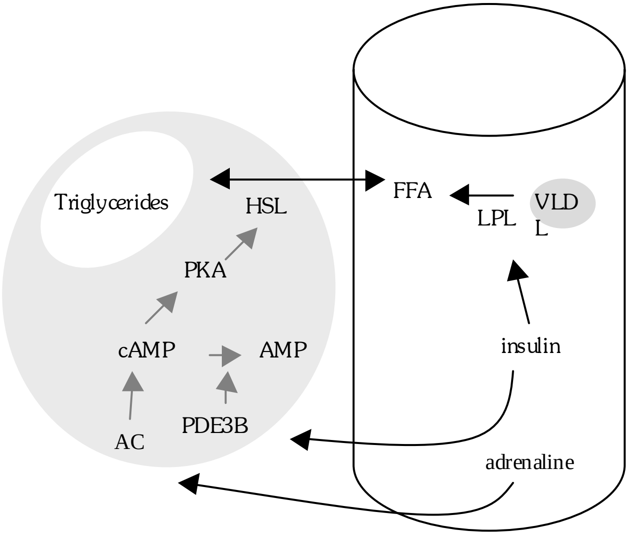


Figure 8. *The regulation of fat storage and FFA flow in the adipocyte.*

Lipolysis is a powerful regulator of fat redistribution. A change in lipolytic activity in tissues could redirect fat from one region to another. During pregnancy the activity of LPL is increased and the activity of HSL in decreased in mammary glands resulting in increased accumulation of fat[126]. If abdominal depots are more sensitive to insulin and more resistant to catecholamines, relative to subcutaneous adipose tissue, this will redirect subcutaneous fat to the abdominal region. This could explain the predisposition to abdominal obesity observed in subjects with type 2 diabetes, in whom visceral fat seems to be more sensitive to insulin than peripheral subcutaneous fat[127]. Disturbed regulation of lipolysis can also lead to alterations in blood lipid levels. Increased lipolytic activity in adipose tissue could increase serum FFA levels, and reduced LPL activity in the sera could induce post-prandial hyperlipidaemia. Lipolysis clearly can be of importance for the maintenance of body fat stores. Reduced lipolytic rate increased adipose tissue mass in the adipocyte lipid/ fatty acid binding protein (aP2) knockout and the monosodium glutamate (MSG) mice.

Neonatal administration of monosodium glutamate destructs the arcuate nuclei in the hypothalamus, and these mice develop increased adiposity despite of normal eating behaviour[128]. Interestingly, the aP2 knockout mouse has disturbed insulin secretion, supporting the existence of an adipoinsular axis[129]. The perilipin[130] and protein kinase A (PKA)-RIIβ isoform[131] knockout mice have constitutively activated lipolysis, small adipocytes, reduced adipose mass, increased lean body mass and energy expenditure, and are resistant to the development of obesity when fed a high-fat diet.

3.5 Energy expenditure and uncoupling proteins

The amount of fat stored after ingestion of food varies between individuals. There are mainly two mechanisms responsible for this -*thermogenesis* and *energy expenditure*[132]. Basal energy expenditure is the energy spent for the maintenance of the basal cellular metabolism, and is measured at isothermal conditions at rest. Energy expenditure is influenced by environmental factors and increases in response to certain dietary factors and a high level of physical activity[133]. Obesity is associated with low energy expenditure, and low energy expenditure predicts future weight gain[134]. Brown adipose tissue (BAT) is rich in mitochondria and actively uncouples the respiratory chain from ATP synthesis. Oxidation of nutrients in the respiratory chain and the synthesis of ATP from ADP take place in the inner mitochondrial membrane. These processes are coupled and electron transport in the respiratory chain is inhibited in lack of ADP. During the electron transport, protons are transported across the inner membrane, building up a proton gradient that drives the ATP synthase. ATP synthesis can be uncoupled from the respiratory chain if the proton gradient is discharged, and instead of storing the energy as ATP it can be released as heat. In fact, despite its unacceptable side-effects, the chemical uncoupler dinitrophenol was used as a weight-lowering agent in the 1930's. The biological uncoupler thermogenin or *uncoupling protein* (UCP1 or simply UCP) was discovered in the late 1970's and is located in brown adipose tissue[135]. Human babies have brown adipose tissue between the scapulae to produce heat during their first year of life, whereas adult individuals only have very small amounts of brown adipose tissue. The discovery of uncoupling proteins located in tissues that are more abundant in the human body (UCP2[136,137] and UCP3[138,139]) reinforced the idea that the uncoupling mechanism could be important for the regulation of thermogenesis and energy expenditure in human adults. The amino acid homology between UCP2/UCP3 and UCP1 is about 55%[139]. In spite of this homology, an uncoupling function of UCP2 and UCP3 has not been clearly established. Although overexpression of UCP2 or UCP3 in yeast resulted in depolarisation of the mitochondrial membrane, uncoupling activity was not increased[140].

Data from animal models suggest that ablation of brown adipose tissue induces hyperphagia and obesity[141]. Although mice without the *UCP1* gene are unable to increase thermogenesis in response to cold, these mice have normal weight[142]. Adipose tissue and skeletal muscle are abundant in mice and men, and one could expect that altered uncoupling activity in these tissues would influence energy expenditure and/ or thermogenesis. This is supported by resistance to weight-gain on a high-fat diet in mice with ectopic expression of UCP1 in white adipose tissue (WAT)[143] or skeletal muscle[144]. UCP2 is expressed in many tissues, most abundantly in white adipose tissue[136,137], whereas UCP3 is expressed in skeletal muscle[139]. Therefore, one could expect that altered UCP2 or UCP3 activity would influence energy expenditure and/ or obesity in human subjects.

4. Candidate genes that may contribute to type 2 diabetes and the metabolic syndrome

Mutations of nearly 1000 genes are known to cause monogenic disease or clearly increase the susceptibility for complex traits. Recent data show that almost half of all disease genes encode enzymes or enzyme co-activators[145]. Mutations in enzyme and enzyme co-activator genes were particularly over-represented in diseases developing during the first year of life and in diseases emerging after the age of 50. Diseases caused by these mutations were often transmitted recessively. Mutations of transcription factors often caused disease that developed *in utero*, and these diseases were often transmitted in a dominant fashion. It is thus highly likely that mutations increasing the susceptibility to type 2 diabetes and the metabolic syndrome may be found in enzymes regulating glucose and lipid metabolism, and that the phenotype may in some cases be more obvious in homozygous carriers.

Several chromosomal regions have been suggested to contain genes that predispose to type 2 diabetes or quantitative measures associated with obesity. Adiposity has been linked to chromosomes 2p (fat mass or leptin levels), 20q (%fat) and 11q21-q22 (%fat), and basal metabolic rate has been linked to chromosome 11q13. Type 2 diabetes has been linked to chromosomes 1q, 2q (*NIDDM1*), 12q (*NIDDM2*) and 20q, and recent data from the Botnia study reported suggestive linkage to chromosomes 9q (type 2 diabetes) and 18p (obese type 2 diabetes)[4]. The *calpain 10* gene was the first example of positional cloning of a gene that might contribute to type 2 diabetes. The *calpain 10* (*CAPN10*) gene is located in the *NIDDM1* region (chromosome 2q) and a haplotype variant of this gene seem to predispose to type 2 diabetes in several but not all populations[146-148]. In the Botnia population, subjects carrying the SNP43 11 or 12 genotypes were more insulin resistant than their gender-matched genotype-discordant siblings, and both diabetic and non-diabetic carriers of this allele had elevated FFA levels, compared to non-carriers (Marju Orho-Melander, unpublished).

Several genes involved in the metabolism of FFA seem to contribute to susceptibility to features associated with type 2 diabetes, the metabolic syndrome and CVD, including the genes encoding the β-adrenergic receptors (*ADRB1-3*, chromosomes 10q24-q26, 5q32-q34 and 8p12-p11.2, respectively) and the fatty acid binding protein 2 (*FABP2*, chromosome 4q28-q31)[4]. Recently, a genome-wide scan of quantitative traits associated with the metabolic syndrome was performed, suggesting that several of these traits link to chromosomes 3q27 and 17p12[149]. Preliminary data of a gene located in the 3q27 region, adiponectin, support a role for adiponectin in the modulation of insulin resistance[90]. In this thesis we have concentrated on five candidate genes that are important for the regulation of energy expenditure, lipolysis and adipose tissue development.

4.1 Uncoupling protein 2 (UCP2)

UCP2 was discovered in 1997[136,137] and has since then been intensely studied with over 300 publications in PubMed. The *UCP2* gene is encoded by 6 coding and 2 non-coding exons, spanning a region of 8.6 kb on chromosome 11q13 (accession number NT_009296 at www.ncbi.nlm.nih.gov) (Figure 18, results, page 51). The mRNA is 2.1 kb and the protein consists of 309 aminoacids (accession number XM_035028). The UCP2 and UCP3 proteins have 71% homology, and UCP2 is 55% homologous with UCP1[139]. Obesity is associated with lower *UCP2* mRNA levels in visceral fat, but no differences in *UCP2* mRNA levels have been seen neither in subcutaneous fat nor in skeletal muscle between lean and obese subjects[140]. *UCP2* mRNA levels increase in response to fasting [150,151] a finding which challenges the view that UCP2 would be a positive regulator of energy waste. In diabetic subjects the upregulation of *UCP2* expression in response to fasting seems to be impaired[151]. However, care should be taken in the interpretation of *UCP2* mRNA levels since it has been shown that *UCP2* is also regulated at translational steps[152].

4.2 Hormone-sensitive lipase (HSL)

HSL is a major regulator of lipolysis in many tissues, catalysing the hydrolysis of mono-, di- and triacylglycerols and cholesterol esters to FFA, glycerol and cholesterol[109]. Adipose tissue lipolysis mainly provides substrates for lipid oxidation in other tissues and organs and is activated by molecules stimulating the cAMP/protein kinase A (PKA) complex such as adrenaline, adrenocorticotrophic hormone (ACTH) and glucagon.

The *HSL* gene is encoded by 9 exons[153], spanning a region of 26 kb on chromosome 19q13.1-q13.2 (accession number NT_011128) (Figure 19, results, page 52). The mRNA is 3.8 kb and the protein consists of 1076 aminoacids (accession number XM_008882). The regulatory region upstream the *HSL* gene seems complex, including at least five alternative first exons and two separate promoters differentially activated in various tissues[154]. This implies that transcriptional regulation of the gene may be important for tissue-specific expression. The *acute regulation* of HSL involves phosphorylation mediated by the cAMP/PKA complex[155], and this phosphorylation seems to increase the propensity of HSL to interact with other proteins[156]. It has been suggested that HSL interacts with a number of proteins, including perilipin[157], aP2[158] and lipotransin[159]. HSL needs to be translocated to the lipid droplet for efficient lipolytic activation[157,160], and this translocation could be dependent on protein-protein interactions. Perilipin seems to protect the triglyceride pool from lipolytic breakdown, as judged from adipose tissue of perilipin deficient mice which has constitutively activated HSL and lipolysis[130]. It has been proposed that the interaction of HSL with aP2 prevents substrate-inhibition and that aP2 would rapidly bind to released FFAs, dissociate from HSL and transport FFA to the plasma membrane[158]. This hypothesis is supported by aP2 deficient mice whom display accumulation of intracellular FFA in adipocytes[129]. The interaction of HSL with lipotransin has only been detected in the presence of insulin and could provide a mechanism preventing the translocation of HSL to the lipid droplet[159]. Furthermore, there is evidence that HSL is functionally active as a homodimer *in vivo*, and that dimerization increases HSL activity[161].

In one mouse strain the lack of HSL is associated with increased abdominal fat stores[162]. However, knocking out the *HSL* gene in mice does not seem to influence total body adiposity[162,163]. This indicates a redundancy of HSL activity in adipose and perhaps other relevant tissues, suggesting that other lipolytic enzymes could be active in the adipocyte and substitute HSL in its absence. Under basal conditions, the rate of lipolysis does not entirely correlate with HSL activity in human subjects[164], indicating that other lipolytic enzymes might be expressed and active. However, β-adrenergic stimulation of adipose tissue lipolysis is highly dependent upon HSL activity[165] and the HSL knockout mice show a markedly reduced β-adrenergic response[162,163]. In addition, it is not known if substitution of HSL activity by other lipolytic enzymes will take place in the presence of a defective HSL protein.

Increased HSL activity in skeletal muscle could contribute to insulin resistance by modulating intracellular FFA metabolism that compete with glucose metabolism. HSL is activated by adrenaline and muscle contractions in skeletal muscle[166,167]. In male reproductive organs HSL cholesterol esterase activity has proven crucial for sperm production, and male mice deficient of *HSL* are infertile[163]. In the *β-cell*, lipolytic activity could modulate glucose-stimulated insulin secretion (GSIS)[168]. In fact, transgenic mice overexpressing HSL in β-cells develop glucose intolerance when challenged with a high-fat diet (Maria Sörhede-Winzell, EASD presentation 308, Jerusalem 2000). In the β-cell lipolysis may also represent an alternative source of energy independent of glucose. The effect of glucagon-like peptide-1 (GLP1) on glucose-stimulated insulin secretion and potentially on the activation of HSL[169] may play an important role in insulin secretion.

4.3 Lipoprotein lipase (LPL)

LPL belongs to another family of lipases including the pancreatic and the hepatic lipase. These lipases have structural similarities, are secreted and adhere to heparan sulphate proteoglycans on the endothelial wall. Their function is to release FFA from circulating lipoproteins, and increase the uptake of FFA in the tissue where they are expressed. Upon heparin treatment they are released into the blood stream. LPL is the primary enzyme responsible for the conversion of lipoprotein triglycerides into FFA. Maximal activity of LPL requires the presence of its co-activator, APOCII, situated on triglyceride-rich lipoproteins. In contrast to HSL which is inhibited by insulin, the activity of LPL is enhanced by insulin.

The *LPL* gene is encoded by 10 exons spanning a region of 28 kb on chromosome 8p22 (accession number NT_008271). The mRNA is 3.5 kb and the protein consists of 475 aminoacids (accession number XM_044682). LPL is most active as a homodimer[170]. Over 80 variants and mutations have been identified in the *LPL* gene, many of which have a substantial influence on the phenotype of the carrier[170]. Type I hyperlipoproteinaemia is an autosomal recessive disorder caused by mutations in the *LPL* gene. Subjects affected by this disorder are particularly sensitive to high-fat diets. Even if LPL deficiency is not lethal in man, *LPL* knockout mice die within their first day of life[171]. When the mice start to suckle they develop severe hypertriglyceridaemia resulting in cyanosis (chylomicrons obstruct the arterioles of the lung). Haploinsufficiency of the *LPL* gene has some influence on lipaemia in human subjects[170]. Several variants of the *LPL* gene have been identified in the general population, including four coding (Asp9Asn, Gly188Glu, Asn291Ser, Ser447X) and two non-coding variants that potentially could interfere with regulatory elements (T-93G and a *HaeIII* polymorphism in intron 8)[170]. These variants have been associated with dyslipidaemia and increased cardiovascular risk, and haplotype analysis suggests that several of them independently influence the lipid profile[170].

It has been proposed that LPL could contribute to *obesity* as the *LPL* gene is upregulated in adipose tissue of obese subjects[172]. This may rather reflect a consequence than a cause of obesity for the following reasons. Firstly, human subjects lacking LPL[173] and mice haploinsufficient for LPL[171] are neither lean nor obese. Secondly, mice overexpressing LPL in adipose tissue are not obese due to upregulation lipolysis (HSL activity)[174]. Interestingly, overexpression of LPL in skeletal muscle results in whole-body insulin resistance[106,175], and sometimes myopathy[106], suggesting that an increased intramuscular FFA metabolism may have harmful effects on muscle glucose metabolism.

4.4 Peroxisome proliferator-activated receptor gamma (PPARγ)

PPARγ is a transcription factor of the orphan nuclear receptor family, which means that the natural ligand is not known. PPARγ was cloned investigating proteins that interact with the 5' flanking region of the *aP2* gene[176]. Ectopic expression of PPARγ in fibroblasts promote adipogenesis[177]. There are at least three PPARs, PPARα (or simply PPAR), PPARγ and PPARδ[178]. PPARα is expressed in the liver and skeletal muscle and controls the regulation of genes involved in fatty acid oxidation. The beneficial influence of fibrates on lipid metabolism is from the activation of PPARα. The function of PPARδ is less defined. PPARγ heterodimerises with another transcription factor, the retinoid X receptor (RXR). Long-chain FFA and prostaglandins have been proposed as naturally activating PPARγ ligands[179]. TZDs are synthetic activating ligands for the PPARγ/RXR heterodimer[180], and PPARγ mediates at least some of the pleiotrophic effects that TZDs have on insulin action, including skeletal muscle glucose uptake[178].

The *PPARγ* gene is encoded by 7 exons[181], including the *PPARγ2*-specific exon B (Figure 21, results, page 54). The gene spans a region of 83 kb on chromosome 3p25 (accession number NT_005718). The gene has two major transcription initiation sites, corresponding to two common isoforms of the mRNA [182]. The PPARγ1 mRNA is 1.6 kb and the protein consists of 477 aminoacids (accession number XM_051559), and the PPARγ2 protein is 28 amino acids longer (encoded by exon B, Figure 21, results, page 54).

Knockout of the *PPARγ* gene in mice is lethal[183]. By generating chimeric PPARγ homozygous knock-out mice it was established that the gene is required for fat cell development, since no cells lacking PPARγ could be identified in the adipose tissue of these animals[184]. In spite of this, mice heterozygous for the PPARγ gene were of normal weight and had normal adipose tissue development[185]. The mice were more insulin sensitive than their wild-type littermates.

PPARγ is predominantly expressed in adipose tissue (mainly PPARγ2), although the PPARγ1 isoform has been identified in skeletal muscle at 10% of the levels in adipose tissue[186]. *PPARγ* mRNA levels have been reported upregulated or unchanged in skeletal muscle of type 2 diabetic patients and in visceral adipose tissue and skeletal muscle of obese individuals[186,187] compared to healthy control subjects. *PPARγ* expression in adipose tissue is downregulated in response to a very-low calorie diet *in vivo*[187]. TZD treatment upregulates *PPARγ1* mRNA and protein expression in skeletal muscle in both diabetic and non-diabetic subjects[188] but the glucose- and insulin-lowering influence of TZDs in mice seems to be dependent upon adipose tissue[189].

4.5 Phosphodiesterase 3B (PDE3B)

Phosphodiesterases hydrolyse cAMP, thus inactivating an important intracellular second messenger. At least eleven families of structurally related phosphodiesterases have been identified[190]. The phosphodiesterase isoenzymes differ in their affinity for cAMP, cellular location, mechanisms of regulation and tissue expression pattern. In most cell types several phosphodiesterases are co-expressed, producing a highly sophisticated regulation of cAMP levels. PDE3B is a membrane-bound protein and probably located in the endoplasmic reticulum. PDE3B is mainly expressed in insulin-sensitive cells like adipocytes, hepatocytes and pancreatic β-cells[111]. The signalling pathway by which insulin regulates PDE3B activity is only partially understood. In the adipocyte, PDE3B activation seems to be dependent on phosphoinositol-3 kinase and protein kinase B (Akt2)[191]. It has a high affinity for cAMP (active at low cAMP concentrations) and can be inhibited by high levels of cGMP.

The *PDE3B* gene is encoded by 16 exons[192,193] covering 220 kb on chromosome 11p15.1 (accession number NT_009062) (Figure 23, results, page 56). The mRNA is 3.3 kb and the protein consists of 1112 aminoacids (accession number NM_000922). The lipolytic effect of TNFα involves the downregulation of PDE3B[194]. In the adipocyte, PDE3B accounts for 90% of the cells total phosphodiesterase activity and mediates the antilipolytic action of insulin[112,195]. In the hepatocyte, insulin inhibits glycogenolysis by activating PDE3B[196,197]. Studies have shown that activation of PDE3 in the β-cell can inhibit insulin secretion stimulated by increased cAMP levels in the β-cell[198,199]. Potentiation of insulin secretion at high glucose levels by an increase in intracellular cAMP levels might represent a mechanism by which the β-cell adapts to an increased insulin demand[200]. Insulin stimulates insulin growth factor-1 (IGF-1) in the liver and IGF-1 is known to inhibit insulin secretion. Both IGF-1 stimulate PDE3B and it has been suggested that PDE3B may mediate negative feedback on insulin secretion[198].

5. The aims of this thesis

The overall aim of this thesis was to identify genetic variation that increase the susceptibility to type 2 diabetes and the metabolic syndrome by investigating candidate genes in adipose tissue and lipid metabolism.

The specific aims were:

I) To identify mutations and variations in the coding region of the *uncoupling protein 2* gene in obese patients with reduced energy expenditure, and to investigate if a common variant in this gene is associated with alterations in energy expenditure.

II) To identify mutations and variations in the coding region of the *hormone sensitive lipase* gene in diabetic patients with hypertriglyceridaemia, and to investigate if a microsatellite marker in the gene is associated with diabetes and/ or abdominal obesity.

III) To investigate if a common variant in the *lipoprotein lipase* gene, Asn291Ser, is associated with dyslipidaemia in prediabetic and diabetic subjects, and to see if the association between Asn291Ser and dyslipidaemia in normoglycaemic subjects is altered by the presence of insulin resistance or obesity.

IV) To study if sixteen previously published associations between genetic variations and type 2 diabetes or prediabetic phenotypes can be replicated using a multi-step family-based approach. To identify mutations and variations in the coding region of the *peroxisome proliferator-activated receptor gamma* gene in diabetic patients.

V) To identify mutations and variations in the coding region of the *phosphodiesterase 3B* gene in diabetic patients, and to investigate if a common variant in the gene is associated with diabetes and/ or prediabetic phenotypes.

6. Methodology

6.1 Study subjects

The study subjects were selected from Finland and Southern Sweden (Figure 9)[12]. Phenotypic characteristics of the study subjects are presented in Tables 1-5. All subjects have given informed consent, and the local ethic committees have approved of the study. Diagnosis of diabetes, IGT and IFG was based upon criteria established by the WHO or by a previous diagnosis of diabetes and treatment with oral agents and/or insulin. WHO criteria from 1985[7] were used in studies 1, 2 and 3 and WHO criteria from 1998[1] in studies 4 and 5. All subjects studied in this thesis are part of the Botnia study which started in 1990 in the Botnia region of western Finland and later extended to other parts of Finland and southern Sweden. To date, the Botnia study includes 1389 families with 9315 subjects, 3379 of whom have type 2 diabetes and 924 that are healthy control spouses. In particular the families from the Botnia region represent a genetically homogenous and young population, which is ideal for studies of complex genetic diseases.

***Figure 9.** The study subjects were selected from Sweden and Finland, including the Botnia region in western Finland.*

Table 1. Phenotypic charateristics of the subjects in study I.

	Metabolic syndrome subjects	*Control subjects*	*Mutation screened subjects*
n (men/women)	55 (28/27)	46 (21/25)	30 (15/15)
Age (years)	61±13	64±11	58±13
Number (%) of MSDR cases[¶]	52 (95)	5 (11)	25 (83)
Number (%) of diabetic cases[§]	42 (76)	-	21 (70)
Age at onset of diabetes	57±12	-	57±10
HbA$_{1c}$ (%)	6.6±1.7	4.9±0.4	6.3±1.5
Free fatty acids (µM)	858±273	713±214	738±264
HOMA-IR index*	22.9±17.7	7.2±6.6	16.3±10.0
BMI (kg/m^2)	32±5	23±3	31±4

Data are numbers or mean ± standard deviation (SD). MSDR is the metabolic syndrome according to the proposal by the World Health Organization (WHO) in 1998. [§]Type 2 diabetes mellitus according the WHO consultation in 1985. *HOMA-IR index is reported only for subjects not treated with insulin.

Table 2. Phenotypic charateristics of the subjects in study II.

	Type 2 diabetic subjects	*Control subjects*	*TDT offspring subjects*	*Mutation screened subjects*
n (men/women)	235 (115/120)	146 (71/75)	42 (23/19)	89 (53/36)
Age (years)	62±9	60±12	37±9	56±14
Number (%) of MSDR cases[¶]	127 (54)	10 (7)	10 (24)	80 (90)
Number (%) of diabetic cases[§]	235 (100)	-	-	62 (70)
Age at onset of diabetes	54±9	-	-	52±11
HbA$_{1c}$ (%)	7.6±1.7	5.3±0.5	5.5±0.6	7.4±2.1
Free fatty acids (µM)	972±379	711±218	717±259	1152±464
HOMA-IR index*	7.0±7.1	1.7±0.9	2.2±1.4	9.6±10.7
BMI (kg/m^2)	27±5	25±3	27±5	30±4

Data are numbers or mean ± standard deviation (SD). MSDR is the metabolic syndrome according to the proposal by the World Health Organization (WHO) in 1998. [§]Type 2 diabetes mellitus according the WHO consultation in 1985. *HOMA-IR index is reported only for subjects not treated with insulin.

Table 3. Phenotypic charateristics of the subjects in study III.

	Type 2 diabetic subjects	*Study III* *Relatives of diabetic subjects*	*Control subjects*
n (men/women)	192 (99/93)	278 (152/126)	226 (109/117)
Age (years)	63±12	55±13	56±10
Number (%) of MSDR cases¶	136 (71)	68 (24)	22 (10)
Number (%) of diabetic cases§	192 (100)	-	-
Age at onset of diabetes	56±12	-	-
HbA_{1c} (%)	7.3±1.5	5.5±0.5	5.6±0.4
Free fatty acids (µM)	975±333	723±211	666±189
HOMA-IR index	6.3±5.3	2.3±1.4	2.1±1.5
BMI (kg/m^2)	28±5	27±3	26±4

Data are numbers or mean ± standard deviation (SD). MSDR is the metabolic syndrome according to the proposal by the World Health Organization (WHO) in 1998. §Type 2 diabetes mellitus according the WHO consultation in 1998. *HOMA-IR index is reported only for subjects not treated with insulin.

6.2 Phenotypic characterisation

The subjects included in the Botnia study have been extensively phenotyped[12]. All subjects have undergone at least one health check to which they arrived fasting. The subjects were weighed and waist circumference, hip circumference and height were measured. Fat mass was assessed with the Futrex infrared and bioelectrical impedance methods (BIA 101, RJL systems, Detroit, MI). Blood pressure (sitting) was measured two to tree times with 5 minutes rest between each reading. Venous blood was sampled for extraction of DNA and for measurement of HbA_{1C} and lipids, and albumin excretion rate was measured from timed overnight urine. The subjects were asked about their medical treatment, family-history of diabetes and related disease. Age at onset of diabetes and diabetic complications were checked from medical records. Most subjects (except those that have very severe diabetes, fasting glycaemia >10 mmol/l) underwent an OGTT. The OGTT was performed by the ingestion of 75 g glucose dissolved in water and venous blood sampling at –10, 0, 30, 60 and 120 minutes to measure glucose, insulin and c-peptide concentrations. Insulin resistance was estimated with the homeostasis model assessment (HOMA-IR) index and calculated as fasting insulin times fasting plasma glucose divided by 22.5. Some subjects (*Study I*) underwent the so-called Botnia clamp, which is an intravenous glucose tolerance test (IVGTT) directly followed by an euglycaemic clamp. The IVGTT was performed with an intravenous bolus-injection of glucose (0.3 g 20% glucose /kg body weight) and venous blood sampling every 2 minutes for the first 10 minutes and then every 5 minutes for 50 minutes. Energy expenditure was measured before and during the IVGTT. The euglycaemic clamp was performed with an intravenous infusion of insulin and simultaneous infusion of glucose to maintain glucose levels at 5.5 mmol/l for 2 hours, and mean glucose infusion rate between 60-120 minutes was used to estimate insulin sensitivity (the M-value). During the clamp glucose levels were measured every 5 minutes and clamped at 5.5 mmol/l.

Table 4A. Phenotypic charateristics of TDT offspring and subjects screened for mutations in study IV.

	TDT offspring		*Mutation*
	T2DM/ IFG/ IGT[†]	*Non-diabetic*	*screened subjects*
n (men/women)	333 (176/157)	379 (187/192)	90[#] (42/48)
Age (years)	39±9	31±10	56±12
Number (%) of MSDR cases[¶]	149 (45)	34 (9)	77 (86)
Number (%) of diabetic cases[§]	126 (38)	-	90 (100)
Age at onset of diabetes	38±12	-	50±11
HbA_{1c} (%)	5.9±1.8	5.1±0.5	7.2±1.4
Free fatty acids (μM)	742±280	661±231	940±297
HOMA-IR index*	5.0±7.1	2.4±3.1	9.1±8.9
BMI (kg/m^2)	27±5	24±5	31±5

Data are numbers or mean ± standard deviation (SD). MSDR is the metabolic syndrome according to the proposal by the World Health Organization (WHO) in 1998. [§]Type 2 diabetes mellitus according the WHO consultation in 1998 and including subjects that have plasma glucose levels above 9.605 mmol/l 2 hours after an oral glucose tolerance test. *HOMA-IR index is reported only for subjects not treated with insulin. [†]The offspring have type 2 diabetes (T2DM), impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). [#] The 90 subjects are from 68 nuclear families.

6.3 Assays

Fasting blood glucose was measured with a glucose oxidase method using a Beckman Glucose Analyser II (Beckman Instruments, Fullerton, CA) (coefficient of variation (CV) <1%). Serum insulin (interassay CV 5%; Pharmacia, Uppsala, Sweden), C-peptide (interassay CV 9%) and leptin (interassay CV 4.5%) concentrations were measured with radioimmunoassay. Glycated haemoglobin (HbA1c) was analysed using high-pressure liquid chromatography (HPLC). Fasting plasma triglycerides (CV 2.2%), total cholesterol (CV 2.1%) and cholesterol in the precipitated HDL subfraction (CV 4.6%) were measured on a Cobas Mira analyser (Hoffman LaRoche, Basle, Switzerland). Apolipoprotein A-I (CV 3.5%) and A-II (CV 3.7%) were measured by turbidoimmunometric methods using commercially available kits (Boehringer Mannheim), and apolipoprotein B (CV 4.4%) concentrations were measured by an immunochemical assay (Orion Diagnostica, Espoo, Finland). Serum FFA was measured with an enzymatic assay and spectrophotometric detection (ACS-ACOD-kit, Wako Chemicals, Neuss, Germany) (CV 1.6%). Basal and isoprenaline-stimulated lipolytic activity in subcutaneous adipose tissue was assessed using glycerol corrected for cell number as a lipolytic index[117]. Genomic DNA was extracted from peripheral blood lymphocytes using standard methods[201]. Briefly, white blood cells were separated from blood by centrifugation in high sucrose. The cells were lysed with proteinase K and sodium dodecyl sulphate (SDS). Proteins were salt-precipitated and separated together with other cell debris by centrifugation. Genomic and mitochondrial DNA from the supernatant was precipitated with isopropanol, washed with ethanol and stored at –20°C in 0.5-3 µg/µl.

Table 4B. Phenotypic charateristics of the sibling pairs and case-control subjects in study IV.

	Sibling pairs[‡]		*Case-control study*	
	Type 2 diabetic siblings	*Non-diabetic sublings*	*Type 2 diabetic subjects*	*Control subjects*
n (men/women)	612 (283/329)	518 (216/302)	481 (252/229)	481 (252/229)
Age (years)	65±10	62±10	61±10	60±10
Number (%) of MSDR cases[¶]	435 (71)	68 (13)	314 (65)	72 (15)
Number (%) of diabetic cases[§]	612 (100)	-	481 (100)	-
Age at onset of diabetes	57±11	-	57±11	-
HbA_{1c} (%)	7.3±1.8	5.4±0.6	7.6±1.7	5.4±0.5
Free fatty acids (µM)	889±394	705±216	912±393	655±217
HOMA-IR index*	6.4±7.1	2.6±2.7	6.2±6.5	2.8±4.2
BMI (kg/m^2)	29±5	26±3	28±5	27±4

Data are numbers or mean ± standard deviation (SD). MSDR is the metabolic syndrome according to the proposal by the World Health Organization (WHO) in 1998. [§]Type 2 diabetes mellitus according the WHO consultation in 1998 and including subjects that have plasma glucose levels above 9.605 mmol/l 2 hours after an oral glucose tolerance test. *HOMA-IR index is reported only for subjects not treated with insulin. [‡]The siblings constitute 877 sibling pairs discordant for diabetes.

6.4 Genotyping

Genotyping was performed with DNA amplification using polymerase chain reaction (PCR). 5-10% of the samples from all cohorts were randomly tested for reproducibility, and genotyping was repeated from stock DNA. Allelic and genotypic frequencies were tested for Hardy-Weinberg equilibrium with χ^2-test: if the frequency of allele 1 is p and allele 2 is q then the number of heterozygotes should be 2pq, and of homozygotes p^2 (allele1) and q^2 (allele 2).

Table 5. Phenotypic charateristics of the subjects in study V.

		Study V	
	Siblings	*TDT offspring subjects*	*Mutation screened subjects*
n (men/women)	1408 (650/758)	108 (59/49)	40 (23/17)
Age (years)	56±17	36±8	56±15
Number (%) of MSDR cases[¶]	635 (45)	32 (29)	25 (63)
Number (%) of diabetic cases[§]	624 (44)	-	20 (50)
Age at onset of diabetes	57±15	-	52±12
HbA_{1c} (%)	6.3±1.6	5.1±0.5	6.2±1.0
Free fatty acids (μM)	813±299	702±275	875±607
HOMA-IR index	4.9±7.6	3.9±3.9	6.8±9.1
BMI (kg/m^2)	27±5	26±4	29±4

Data are numbers or mean ± standard deviation (SD). MSDR is the metabolic syndrome according to the proposal by the World Health Organization (WHO) in 1998. [§]Type 2 diabetes mellitus according the WHO consultation in 1998. *HOMA-IR index is reported only for subjects not treated with insulin.

6.4.1 PCR

The PCR reactions were performed with initial denaturation (96 °C for 3 min) followed by 30 or 35 cycles of denaturation (96 °C for 30 s), annealing (30 s) and extension (72 °C for 30 s), followed by final extension (72 °C for 10 min). Annealing temperature was generally set two degrees below the T_m (T_m= [2 x n(A+T)] + [4 x n(G+C)]) of the primer with the lowest melting temperature. The reactions were performed in a total volume of 20 μl in an ammonium sulphate buffer (16 mmol/l $(NH_4)_2SO_4$, 67 mmol/l Tris (pH 8.8), 0.01% Tween 20) or Tris-buffer for Taq polymerase from Perkin-Elmer, Foster City, CA or Pharmacia, Uppsala, Sweden (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.01% w/v gelatine). The reactions were performed with 0.13 mmol/l dNTP, 1.5 mmol/l $MgCl_2$, 0.2 μmol/l of both primers and 0.5 U Taq polymerase using 25 ng genomic DNA as template. Either 3% dimethylsulphoxide (DMSO), 1.5% formamide or 5% glycerol were used to increase PCR-sensitivity.

6.4.2 Genotyping by PCR-RFLP

The *UCP2 Ala55Val* polymorphism was genotyped by PCR-amplification with a mismatch primer followed by *HincII* digestion. The mismatch created a *HincII* site in sequences coding for a valine (GTC) and allowed electrophoretical separation of valine allele (216 and 25 bp) and alanine allele (241 bp) on agarose gels.

All HSL gene single nucleotide variants were amplified with PCR and cut with restriction enzymes (PCR-RFLP): The C2268T variant (exon 9) was cut with *BcnI* and the G41T variant (exon B) was cut with *DpnII*.

The *PDE3B G1389A* variant (exon 4) was PCR-amplified and the PCR product was cut with *HaeIII*, which cuts the G allele into two fragments of 165 and 88 bp (Figure 10). The *ABCC8* (*SUR1*) G3819A variant (exon 9; also referred to as exon 31 in the literature) was PCR-amplified with a mismatch primer and the PCR-products were digested with *NlaIV* digestion. The mismatch creates a *NlaIV* site in sequences coding for G and the G alleles were cut into two fragments of 125 and 25 bp.

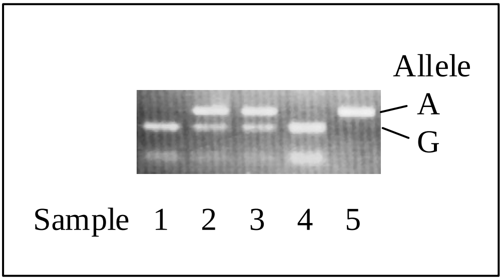


Figure 10. *Genotyping the G1389A variant of the PDE3B gene by PCR-RFLP. The figure illustrates genotyping of five samples (1-5). Lanes 1 and 4 represent individuals homozygous for the G allele, lanes 2 and 3 heterozygous individuals and lane 5 an individual that is homozygous for A.*

6.4.3 Genotyping by radioactive PCR

The *LIPE marker* is a $(CA)_n$ dinucleotide repeat, located in intron 7 of the *HSL* gene[202]. It was genotyped with radioactive PCR using γ–ATP end-labelled primer (Figure 11). PCR was performed in a total volume of 15 μl. The amplified products were separated on a denaturing polyacrylamide gel.

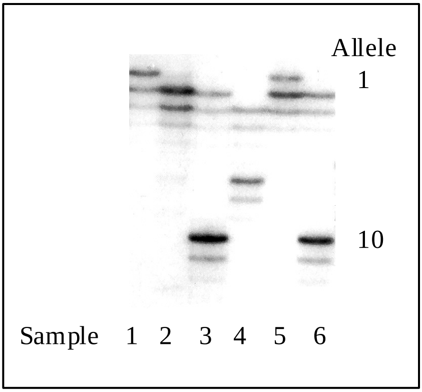


Figure 11. *The LIPE microsatellite marker was genotyped with radioactively labelled primer and the alleles separated by size on a polyacrylamide gel. The Figure shows the genotypes of six samples (1-6) with the following genotypes: 1/1, 2/2, 2/10, 3/7, 1/2, and 2/10.*

6.4.5 Genotyping by radioactive PCR-SBE

The *LPL Asn291Ser* variant was genotyped with an allele-specific mini-sequencing method (single-base extension, SBE), using a biotinylated downstream primer for PCR-amplification[203]. The biotinylated PCR-fragment was linked to a streptavidin-coated well, incubated with detection primer and [3H]-dGTP, and a liquid scintillation counter was used to detect allelic variants with an A at the second position of codon 291.

6.4.6 Genotyping by PCR-SBE with fluorescent detection

In *study IV*, genotyping was performed with SBE using fluorescence polarization (SBE-FP) or fluorescence resonance energy transfer (SBE-FRET)[204,205]. After PCR, shrimp alkaline phosphatase and exonuclease were added to degenerate excess dNTP and primers. Thereafter the variable nucleotide was PCR-amplified with a fluorescently labelled base. The genotypes were analyzed with an Analyst Fluorescence Plate-reader (Figure 12).

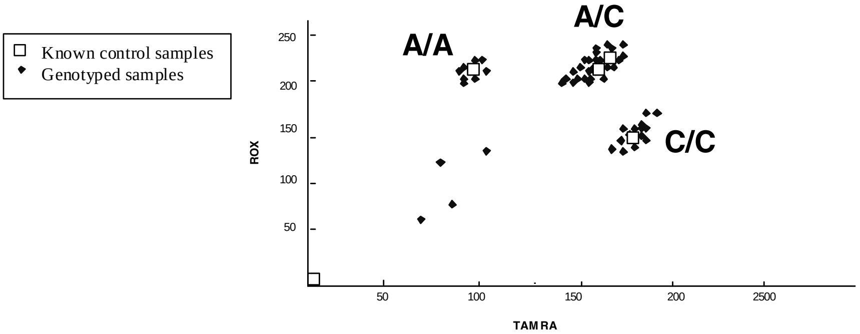


Figure 12. *Scatterplot of genotypes determined with the SBE-FP method. The samples were irradiated with plane-polarized light. The light emitted from the fluorescent markers is of different wavelengths and the spread of the emitted light(s) determines the genotype. The light is more scattered from the fluorescently labelled nucleotides that have not been incorporated onto the SBE-primer. The output signals were plotted in a scatterplot and genotypes that lie within a certain distance from known control samples were determined.*

For the SBE-FRET method the variable nucleotide was PCR-amplified with a fluorescently labelled base in an ABI 7700. The differences in fluorescence (read during the extension phase) between cycle 6 and cycle 1 were calculated for ROX and TAMRA after matrix correction for spectral overlap and these differences were used to assign genotypes (Figure 13). In both methods, genotypes were assigned by clustering the data from 96 to 960 individuals and assignments were reviewed by at least two individuals.

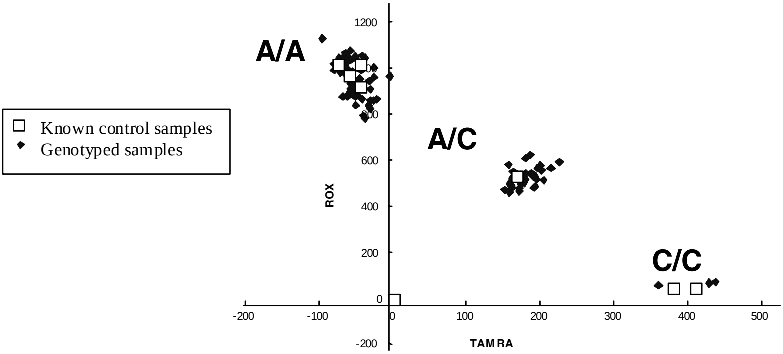
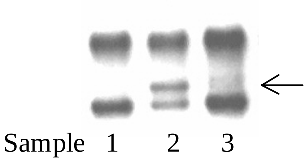


Figure 13. *Each PCR-cycle was monitored in a Taqman and after each cycle the fluorescent markers were irradiated with plane-polarized light. The light emitted from the fluorescent markers is of different wavelengths and the spread of the emitted light(s) determines the genotype.*

6.5 Mutation screening (SSCP) and sequencing

For the mutation screening we used the single-strand conformational polymorphism (SSCP) technique[206]. PCR was performed with 0.5 μCi α-^{32}P-dCTP using intronic primers for amplification of coding and non-coding exons (Figure 14). A minimum distance of 25 bp between the intronic primer and the exon border was used when possible to allow detection of variants that could alter splicing. PCR products were separated on non-denaturing polyacrylamide gels in two conditions. When differences in band pattern were observed, PCR-products were sequenced bidirectionally. Heterozygous variants were confirmed by PCR-RFLP. Using this SSCP procedure, the estimated degree of mutation detection is around 95% in our laboratory (data from studies on monogenic disorders).

Figure 14. *The mutation of the HSL exon B (nucleotide 41) as detected by SSCP. The picture shows the single-strand conformations of HSL exon B amplified by PCR with radioactively labelled nucleotides. Sample 2 has an alternative conformation (indicated by the arrow), which is due to a rare T allele at position 41.*



6.6 Determination of exon/intron boundaries of the *UCP2* gene

Using information about the exon/intron boundaries of the human *UCP1* gene, introns of the human *UCP2* gene were positioned while assuming conserved exon/intron structure between the genes. Exonic primers were constructed on both sides of each intron, and the introns were amplified by PCR and sequenced bidirectionally. Exon/intron borders were positioned by applying the GT/AG rule.

6.7 Determination of the region upstream of the first coding exon of the *UCP2* gene

Genomic DNA was cut at nucleotide 77 of the second coding exon of *UCP2* and at an unknown position upstream the coding region using *NcoI*. The fragmented genomic DNA was religated, generating circularised fragments of exon 1 extended with an unknown segment upstream of the gene. A 2.3 kb long circular fragment containing approximately 2 kb upstream of the first coding exon was generated. Two rounds of PCR were then performed using nested primers, and 295 bp of the region upstream of the first coding exon was determined by sequencing.

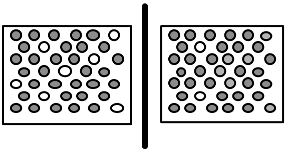
6.8 Statistical analyses

Statistical analyses were performed using the BMDP New System for Windows statistical package (Biomedical Data Processing, Los Angeles, CA). Descriptive clinical data were compared with the Student's t-test (mean ± standard deviation (SD)). Differences in clinical characteristics of different genotype carriers were tested by Student's t-test or Mann-Whitney non-parametric test (mean ± standard error of the mean (SEM) or median (interquartile range)). Bonferroni correction for multiple comparisons was used and reported when specified, although the corrected p-value can be considered too conservative when variables are not independent. Genetic interaction with physiological parameters was calculated by two-way analysis of variance (ANOVA). P-values of <0.05 were considered statistically significant.

6.8.1 Case-control studies

The case-control design is illustrated in Figure 15. The significance of differences in allele frequencies and allele frequency distributions were tested with Pearson χ^2-analysis, and Fisher's exact test (two-tailed) when expected numbers were too low (<5) to ensure the validity of the χ^2. In study 2 alleles with expected frequencies of less than 5.0 were pooled to assure the validity of the χ^2-test. Comparative risk was estimated with the odds ratio (OR) and 95% confidence intervals ($CI_{95\%}$) were calculated.

Figure 15. *A schematic representation of the case-control design. Unrelated subjects matched (pair- or group-wise) for age and ethnic origin but discordant for the disease are compared for allele frequencies.*



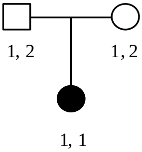


Figure 16. *A schematic representation of the TDT analysis. The parental affection status is not necessary for the analysis and parental transmissions are informative only from heterozygote parents. In this example, both parents transmitted the 1-allele. If 1-alleles are significantly over-transmitted to affected offspring in a large sample of parent-offspring trios this suggests that the 1-allele is associated with the disease.*

6.8.2 Transmission disequilibrium test (qualitative) (TDT)

The transmission disequilibrium test is illustrated in Figure 16. In *study II,* the extended TDT (ETDT) analysis was performed to follow transmission of multiple alleles. The ETDT-program (version 1.4), which logistically regresses counts of transmitted and non-transmitted alleles in an allele-wise or genotype-wise model[207] was used. Results are given as χ^2-values and non-significant comparisons between the results from the two models indicate that the material is well fitted for the analysis. In *study IV*, TDT was performed with χ^2 tests. In *study V*, TDT was performed using Genehunter 2.1[208] implementing the TDT2 function to analyse haplotype data.

6.8.3 Quantitative transmission disequilibrium test (QTDT)

The quantitative transmission disequilibrium test (QTDT) was analysed using the variance components model with age, gender and BMI as covariates. In *study IV* the TDTQ5[209] and in *study V* the Abecasis model[210] (www.well.ox.ac.uk/asthma/QTDT) were used.

6.8.4 Genotype-discordant sibling pair analysis

Analyses comparing siblings are illustrated in Figure 17. Phenotypic differences between genotype discordant sibling pairs were compared using a simulation-based permutation test. The observed sum of differences (OSD) is the sum of differences between sibling pairs (Σ(sibling1 - sibling2). The OSD is compared to the total distribution of 10^6 permutations of the data set to determine the level of significance.

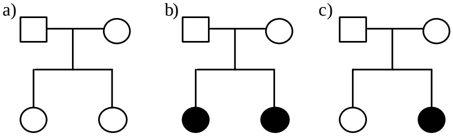


Figure 17. *Analyses comparing siblings. The sibling pairs can be matched for affection status, both either being non-affected (a) or affected (b). Siblings discordant for a specific genotype can be compared with regards to their phenotype. Siblings discordant for the phenotype (one affected and one non-affected) (c) can be compared for allele frequency (discordant alleles test, DAT).*

6.8.5 Discordant alleles test

The discordant alleles test (DAT)[211] is a pair-wise test comparing the frequency of alleles in phenotypically discordant siblings using a χ^2 test (Figure 17).

6.8.6 Population attributable risk

Population attributable risk (PAR) was calculated assuming a multiplicative model (PAR = (X-1)/X, where $X = (1-f)^2 + 2f(1-f)\gamma + f^2\gamma^2$). PAR is largely determined by the frequency of the risk allele (f), and the estimated genotype relative risk (GRR, γ).

7. Summary of studies I-V

7.1 *Study I*: No relationship between identified variants in the uncoupling protein 2 gene and energy expenditure

The aim of this study was to explore if genetic variability of the *UCP2* gene contributes to reduced energy expenditure in lean and obese subjects, and whether it increases the susceptibility to develop obesity. To accomplish this we included subjects with information on basal metabolic rate (BMR). To screen the gene for mutations we first identified the exon/intron boundaries by sequencing. The human *UCP2* gene was similar to the *UCP1* gene in its exon/ intron structure, with six exons covering 5 kb genomic sequence (Figure 18). The coding regions of the gene were screened for mutations in 30 obese subjects with low BMR, revealing a common polymorphism in exon 2, changing alanine to valine in codon 55 (Figure 18). The Ala55Val variant was not associated with BMR in 51 healthy controls from Finland or in 55 patients with the metabolic syndrome from Sweden. Neither was there any difference in allele or genotype frequency distribution between patients with the metabolic syndrome and 46 healthy controls from Sweden. The results suggest that mutations in the coding regions of the *UCP2* gene do not affect BMR and do not contribute to increased susceptibility to obesity or the metabolic syndrome.

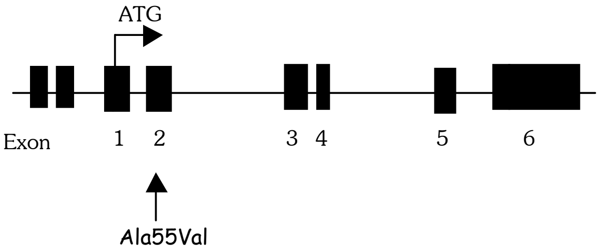


Figure 18. *The UCP2 gene consists of 6 coding exons and 2 non-coding exons. The Ala55Val variant is located in exon 2.*

7.2 *Study II*: The putative role of the hormone-sensitive lipase gene in the pathogenesis of Type II diabetes mellitus and abdominal obesity

The aim of this study was to investigate if genetic variability of the *HSL* gene contribute to abdominal obesity and type 2 diabetes. The subjects were selected based upon presence or absence of abdominal obesity and low HDL cholesterol levels for association studies and mutation screening of the *HSL* gene. We used the LIPE microsatellite marker in intron 7 of the gene (Figure 19) to investigate association between the gene and type 2 diabetes. The allele frequency distribution of the LIPE marker was different between 235 diabetic subjects and 146 healthy control subjects (p=0.002), suggesting that the LIPE marker was associated with type 2 diabetes. In a transmission disequilibrium test the transmission of LIPE marker alleles to 42 abdominally obese subjects with first degree family history of diabetes was distorted (p<0.05). There was no significant difference in allele frequency distribution of LIPE among non-diabetic subjects with high and low lipolytic rate in subcutaneous adipose tissue (p=0.07).

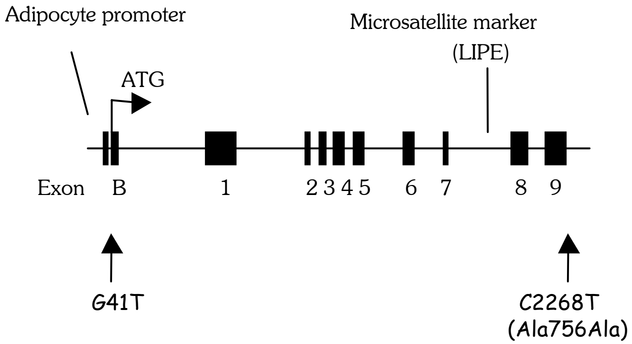


Figure 19. *The HSL gene consists of 9 coding exons and two upstream exons. The LIPE marker is located in intron 7. Two silent variants were identified, one in exon B and another one in exon 9.*

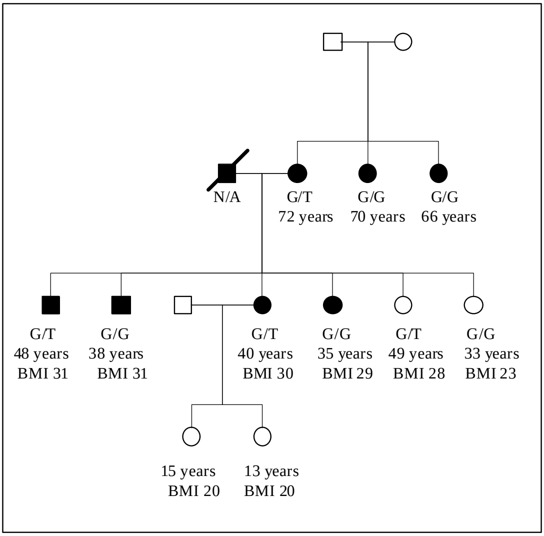


Figure 20. *A family with a silent mutation in HSL exon B. Open symbols represent normoglycaemic subjects and filled symbols type 2 diabetic cases.*

Mutation screening of abdominally obese subjects with the metabolic syndrome and subjects with high plasma triglycerides revealed two novel silent variants. In exon 9, a relatively common C→T change in codon 756 (alanine→alanine, nucleotide 2268) was detected. The variant did not co-segregate with diabetes or obesity, and allele and genotype frequencies did not differ between patients with the metabolic syndrome and healthy controls. In the non-coding exon B, a G→T change in nucleotide 41 was detected in one subject. As illustrated in Figure 20, the variant did not co-segregate with diabetes or obesity. No other carriers of the variant were detected among patients with the metabolic syndrome or healthy controls.

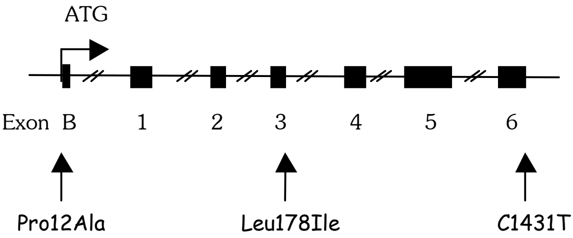
7.3 *Study III*: Interaction between the Asn291Ser variant of the *LPL* gene and insulin resistance on dyslipidaemia in high risk individuals for Type 2 diabetes mellitus

In this study we examined whether the Asn291Ser variant in the *LPL* gene was associated with dyslipidaemia in insulin resistant and type 2 diabetic subjects using a case-control design including 192 diabetic subjects, 278 non-diabetic subjects with first-degree family history of diabetes and 226 healthy control subjects with no known family history of diabetes. There was no difference in allele or genotype frequencies between diabetic subjects, first-degree relatives of diabetic subjects and controls. The serine allele was more common among normoglycaemic subjects with high plasma triglyceride levels than among subjects with normal triglyceride levels ($p<0.05$). Normoglycaemic serine carriers had three times greater risk of hypertriglyceridaemia than non-carriers ($p<0.001$). Of the normoglycaemic serine carriers, 46% had plasma triglyceride levels in the top quartile. There was a significant interaction between the Asn291Ser variant and insulin resistance, so that insulin resistant serine carriers developed more severe hypertriglyceridaemia than insulin sensitive carriers ($p=0.05$). In the diabetic subjects serine carriers were evenly distributed between all quartiles and they had no increased risk for hypertriglyceridaemia, even if they were insulin resistant.

7.4 *Study IV*: The common PPARγ Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes

The aim of this study was to investigate whether earlier reported associations could be replicated in Scandinavian subjects using a family-based approach. For this purpose we studied the role of 16 variants in 14 genes, that previously had been associated with type 2 diabetes, in Scandinavian parent-offspring trios. All offspring had type 2 diabetes, IGT or IFG. Variants that were associated with the trait in the trios were further tested in three independent replication sets: two samples of cases and controls from Scandinavia and French Canada and in Scandinavian sibling pairs discordant for diabetes. Variants that were associated with diabetes in these sets of patients were tested for association with quantitative variables in a set of parent-offspring trios with abdominally obese or lean normal glucose tolerant offspring. In addition, we tried to identify new variants in the coding region of the *PPARγ* gene in diabetic subjects belonging to families with some evidence of linkage to chromosome 3p25 in a previous genome-wide scan.

Figure 21. *The PPARγ gene consists of 6 coding exons and one upstream (PPARγ2 specific). A Leu178Ile missense mutation was identified in one diabetic subject.*



Three of the 16 tested gene variants were rare or absent and could not be tested in this material of subjects (Gly40Ser of the glucagon receptor gene, Val985Met of the insulin receptor gene and Ser20Gly of the islet amyloid polypeptide gene). Of the remaining thirteen variants, only two were associated with diabetes, Pro12Ala of the *PPARγ* and a silent C/T variant in exon 22 of the sulphonylurea receptor gene (*ABCC8*). Of the 12Pro alleles in the *PPARγ* gene, 104 were transmitted and 81 were not transmitted from heterozygous parents (p=0.045, one-sided). Of the T alleles of the *ABCC8* gene 26 were transmitted and 12 were not transmitted from heterozygous parents (p=0.012, one-sided). This indicates a significant over-transmission of these alleles to offspring with abnormal glucose tolerance. Only the association with the *PPARγ* variant was confirmed in the replication sets. The *PPARγ* 12Pro allele was also more frequent in diabetic subjects compared with their non-diabetic siblings (discordant alleles test (DAT); of the diabetic siblings 37 had alanine alleles and of the non-diabetic siblings 50 had alanine alleles, p=0.016, one-sided). The *PPARγ* 12Pro allele was more frequent in Scandinavian (85% vs. 83%, p=0.11, one-sided) and French Canadian (91% vs. 87%, p=0.10, one-sided) diabetic patients compared to control subjects. Although all replication studies were not statistically significant by themselves, the data was significant after pooling (p=0.012). In the replication data of the ABCC8 variant, the T allele was slightly less frequent in Scandinavian diabetic siblings compared to non-diabetic siblings (3.5% vs. 4.9%, p=0.06, one-sided). This was in contrast with the overtransmission of the T allele in the TDT data and the ABCC8 variant was not studied further. The quantitative transmission disequilibrium test (QTDT) did not show any quantitative correlations with insulin sensitivity (HOMA-IR index), BMI or percent body fat between parental and offspring generations and the transmission of 12Pro or 12Ala alleles. Meta-analysis of all published reports on this variant in relation to diabetes showed that the frequency of the 12Ala allele was consistently less frequent in diabetic than control subjects (p=0.00007), although the individual study groups were mostly too small to show a statistical difference. The risk associated with the 12Ala allele was 0.8 ($CI_{95\%}$=0.7-0.9) in the pooled data, indicating that the 12Ala allele protects against development of type 2 diabetes. Since the risk allele (12Pro) is common, the risk attributed to the 12Pro allele in the general population was estimated to be approximately 25% (genotype relative risk 1.25 and allele frequency about 85%). Mutation screening of 90 diabetic subjects from 68 families identified one novel variant in one subject, changing a leucine to an isoleucine at codon 178 (Figure 21), but the variant did not co-segregate with diabetes or obesity (Figure 22).

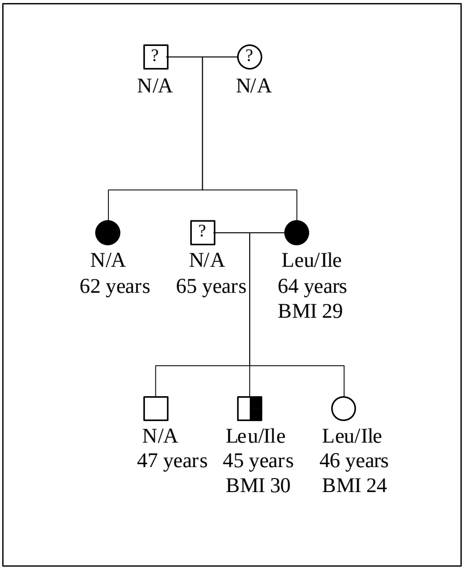
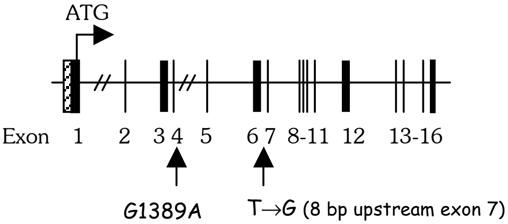


Figure 22. *A family with the Leu178Ile mutation in exon 3 of the PPARγ gene. Open symbols represent normoglycaemic subjects, filled symbols type 2 diabetic patients and two-coloured symbols subjects with impaired glucose tolerance.*

7.5 *Study V*: Association between a variant in the phosphodiesterase 3B gene and hyperinsulinemia in genotype-discordant sibling pairs

The aim of this study was to investigate if genetic alterations in the coding region of the *PDE3B* gene contribute to adiposity, insulin response or insulin sensitivity and increase the risk to develop type 2 diabetes and/ or impaired glucose tolerance. The coding region of the gene was screened for mutations in 40 subjects, including 20 subjects with type 2 diabetes and 10 sibling pairs discordant for the PDE3B G1389A variant. One novel variant was identified in intron 6 of one diabetic subject (Figure 23). A silent common polymorphism in exon 4, changing a nucleotide in position 1389 from guanine to adenine, was studied further using a genotype-discordant sibling pair design.

***Figure 23.** The PDE3B gene consists of 16 exons. An intronic variant was identified upstream of exon 7 in one subject.*



The G1389A variant was investigated in relation to quantitative variables using a genotype-discordant sibling pair design. Siblings matched with at least one other sibling for gender and diabetic status were genotyped. This identified 266 sibling pairs discordant for the variant. Siblings discordant for the variant had similar fasting glucose and lipid levels and similar body composition. Siblings homozygous for the A allele had lower insulin levels 2 hours after OGTT compared to siblings carrying one or two G-alleles ($p=0.0015$). Sibling pairs discordant for PDE3B G1389A but matched for a variant in the sulphonylurea receptor gene (ABCC8) that previously has been associated with hyperinsulinaemia (G3819A), differed in 2-hour insulin levels ($p=0.048$). The association appeared stronger in sibling pairs discordant for both PDE3B/G1389A and ABCC8/G3819A ($p=0.000035$). The transmission of G1389A to offspring with IGT or IFG was tested in 108 parent-offspring trios. Two-hour insulin levels did not differ between carriers of A and G alleles in a quantitative TDT analysis. However, haplotype TDT including several variants in the region (Figure 24) showed that 1389A haplotypes were over-transmitted ($p<0.05$) and 1389G haplotypes were under-transmitted ($p<0.05$) to offspring with IGT or IFG.

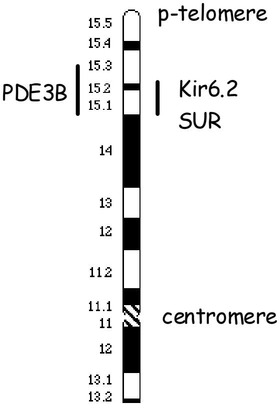


***Figure 24.** The short arm of chromosome 11. PDE3B, ABCC8 (the sulphonylurea receptor gene, SUR) and KCNJ11 (the inwardly rectified potassium channel, Kir6.2) are located in the same chromosomal band.*

8. General discussion

Few genetic factors that contribute to common forms of type 2 diabetes or the metabolic syndrome in humans have so far been identified. Some factors that may confer increased risk for the syndrome have been reported, but the interpretation of these different studies has been comprised by variable results in different ethnic populations. Small data sets, different selection criteria, different methods to assess phenotypic variables and ethnic differences may contribute to the discrepancies[212]. There are several approaches that can be used in the search for genes that contribute to complex diseases. In the data presented in this thesis, we have utilised the *candidate gene* search, investigating genes of potential importance for the regulation of physiological mechanisms that are altered in the disease. This requires knowledge about the underlying pathogenic mechanisms, which is not always the case. Other approaches can be used to explore the whole genome, transcriptome, or proteome of a cell or an individual.

The *genome-wide scan* approach can be used to identify chromosomal regions linked to disease. Evidence for linkage is implied when affected members in families with the disease share alleles at a chromosomal region. The genome-wide scan approach has been important for the identification of genes that cause diseases with a clear Mendelian pattern of inheritance, e.g. diastrophic dysplasia (*DTD*). Diastrophic dysplasia is an autosomal recessive disease that was linked to chromosome 5q in 1991[213]. The gene, which encodes a sulphate transporter, was cloned by linkage disequilibrium mapping in 1994[214]. The success of this approach in type 2 diabetes has been limited. Recently, a novel gene, *calpain 10*, was identified by linkage disequilibrium (LD) mapping of a region on chromosome 2q in Mexican Americans[146]. Surprisingly, linkage was explained by three intronic single nucleotide polymorphisms (SNPs). The reproducibility of genome wide scans has been poor which could reflect different contribution of genes in different populations. Allele frequency and interaction with other genetic and environmental factors will influence the power to detect linkage and may differ between populations. It is also likely that the genes that contribute to diabetes and obesity have only moderate influence on the phenotype, and therefore more sensitive methods than linkage analysis are needed for their identification. For this reason genome-wide association studies have been suggested. This type of studies have so far been hampered by the enormous amount of data that they would generate, as single nucleotide polymorphisms would be required at 2 cM distances over the entire genome. It is also uncertain what level of significance that would be required to distinguish statistically reliable data in such a setting.

Another approach is *gene expression profiling* in tissues of importance for the disease. An example of a protein that was identified in this fashion and which may contribute to type 2 diabetes is Rad (ras-related protein associated with diabetes). *Rad* was identified using a cDNA substraction technique as being highly overexpressed in skeletal muscle of type 2 diabetic subjects[215]. Today, microarray expression chips with probes for most mRNA expressed in human tissues have been developed. The interpretation clearly take longer than the generation of such data.

Heterogeneity of the disease is another challenge for genetic analysis. In the studies presented in this thesis we have tried to circumvent problems related to heterogeneity by studying relatives of subjects with type 2 diabetes, and/ or features related to the metabolic syndrome that precede the onset of diabetes. Type 2 diabetes and the metabolic syndrome are late-onset diseases and parents of affected individuals usually are not available. This complicates the selection of subjects for intra-familial studies like the TDT. Nuclear families are useful for intra-familial association studies, which tend to be less sensitive to ethnic admixture than case-control studies. To avoid phenocopies we have selected subjects who developed diabetes before the age of 60 to 65 years, and control subjects who were at least 40 years old.

A difficulty with polygenic diseases is that several genes may contribute to the phenotype, and that the effects may show variable penetrance. For example, only 1% of heterozygous and 10% of homozygous carriers of the ε2 allele of the *APOE* gene develop dyslipidaemia[216]. There are several statistical problems with genetic studies, most of which relate to multiple testing; either of a large number of genetic markers or multiple phenotypes. Several approaches have been suggested to deal with these problems. However, the most important approach is still replication in an independent data set. In addition, to conclusively show that a genetic variant increases risk for developing the disease, cross-sectional data should be confirmed in prospective studies.

Other problems could arise from the phenotypic assessments. Triglycerides and FFA are known to have a high intra-individual biological variability and fasting levels of these may not always reflect the post-prandial state of lipemia. In addition, it may be more relevant to study FFAs which are transported from abdominal fat to the liver via the portal vein (“splanchnic FFA”). Careful assessment of insulin secretion and insulin sensitivity would require time-consuming and invasive studies like the hyperglycaemic and euglycaemic clamps. Therefore most genetic and epidemiological studies use surrogate measures like fasting insulin levels and the HOMA indices.

9. Specific discussion

9.1 *Study I*: Uncoupling protein 2 (UCP2)

Concerning our results on the *UCP2* gene, other studies have confirmed that the Ala55Val variant does not associate with diabetes or obesity[217-221]. A Gly85Ser mutation was detected in one morbidly obese diabetic French subject, but it did not segregate with obesity or diabetes[218]. However, two non-coding exons upstream the first coding exon and a large 3'-untranslated region of the last coding exon (number 6 in our data but also referred to as exon 8 in the literature) and a common variant in the 3'- untranslated region (exon 8 insertion/ deletion) were identified[222]. The exon 8 insertion allele was associated with increased metabolic rate and low BMI in Pima Indian subjects[222] and with increased BMI in other studies[223,224]. Recently, a G-866A variant was identified in the promoter region of the *UCP2* gene[225]. This variant was in linkage disequilibrium with the 3' insertion/ deletion variant, and associated with obesity.

The *UCP2* gene is located on chromosome 11q13, very close to the *UCP3* gene which is expressed in skeletal muscle[226]. Several variants have been identified in the *UCP3* gene, including the common C-55T substitution in the putative promoter region[227] and a splice donor variant in exon 6[228,229]. The splice donor variant was associated with reduced fat oxidation and obesity, but the association seems restricted to subjects of African descent[228,229]. The TT genotype of the promoter variant has been associated with increased BMI[230], increased WHR[231], dyslipidaemia[232] and type 2 diabetes[232]. This variant is in tight linkage disequilibrium with the insertion/ deletion variant in exon 8 of the *UCP2* gene, such that the T-allele of *UCP3* C55T often is co-inherited with the deletion allele of the *UCP2* gene[231]. Therefore it is less likely that linkage disequilibrium between these variants would contribute to both findings.

We can only speculate about the physiological role of uncoupling proteins expressed in white adipose tissue and skeletal muscle. The expression of *UCP2* and *UCP3* genes seems to be tightly regulated, and increase during fasting and endurance training and decrease after re-feeding[150,233]. This suggests that the uncoupling proteins could be regulated by FFA, and play a role in fuel partitioning. This is in line with results on the splice variant of the *UCP3* gene that was associated with fat oxidation. Lately it has also been recognised that UCP2 may be important in the β-cell and play a role in insulin secretion. The *UCP2* gene is normally expressed at low levels in pancreatic β-cells and overexpression of the gene in β-cells was associated with impaired glucose-stimulated insulin secretion[234]. Correspondingly, the *UCP2* knockout mice have lower plasma glucose levels due to increased insulin secretion[235]. It has also been recognised that UCP2 and UCP3 may have a role in the regulation of the level of reactive oxygen species (ROS), since the level of these substances is increased in UCP2[235,236] and UCP3[237] knockout mice. This might explain why *UCP2* knockout mice are resistant to toxoplasmosis, and point at the possibility that the uncoupling proteins may be important for the prevention of complications associated with the metabolic syndrome.

9.2 *Study II*: Hormone-sensitive lipase (HSL)

In agreement with our results on the *HSL* gene, other studies have shown that this region on chromosome 19q13 might harbour genetic variability that increase the susceptibility to obesity and/ or type 2 diabetes. Studies including French and Pima Indian families with type 2 diabetes have displayed some evidence for linkage to this chromosomal region[238]. An association study using a microsatellite marker in intron 6 of the *HSL* gene indicated association to diabetes and obesity[239], very similar to the results presented in our study. To identify variants that contribute to these findings, we screened the coding region of the gene for mutations, detecting one common silent variant in the C-terminal region of the protein (C2268G). However, this variant was not associated with diabetes or the metabolic syndrome. Recently, the 5´ and 3´ untranslated and regulatory regions of the *HSL* gene were screened for mutations, identifying a C-60G substitution in the minimal promoter and a G3138A variant in the 3´-untranslated region[240]. Later, another relatively common variant in exon 9 encoding the C-terminal region was identified in Swedish subjects, substituting glycine for alanine at codon 772 (Valerie Large, personal communication). Homozygosity for the –60G allele was associated with increased BMI in healthy Finnish women[241] and reduced transcriptional activity *in vitro* in COS cells[240]. However, preliminary data from 214 Swedish subjects indicate that the variant is not associated with obesity, dyslipidaemia or disturbed lipolysis and that the C and G alleles are equally transmitted to abdominally obese offspring of diabetic subjects (Mia Klannemark, unpublished). Investigating allelic association between the identified variants, testing for transmission disequilibrium in the originally analysed trios with abdominally obese offspring as well as functional studies of the G-60C, C2268G, G3138A and Gly772Ala variants will be needed to clarify if these variants contribute to the original finding.

It is possible that genetic factors in other genes nearby the *HSL* gene contribute to the association between the *HSL* gene and diabetes/ abdominal obesity. Recent data in Caucasian populations have indicated that linkage disequilibrium can stretch over large chromosomal regions and that the degree of linkage disequilibrium can substantially vary between different regions of the genome. In *study V* we demonstrated a certain degree of linkage disequilibrium across the entire short arm of chromosome 11, a distance covering at least 15 Mb (between the KCNJ11 and the insulin genes). Several genes of interest are located on the long arm of chromosome 19. These include the *CEBP*α, the *APOE/CI/CIV/CII* gene complex and the *muscle glycogen synthase* (*GYS1*) genes. The *APOE/CI/CIV/CII* gene complex is located 3 Mb telomeric to the *HSL* gene (accession number NT_011240). Since these genes encode proteins involved in the regulation of lipid metabolism, variability in these genes could contribute to any of the phenotypes investigated in this study. The *GYS1* gene is located ~7 Mb telomeric to the *HSL* gene. The XbaI polymorphism of the *GYS1* gene has been associated with diabetes and features of the metabolic syndrome, particularly with hypertension[242,243]. Although the distance between the *HSL* and the *GYS1* gene is relatively large, it would be interesting to know if the *HSL* LIPE marker and the *GYS1* XbaI variant are in allelic association. The *CEBP*α gene is located 10 Mb centromeric to the *HSL* gene.

9.3 *Study III*: Lipoprotein lipase (LPL)

Our data on the *LPL* gene show that carriers of the 291Ser allele have a 3-fold increased risk to develop dyslipidaemia. Diabetic subjects commonly have reduced LPL activity[244], and the type of dyslipidaemia seen in subjects with type 2 diabetes is very similar to that associated with the 291Ser allele. However, we did not observe an association between the 291Ser allele and plasma triglyceride or HDL cholesterol levels in diabetic subjects, suggesting that the diabetic state itself has a strong influence on the lipid profile. In fact, up to 60% of diabetic subjects compared to less than 20% of normoglycaemic subjects are dyslipidaemic[18], implying that development of diabetes confers a 3-fold increased risk for dyslipidaemia. There are several possible explanations for this, including mechanisms dependent on LPL activity. LPL activity is commonly reduced in diabetic subjects [244,245]. Insulin upregulates LPL activity and lack of insulin could account for the reduced activity in type 1 diabetic subjects. As type 2 diabetic subjects are hyperinsulinaemic some other mechanism would be expected. LPL activity is inhibited by TNFα[246] and TNFα levels are highly correlated with insulin resistance[92]. Therefore, reduced LPL levels in adipose tissue of type 2 diabetic subjects could be partially explained by the inhibitory effect of TNFα, or by other factors associated with insulin resistance. However, such a mechanism would be expected to enhance rather than attenuate the difference between diabetic 291Ser carriers and non-carriers. Dyslipidaemia in diabetic subjects could be related to increased VLDL production in the liver. In fact, most studies seem to favour a high hepatic output of VLDL rather than reduced clearance, which is LPL dependent. Abdominal obesity and/ or dysregulation of intracellular lipolysis in the visceral fat would lead to increased hepatic influx of FFA, known to stimulate hepatic output of VLDL. The net flux of FFA will be outward if intracellular lipolysis (i.e. HSL activity) is higher than the LPL-mediated lipolysis in the plasma. If dyslipidaemia develops independently of LPL it is unlikely that small variations in LPL function will further influence the phenotype.

9.4 *Study IV*: Peroxisome proliferator-activated receptor gamma (PPARγ)

The Pro12Ala variant of the *PPAR*γ gene was identified by Yen *et al.*[247] and was originally reported to be associated with a reduced risk for diabetes and a more insulin-sensitive phenotype[248]. *In vitro* studies showed that the alanine allele had a reduced transcriptional transactivation activity, compared to the proline allele[248]. Most follow-up studies could not replicate the finding[249-252]. Instead, the Pro12Ala polymorphism was reported to be associated with obesity in some but not all populations [249,253-260]. In *study IV*, we used several approaches including family-based association studies to test the hypothesis that the Pro12Ala is associated with diabetes. We could show that the 12Pro allele was associated with an increased risk for diabetes. Importantly previous studies support the data although they seem to lack power to detect significant differences, and recent studies published on the Pro12Ala variant support a protective role for the 12Ala allele[261]. To dissect the clinical phenotype we performed a quantitative test of phenotypic variables using the QTDT method. In contrast to some association studies[248,262,263], this test did not show any correlations between genotype and insulin sensitivity (HOMA-IR index) or obesity. To our knowledge this is the first QTDT on phenotypes related to the Pro12Ala variant of the *PPARγ* gene. While the QTDT represents a specific test of a transmitted phenotype, it has not been established how sensitive it is. PPARγ has been detected in pancreatic β-cells, and carriers of the 12Ala allele have been shown to have reduced insulin secretory capacity after lipid challenge[264]. Reduced insulin secretory capacity might appear discrepant with a reduced risk for diabetes. However, it seems that once diabetes develops in 12Ala carriers, it is associated with a less favourable progress[264]. FFAs have been proposed as natural ligands for PPARγ. Dietary FFAs represent a mixture of fatty acids that vary in length and degree of saturation. It seems that the phenotype of Pro12Ala genotype carriers is dependent upon the fatty acid composition of the diet. In subjects with a high intake of saturated fat, the 12Ala allele was associated with increased BMI, whereas the opposite was seen when intake of unsaturated fat was low[265].

9.5 *Study V*: Phosphodiesterase 3B (PDE3B)

We provide novel evidence that genetic variability in the *PDE3B* gene may contribute to dysregulated insulin secretion. Several genes of relevance for insulin secretion and insulin sensitivity are located in this region of chromosome 11. These include the genes for the sulphonylurea receptor (ABCC8), the inwardly rectified potassium channel (KCNJ11) and the phosphoinositol 3-kinase subtype 2A. These genes are located within a 1 Mb segment close to the centromere on the short arm of chromosome 11. Data from haplotype TDT and from siblings discordant for both the *PDE3B* and ABCC8 variants suggested that there may be an interaction between variants in these two genes. Furthermore, preliminary data propose an interaction between PDE3B and the sulphonylurea receptor in the β-cell.

Many polygenic phenotypes could be associated with inherited haplotypes or haplotype combinations rather than with specific alleles. Sibling analysis has the advantage of comparing haplotypes since siblings share stretches of alleles. The use of permutations generates a distribution specific to the data set under analysis, yielding very high specificity and a small risk for false positive results.

10. Summary and conclusions

➟Variants in the coding region of the *UCP2* gene were not associated with BMR and did not contribute to increased susceptibility to obesity or the metabolic syndrome. It is thus unlikely that variation in the *UCP2* gene contributes to obesity or the metabolic syndrome.

➟ The LIPE marker in the *HSL* gene was associated with abdominal obesity and type 2 diabetes. This suggests that variation in the *HSL* gene may increase susceptibility to type 2 diabetes and the metabolic syndrome.

➟ The Asn291Ser variant of the *LPL* gene was associated with dyslipidaemia in normoglycaemic subjects, and the dyslipidaemic phenotype was more severe in insulin-resistant subjects. This association was not seen in diabetic subjects. The results suggest that the Asn291Ser variant of the *LPL* gene may predispose to dyslipidaemia in insulin resistant subjects.

➟ The Pro12 allele of the *PPAR*γ gene was associated with a modest but highly reproducible risk for type 2 diabetes. Due to the high frequency of the risk allele the variation in the *PPAR*γ gene may explain as much as 25% of the increased risk of type 2 diabetes.

➟ Variability in or near the *PDE3B* gene may contribute to elevated postprandial insulin levels. This suggests that genetic variability in this region of chromosome 11 may be important in the regulation of the insulin response to different stimuli.

Taken together, variability in genes regulating lipolysis and adipogenesis seem to predispose to type 2 diabetes and the metabolic syndrome.

11. Populärvetenskaplig sammanfattning

Typ 2 diabetes (åldersdiabetes eller sockersjuka) är en av de vanligaste folksjukdomarna, som ofta medför allvarliga komplikationer. Typ 2 diabetes utvecklas under en lång tid och uppträder ofta tillsammans med andra riskfaktorer för hjärt- och kärlsjukdomar, såsom fetma, förhöjt blodtryck och förhöjda blodfetter. Denna sjukdomsbild kallas det metabola syndromet. Individer som har det metabola syndromet har 2-3 gånger ökad risk att drabbas av hjärt- och kärlsjukdomar. Typ 2 diabetes och det metabola syndromet är komplexa polygena sjukdomar, d.v.s. de är sannolikt resultatet av en kollision mellan riskfaktorer som är ärvda och som finns i livsstilen.

Direkta orsaker till varför somliga individer utvecklar typ 2 diabetes är inte kända. Målet med de studier som presenteras i den här avhandlingen var att identifiera genetiska riskfaktorer som ökar risken att drabbas av typ 2 diabetes eller metabola syndromet. Vi studerade kandidat-gener som reglerar lipolys (nedbrytningen av fett), basal energiförbrukning och fettcells-utveckling, eftersom lipid- och fettcells-metabolism är av avgörande betydelse för upprätthållande av en normal glukosmetabolism. Vi identifierade mutationer och variationer i dessa kandidat-gener. Vidare studerade vi om någon specifik allel (en av flera alternativa former av en gen) eller haplotyp (en kombination av alleler på samma kromosom) av dessa gener var associerade med typ 2 diabetes eller det metabola syndromet.

Uncoupling protein 2 (UCP2) är en transport-kanal i mitokondrien som reglerar frånkopplingen av elektrontransport-kedjan från ATP-syntesen. Vi visade att en vanlig variation i genen (Ala55Val; ena allelen har en alanin och den andra en valin i kodon 55) inte är associerad med förändrad basalmetabolism och att allelerna var lika vanliga hos patienter med metabola syndromet som hos friska kontroller. Vi kunde inte identifiera några ovanliga mutationer eller mer vanliga variationer i genen hos patienter med metabola syndromet. Resultaten tyder på att variationer i genen inte predisponerar för metabola syndromet eller nedsatt basalmetabolism.

Hormon-känsligt lipas (HSL) är ett enzym som finns i fettväven och som reglerar rekryteringen av fett som energikälla under fasta. Vi identifierade en mutation och en relativt vanlig varation i genen hos patienter med bukfetma och förhöjda triglyderider, och visade att dessa inte var associerade med metabola syndromet. Genom att studera en genetisk markör i genen (LIPE mikrosatellit) kunde vi visa att genen var associerad med typ 2 diabetes och bukfetma hos släktingar till diabetiker. Resultaten tyder på att vissa oidentifierade varianter av genen eller närliggande gener kan predisponera för typ 2 diabetes och bukfetma.

Lipoproteinlipas (LPL) är ett enzym som sitter på insidan av blodkärlen och som spjälker det fett vi äter så att kroppens vävnader kan tillgodogöra sig det. Vi studerade en variation i genen (Asn291Ser; ena allelen har aminosyran aspargin och den andra serin i kodon 291) och kunde visa att individer vars vävnader ger ett sämre svar på insulin (insulin-resistenta) hade en högre risk att utveckla störningar i fett-metabolismen om de var bärare av serin-allelen. Däremot bidrog inte serin-allelen till ökad risk för störningar i fett-metabolismen hos typ 2 diabetiker. Resultaten tyder på att serin-allelen kan vara en speciellt viktig risk-faktor för störningar i fettmetabolismen hos insulin-resistenta individer som inte har diabetes.

Peroxisome proliferator-aktiverad receptor gamma (PPARγ) är en transkriptionsfaktor som reglerar uttrycket av gener som är viktiga för fettcells-utvecklingen. Vi studerade en vanlig variant i genen (Pro12Ala; ena allelen har prolin och den andra alanin i kodon 12) och visade att prolin-allelen var mer frekvent hos diabetiker och nedärvdes oftare till diabetiker, vilket tyder på att prolin-allelen ökar risken att insjukna i typ 2 diabetes. Meta-analys av alla publicerade data visade övertygande att prolin-allelen var förenad med 1.25 gånger ökad risk att drabbas av diabetes. Eftersom risk-allelen är vanlig har den stor betydelse för risken av typ 2 diabetes i befolkningen.

Fosfodiesteras 3B (PDE3B) finns i fettväven, levern och bukspottskörtelns insulin-producerande celler. Vi studerade en vanlig variant i exon 4 av genen, G1389A (ena allelen har en guanin och den andra en adenosin i nukleotid 1389). Homozygota bärare av A allelen hade lägre insulin-nivåer i plasma två timmar efter ett oralt glukos-tolerans-test, jämfört med syskon som har en eller inga A-alleler. Om man samtidigt har förändringar i andra gener på samma kromosom är risken ännu högre. Resultaten tyder på att haplotyper på den korta armen av kromosom 11 kan predisponera till störd insulinsekretion.

Sammantaget tyder detta på att variationer i gener som reglerar lipolys och fettcells-utvecklingen kan bidra till bukfetma, typ 2 diabetes och det metabola syndromet. Prospektiva studier kommer att behövas för att fastställa om individer som bär på dessa genvarianter utvecklar diabetes tidigare och oftare än de som inte har dem.

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No relationship between identified variants in the uncoupling protein 2 gene and energy expenditure

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Abstract

Objective: The uncoupling protein 2 (UCP2) uncouples respiration from the oxidative phosphorylation in most cell types, predominantly in white fat and skeletal muscle. Since a decreased basal metabolic rate (BMR) would increase the susceptibility to weight gain, genetic alterations in the *UCP2* gene could contribute to the pathogenesis of obesity and the metabolic syndrome (MSDR).

Design and methods: To test this hypothesis, we PCR amplified the introns of the *UCP2* gene and sequenced the exon/intron boundaries. This information was used to construct intronic primers and to screen obese patients with low BMR for mutations in the coding regions of the *UCP2* gene, using the single-strand conformational polymorphism technique. Furthermore, we examined whether there is an association between a biallelic marker in the *UCP2* gene and BMR or MSDR.

Results: The *UCP2* gene is composed of six coding exons, covering 5 kb of chromosome 11q13. One polymorphism, but no mutations, were identified in the coding regions of the *UCP2* gene. There were no significant differences in the allele or genotype frequencies of the Ala55Val polymorphism between 55 patients with MSDR and 46 healthy controls. No association was found between the *UCP2* gene and BMR in patients with MSDR or in healthy controls.

Conclusions: Mutation screening and association studies suggest that mutations in the coding regions of the *UCP2* gene do not affect BMR and do not contribute to increased susceptibility to obesity or MSDR. The results cannot, however, exclude the possibility that variants in regulatory elements of the gene could contribute to the development of obesity or MSDR.

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Introduction

The uncoupling proteins (UCP) represent a family of proteins that uncouple the respiration from the oxidative phosphorylation in the inner mitochondrial membrane (1). For a long time, UCP1 (2, 3) has been a marker for brown fat, a relatively abundant tissue in hibernating animals and newborns but less abundant in adults. The discovery of UCP2 and UCP3 isoforms in white fat and skeletal muscle (4–7) has relaunched the *UCP* genes as candidate genes for obesity, particularly as white fat and skeletal muscle can be expected to contribute more to variations in energy expenditure than the scarce brown fat (8–12).

UCP2 is expressed in most tissues, with the highest mRNA concentrations found in white fat, skeletal muscle and cells of the immune system (4). It is known to be upregulated by leptin (13, 14), high-fat feeding (4) and the peroxisome proliferator-activated receptor-gamma agonist troglitazone (15). Recent results suggest that intracellular metabolism of free fatty acids may regulate the expression of UCP2 in adipose tissue (16). The *UCP2* gene is located on chromosome 11q13 (4), a locus that has recently been linked to resting metabolic rate in the Quebec Family Study (17).

Abdominal obesity and dyslipidaemia with high very-low-density lipoprotein-triglyceride and low high-density lipoprotein (HDL)-cholesterol levels are strongly associated with non-insulin dependent diabetes mellitus (NIDDM) (18–25). This particular clinical picture is often referred to as the metabolic syndrome (MSDR; insulin resistance syndrome/syndrome X), which predisposes to the development of atherosclerosis and macrovascular disease (26) and is considered to have a strong genetic background. MSDR clusters in families and represents a genetic adaptation towards an energy-saving phenotype with survival advantage during periods of famine (27). Therefore, genetic alterations in genes influencing energy expenditure seem likely to be involved in the polygenic background of MSDR.

To study whether alterations in the *UCP2* gene contribute to the development of obesity or MSDR, we determined the exon/intron boundary sequences of the *UCP2* gene and screened the coding regions and the exon/intron junctions for mutations in obese subjects with low basal metabolic rate (BMR). Using an amino

Table 1 Clinical characteristics of subjects participating in the association study. Data are means ± S.D.

	MSDR	Controls
Number	55	46
Sex (F/M)	28/27	21/25
Age (years)	61 ± 13	64 ± 11
BMI (kg/m^2)	31.6 ± 4.6[‡]	23.3 ± 2.7
WHR; men	0.99 ± 0.06[§]	0.91 ± 0.05
WHR; women	0.91 ± 0.08[†]	0.79 ± 0.06
Fatmass (%); men	29.6 ± 8.9[§]	18.9 ± 5.2
Fatmass (%); women	37.1 ± 5.7[§]	29.1 ± 4.0
Triglycerides (mmol/l)	2.6 ± 1.8[†]	1.2 ± 0.5
Cholesterol (mmol/l)	5.9 ± 1.2	5.8 ± 1.0
HDL cholesterol (mmol/l); men	0.89 ± 0.2*	1.2 ± 0.3
HDL cholesterol (mmol/l); women	1.0 ± 0.3[§]	1.6 ± 0.5
Systolic blood pressure (mmHg)	148 ± 20*	137 ± 22
Diastolic blood pressure (mmHg)	82 ± 8.6*	78 ± 9.7
Fasting blood glucose (mmol/l)	7.5 ± 2.5[†]	4.9 ± 0.5
HbAlc (%)	6.6 ± 1.7[†]	4.9 ± 0.4
Fasting serum insulin (nmol/l)	13.0 ± 8.1[†]	8.1 ± 11
BMR/LBM (kcal/kg LBM)	27.4 ± 3.0	–

* $P < 0.05$, [§] $P < 0.0005$, [†] $P < 1 \times 10^{-5}$ and [‡] $P < 1 \times 10^{-14}$ vs control subjects.

acid polymorphism identified in the second coding exon of the *UCP2* gene, we examined whether this variant is associated with MSDR or decreased BMR.

Materials and methods

Subjects and study design

MSDR was defined in accordance with the recent proposition adopted by the World Health Organization (unpublished, 1997) suggesting that patients fulfilling at least three of the following criteria have MSDR: abdominal obesity, glucose intolerance, elevated blood pressure, elevated plasma triglyceride concentrations, decreased plasma HDL-cholesterol concentrations, microalbuminuria and insulin resistance. In this study, each patient defined as MSDR fulfilled at least three of the following criteria: waist-to-hip ratio (WHR) >0.95 for men and >0.85 for women, systolic/diastolic blood pressure >160/90 mmHg, plasma triglycerides >1.7 mmol/l, plasma HDL-cholesterol <1.0 mmol/l for men and <1.1 mmol/l for women, albumin excretion rate >20 μg/min and impaired glucose tolerance or NIDDM (28).

Mutation screening Thirty (16 males, 14 females) unrelated Swedish obese patients with low BMR were selected (age 58.2 ± 13 years, body mass index (BMI) 31.1 ± 3.6 kg/m^2, BMR 25.9 ± 1.8 kcal/kg lean body mass (LBM)). BMR was in the lowest quartile of a control population and BMI was >27 kg/m^2 for these patients. Twenty-five of them fulfilled the criteria for MSDR as given above.

Association study Fifty-five unrelated patients with MSDR (including the 25 from the mutation screening) and 46 unrelated healthy controls without signs of MSDR were selected from southern Sweden. Clinical characteristics of both study groups are shown in Table 1. Measurements of BMR were available from patients (range 21.1–39.6 kcal/kg LBM) but not from controls.

Assessment of the effect of the Ala55Val genotype on BMR Fifty-one (26 males, 25 females) unrelated healthy controls with normal glucose tolerance and without known family history of diabetes, were selected from the Botnia region in western Finland. The clinical characteristics of these healthy subjects were: age 45 ± 15 years, BMI 24.0 ± 2.6 kg/m^2, WHR 0.88 ± 0.06, fatmass 18.8 ± 6.5% and BMR 30.7 ± 4.9 kcal/kg LBM (range 24.4–47.5 kcal/kg LBM). Of note, the MSDR patients included in the association study (see above) have significantly lower BMR than these controls (27.4 kcal/kg LBM vs 30.7 kcal/kg LBM, $P < 0.0001$).

Determination of exon/intron boundaries with PCR and direct sequencing

Using information about the exon/intron boundaries of the human *UCP1* gene (2, 3), introns were positioned in the human *UCP2* gene while assuming conserved exon/intron structure between these genes. Exonic primers were constructed on both sides of each intron, and the introns were amplified by PCR (Table 2). The PCR reactions were performed with initial denaturation (96 °C for 1 min) followed by 30 cycles of denaturation (94 °C for 20 s), annealing (62 °C or 64 °C for 30 s; Table

Table 2 Sequences of exonic primers and PCR conditions for amplification of the introns of the *UCP2* gene. All the primer sequences are given in 5′ to 3′ direction. The number in parenthesis after each primer sequence indicates the exon from which it was derived.

Forward primer sequence	Reverse primer sequence	Intron amplified (product size, bp)	Annealing temperature (°C)
GTTCAAGGCCACAGATGTGCC (1)	ACCATGGTCAGAATGGTGCCC (2)	1 (357)	64
TGGTTGCCGGCCTGCAGCG (2)	TCCTCTCGGGCAATGGTCTTG (3)	2 (~1100)	64
CATGCCAGCATTGGGAGCCG (3)	AGGAGGGCATCCTTGATGAGG (4)	3 (359)	64
TGTTGCTCGTAATGCCATTGTC (4)	TCATGTATCTCGTCTTGACCAC (5)	4 (~1200)	62
AGACGAGATACATGAACTCTGC (5)	TCAGAAGGGAGCCTCTCGGG (6)	5 (584)	62

2) and extension (72 °C for 1 min), followed by final extension (72 °C for 15 min). The reactions were performed in a total volume of 20 μl with Tris buffer for Taq polymerase (10 mmol/l Tris–HCl, pH 8.3; 50 mmol/l KCl; 0.01% w/v gelatin) and 1.5% formamide; 0.13 mmol/l dNTP; 1.5 mmol/l $MgCl_2$; 0.2 μmol/l of both primers and 0.5 U Taq polymerase (Perkin Elmer, Foster City, CA, USA) using 25 ng genomic DNA as a template. PCR products were sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer) and analysed on an automated sequencer (ABI, model 373, Perkin Elmer). Introns 1, 3 and 5 were sequenced bidirectionally in their entirety, whereas only the exon/intron border sequences were sequenced for introns 2 and 4. Exon/intron borders were positioned by applying the GT/AG rule (29).

Determination of the region upstream of the first coding exon by inverse PCR and direct sequencing

Five micrograms genomic DNA isolated from a 50-year-old healthy Caucasian male were cut with 25 U *NcoI* (New England Biolabs, Herts, UK) for 4 h in a total volume of 30 μl using conditions recommended by the manufacturer. *NcoI* was then inactivated at 65 °C for 20 min, and 6 μl of the digest were ligated in a total volume of 230 μl with 50 U T4 DNA ligase (5 U/μl, Appligene, Gaithersburg, MD, USA) at 16 °C for 24 h. In this procedure, the genomic DNA was cut at nucleotide 77 in the second coding exon and at an unknown position upstream of the coding region. Thus, a 2.3 kb long circular fragment containing approximately 2 kb upstream of the first coding exon was generated. Two rounds of PCR were then performed using nested primers; PCR1 (annealing temperature 62 °C, 35 cycles) using the forward primer for fragment 2A (Table 3) and 5′-TGATGAGATCTGCGATGCAGG-3′ as the reverse primer and 2.0 μl ligation mixture as a template; PCR2 (annealing temperature 56 °C, 25 cycles) using 5′-TGGGAGTCTTGATGGTGTC-3′ as the forward and 5′-AACTTCACAGTGGCAGTAGG-3′ as the reverse primer and 0.5 μl amplification product from PCR1 as a template. PCR reactions were performed as for the exon/intron boundary PCR, but using NH_4^+ buffer (16 mmol/l $(NH_4)_2SO_4$; 67 mmol/l Tris, pH 8·8; 0.01% Tween 20) instead of the Tris–HCl and 5% glycerol instead of 1.5% formamide. When sequencing the PCR product from PCR2, the sequence of 295 bp of the region upstream of the first coding exon was acquired.

Single-strand conformational polymorphism (SSCP)

For the SSCP analysis (30), the six coding exons of the *UCP2* gene were amplified with intronic primers (Table 3). For exons 1 and 2, overlapping sets of two (exon 1) or three (exon 2) primer pairs were used. A minimum distance of 25 bp between the intronic primer and exon border was used for all fragments. PCR was performed as for the exon/intron boundary PCR, with the following changes: 0.5 μCi [α-^{32}P]dCTP was added to each reaction; initial denaturation was set at 3 min, cycle denaturation at 30 s, cycle extension at 30 s and final extension at 10 min. See Table 3 for variable annealing temperatures and buffers. The reactions were stopped with 95% formamide buffer (1:1), denatured, cooled and electrophoresed on glycerol-free (35 W for 3.5 h at 4 °C) and 5% glycerol (8 W for 12 h at room temperature), non-denaturing 5% polyacrylamide gels (acrylamide/bisacrylamide 49:1). When differences in band pattern were observed, PCR products were sequenced bidirectionally.

Genotyping the Ala55Val polymorphism in the UCP2 gene

One polymorphism was identified in codon 55 of the

Table 3 Primer sequences and variable conditions for PCR-SSCP analysis of the *UCP2* gene. All the primer sequences are given in 5′ to 3′ direction. The primers for fragments 1A and 1B amplify two overlapping fragments of exon 1. The primers for fragments 2A–2C amplify three overlapping fragments of exon 2. The reverse primer for amplification of exon 6 is located at the end of the coding sequence.

Forward primer sequence	Reverse primer sequence	Exon amplified (product size, bp)	Annealing temperature (°C)	Buffer*
TGAGGGACTTGGTTCTATTAGG	AACTTCACAGTGGCAGTAGG	1A (248)	58	TB
GTTCAAGGCCACAGATGTGCC	AGCTACAGGGATAAGCATGTTGC	1B (254)	64	TB
TACTGCTAAAGTCCGGTTACAG	TGGTTGCCGGCCTGCAGCG	2A (247)	62	NB
TGTCTTGGCCTTGCAGATCC	TTGGTGTAGAACTGTTTGCAG	2B (215)	58	NB
TCCTCTCGGGCAATGGTCTTG	AGGTGCTTAGGAACTATGTGG	2C (256)	60	NB
AGGTAGAAAATGAGTGCAAGC	GGGTGAGACCAGAGTATCG	3 (308)	58	TB
CCTCTGGAAAGGTGTGTACCA	CGCACCTGCTCCTGGCATG	4 (255)	62	NB
GAATGATGGGTGAAGATCTTGA	CATGCATAGCCAAGAGGCCTG	5 (277)	60	TB
GGAAGTGGGAGGTGGAGGTG	TCAGAAGGGAGCCTCTCGGG	6 (165)	64	TB

* TB, tris–HCl buffer for Taq polymerase; NB, ammonium sulphate buffer.

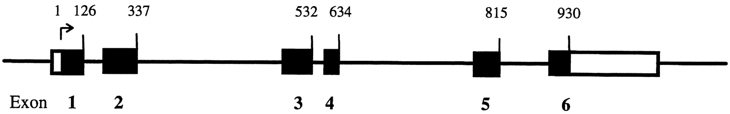

Figure 1 The exon/intron structure of the coding region of the human *UCP2* gene. Exons are numbered starting from the first coding exon and shown with boxes. Filled boxes represent translated sequences and open boxes untranslated sequences. Introns are illustrated with black lines. Vertical lines indicate the last nucleotide in each exon, with the nucleotide sequence numbered from the first nucleotide in the translation initiation codon. The translation initiation codon is indicated with an arrow. The first coding exon starts 99 bp upstream of the translation initiation codon, and exon 6 contains a 644 bp non-translated region (5).

UCP2 gene changing a GCC (alanine) to a GTC (valine). This polymorphism was genotyped by PCR amplification of the genomic DNA using the forward primer for fragment 2A together with a mismatch primer as the reverse primer (5′-CATCACACCGCGGTACTGGGCGTTG-3′, mismatch underlined) that created a *HincII* site in sequences coding for a valine, followed by digestion with the *HincII* enzyme using conditions recommended by the manufacturer (Appligene), and finally electrophoretic separation on an agarose gel. PCR was performed as for the exon/intron boundary PCR, with the annealing temperature set at 62 °C and the initial denaturation set at 3 min, cycle denaturation at 30 s, cycle extension at 30 s and final extension at 10 min.

Statistical analyses

Differences in allele and genotype frequencies between MSDR patients and control subjects were tested by χ^2 analysis, and differences in clinical characteristics by the Mann–Whitney non-parametric test using the BMDP New System for Windows (Biomedical Data Processing, Los Angeles, CA, USA). A P value of <0.05 was considered statistically significant.

GenBank accession numbers

The nucleotide sequences of the *UCP2* gene were submitted to the EMBL Nucleotide Sequence Database with accession numbers AJ223477–AJ223479.

Coding exon no.	5′ splice donor	Intron size (bp)	3′ splice acceptor	Coding exon no. (exon size/ bp)
			...cttggcttagATTCCGGCAG	1 (225)
1	CGG TTA CAGgtgaggggat.......	156	.......ggccttgcagATC CAA GGA	2 (211)
	R L Q^{42}		I^{43} Q G	
2	GGC TCT GAG Cgtgagtatgg....	~1000	....gcccacagAT GCC AGC ATT	3 (195)
	G S E^{112}		H^{113} A S I	
3	CTC TGG AAA Ggtgtgtacca.....	80	.....cctcctacagGG ACC TCT CCC	4 (102)
	L W K^{177}		G^{178} T S P	
4	CTC ATG ACA Ggtgagtcatg...	~1000	...tccttggcagAT GAC CTC CCT	5 (181)
	L M T^{211}		D^{212} D L P	
5	TTC TAC AAA GGgtgagcctct.....	369	....tctcctctagG TTC ATG CCC	6 (759)
	F Y K^{271}		G^{272} F M P	

Figure 2 Sequences at the exon/intron junctions of the coding region of the human *UCP2* gene. Exon sequences are given in uppercase letters, with encoded amino acids shown below the nucleotide sequence. Intron sequences are given in lowercase letters. Amino acids that precede, follow or are interrupted by introns are indicated with their codon number. Intron sizes were determined by sequencing (introns 1, 3 and 5) or by size estimation of the PCR product on an agarose gel (introns 2 and 4). When comparing the coding region of the *UCP2* gene with that of the human *UCP1* gene (3), the exon/intron structure was conserved except that exon 2 of the *UCP1* gene is four amino acids shorter and exon 3 is two amino acids longer than corresponding exons of the *UCP2* gene. Thereby, exon 2 of the human *UCP1* gene is interrupted by intron 2 at Thr^{109} instead of at His^{113}. The exon/intron structure of the coding region of the *UCP2* gene is conserved also with the human *UCP3* gene (31), except that exon 3 of the *UCP3* gene contains three additional amino acids and thus is interrupted by intron 3 at Gly^{181} instead of at Gly^{178}.

Table 4 Basal metabolic rate (kcal/kg LBM) in MSDR patients and healthy controls according to the Ala55Val genotype of the *UCP2* gene.

Genotype	MSDR (Sweden $n = 55$)	Controls (Finland $n = 51$)
Ala/Ala	27.6 ± 3.0	29.0 ± 3.1
Ala/Val	28.2 ± 2.6	30.7 ± 5.2
Val/Val	26.8 ± 2.2	30.6 ± 2.7

Results

The *UCP2* gene was found to be composed of six coding exons covering 5 kb of chromosome 11q13 (Fig. 1). All the exon/intron boundaries and exon sizes (coding exons) were similar to the structures of the human *UCP1* (3) and *UCP3* (31) genes (Fig. 2).

Mutation screening of the *UCP2* gene in 30 obese patients with low BMR revealed one polymorphism (Ala55Val) in codon 55 of the *UCP2* gene, changing an alanine (GCC) to a valine (GTC).

In healthy Finnish controls, the frequency of the alanine allele was 60.8% and that of the valine allele 39.2%. There were no significant differences in BMR between healthy control subjects or MSDR subjects with different Ala55Val genotypes (Table 4). The allele frequencies and genotype distributions of the Ala55Val polymorphism did not differ significantly between 55 subjects with MSDR and 46 healthy controls (Table 5). Genotype frequencies were in Hardy–Weinberg equilibrium in all groups studied.

Discussion

The exon/intron structure of the coding region of the human *UCP2* gene was similar to the structures of the human *UCP1* and *UCP3* genes. Information on the exon/intron boundaries of the six coding exons of the *UCP2* gene allowed us to screen coding exons and flanking intronic regions for mutations in obese patients with low BMR.

Table 5 Allele and genotype frequencies of the Ala55Val polymorphism in Swedish MSDR patients and healthy controls.

Allele or genotype	MSDR % (*n*)	Controls % (*n*)
Allele		
Ala	50.0 (55)	53.3 (48)
Val	50.0 (55)	46.7 (42)
Total	100 (110)	100 (90)
Genotype		
Ala/Ala	29.0 (16)	26.1 (12)
Ala/Val	41.8 (23)	52.2 (25)
Val/Val	29.0 (16)	19.6 (9)
Total	100 (55)	100 (46)

Neither allele nor genotype frequencies differ between MSDR patients and controls.

Basal energy expenditure accounts for 60–70% of total energy expenditure (9), the rest being generated during exercise and food-induced thermogenesis. The variability of BMR is largely dependent on processes in non-adipose tissues (60–80%) (9). Nevertheless, white adipose tissue could contribute to the small decrease needed to gain weight over time, and low BMR is a risk factor for weight gain (32).

Mice deficient in brown adipose tissue become obese and hyperinsulinemic (33). Surprisingly, targeted disruption of the *UCP1* gene in mice does not result in obesity, a finding which led to the postulation of other *UCP* genes involved in this mechanism (34). No mutations that increase the susceptibility to obesity have been found in the coding region of the *UCP1* gene in obese humans (35). However, one polymorphism near the *UCP1* gene, at nucleotide position −3826, has been associated with increased weight gain during adult life (36), and the effect of this variant was enhanced when it occurred together with the Trp64Arg mutation in the β3-adrenergic receptor gene (37).

In the present study, mutation screening of 30 obese patients with low BMR revealed one common variant in exon 2, Ala55Val, but there was no difference in allele frequency between patients with MSDR and healthy controls. This is in accordance with a recent Danish study, in which no association was found between the same polymorphism and juvenile obesity (38). However, this study did not include measures of energy expenditure, a quantitative trait which may be more relevant to the genotype.

In accordance with these findings, highly polymorphic markers flanking the *UCP2* and *UCP3* genes (4, 33) were not linked to BMI or WHR in a study of sibling pairs (39). In contrast to this, the highly polymorphic marker D11S611 was reported to be tightly linked to resting metabolic rate in the Quebec Family Study (17). Consequently, it seems that this chromosomal region links to resting metabolic rate but not to obesity. However, in the present study the Ala55Val polymorphism was associated neither with decreased BMR (in patients with MSDR or in healthy controls) nor with obesity in patients with MSDR. There are several potential explanations for the discrepancy of these findings. First, the Ala55Val variant might not be in linkage disequilibrium with the variants responsible for the linkage found in the Quebec Family Study. Thus, the *UCP2* gene may still be important for the variability of metabolic rate, although in such cases this does not seem to influence susceptibility to obesity. Secondly, regions important for gene regulation were not included in our screening. Thus alleles responsible for the linkage found in the Quebec Family Study could be present in the promoter region or other regulatory parts of the *UCP2* gene or in the very closely located *UCP3* gene. On the other hand, if the linkage in the Quebec Family

Study was explained by a variant in the promoter of the *UCP2* gene, one would expect to find decreased expression of UCP2 in obese individuals or in individuals with reduced BMR. However, in a recent study of UCP2 mRNA levels in adipose tissue and skeletal muscle, no difference was observed between lean and obese subjects and the level of expression did not correlate with BMR (16).

In conclusion, mutation screening and association studies suggest that mutations in the coding regions of the *UCP2* gene do not affect BMR and do not contribute to increased susceptibility to obesity or MSDR. The results cannot, however, exclude the possibility that variants in regulatory elements of the gene could contribute to the development of obesity or MSDR.

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The putative role of the hormone-sensitive lipase gene in the pathogenesis of Type II diabetes mellitus and abdominal obesity

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Summary Impaired lipolysis has been proposed as a pathogenic factor contributing to clustering of abdominal obesity and dyslipidaemia in Type II (non-insulin-dependent) diabetes mellitus – that is, the metabolic syndrome (MSDR). As this syndrome clusters in families, alterations in the hormone-sensitive lipase (HSL) gene could contribute to the genetic predisposition to MSDR. To test this hypothesis we carried out population and intrafamily association studies in individuals with MSDR, using a polymorphic marker (LIPE) in the HSL gene. There was a significant difference in allele frequency distribution between 235 Type II diabetic patients and 146 control subjects ($p = 0.002$), particularly between 78 abdominally obese Type II diabetic patients with MSDR and the control group ($p = 0.010$). An extended transmission disequilibrium test (TDT) showed transmission disequilibrium of 66 alleles to 42 nondiabetic, abdominally obese offspring in families with Type II diabetes ($p < 0.05$). A slight difference in allele frequency distribution was seen between 71 individuals from the lowest and 71 from the highest tertile of isoprenaline-induced lipolysis in fat tissue ($p = 0.07$). No missense mutations were found with single-strand conformational polymorphism (SSCP) in 20 abdominally obese subjects with MSDR. In conclusion, our population and intrafamily association studies suggest that the LIPE marker in the HSL gene is in linkage disequilibrium with an allele and/or gene which increases susceptibility to abdominal obesity and thereby possibly to Type II diabetes. [Diabetologia (1998) 41: 1516–1522]

Keywords Hormone-sensitive lipase, metabolic syndrome, insulin resistance syndrome, syndrome X, LIPE, dyslipidaemia, Type II (non-insulin-dependent) diabetes mellitus, abdominal obesity.

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Abbreviations: cAMP, cyclic adenosine monophosphate; NEFA, non-esterified fatty acids; HOMA, homeostasis model assessment; HSL, hormone-sensitive lipase; IGT, impaired glucose tolerance; MSDR, metabolic syndrome; NGT, normal glucose tolerance; DMSO, dimethylsulphoxide; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; Tg, plasma triglycerides; WHR, waist-to-hip ratio; WHO, World Health Organisation; TDT, transmission disequilibrium test; D. f., degrees of freedom; RFLP, restriction fragment length polymorphism.

Abdominal obesity, hypertension and dyslipidaemia are strongly associated with Type II (non-insulin-dependent) diabetes mellitus [1, 2], and this cluster is often referred to as the metabolic syndrome (MSDR), also called insulin resistance syndrome or Syndrome X. According to a preliminary definition by the World Health Organisation (WHO) [3], diabetic patients, patients with impaired glucose tolerance (IGT) and/or insulin resistance have MSDR if they present at least two of the following features: abdominal obesity, increased plasma triglyceride concentrations, reduced HDL cholesterol concentrations, hypertension and/or microalbuminuria. MSDR is associated with an increased risk of atherosclerosis and macrovascular disease [4, 5] and is considered to have a strong genetic background [6, 7]. In support

Table 1. Clinical characteristics of the subjects analysed in the association study of the HSL gene and characteristics of MSDR

	NGT		Type II diabetic	
	control subjects	MSDR	nonobese	MSDR
n	146	37	157	78
Sex (males/females)	71/75	26/11	81/76	34/44
Age at visit (years)	58.5 ± 11.9	57.7 ± 12.1	62.6 ± 9.4^{a}	$62.2 \pm 8.5^{a,c}$
Age at onset of diabetes (years)	–	–	52.4 ± 9.3	54.5 ± 7.2
BMI (kg/m^2)	24.5 ± 3.3	29.4 ± 3.2^{b}	24.9 ± 3.0	$31.1 \pm 5.0^{b,f}$
WHR; men	0.93 ± 0.05	1.03 ± 0.04^{b}	0.92 ± 0.04	$1.04 \pm 0.04^{b,f}$
WHR; women	0.80 ± 0.05	0.93 ± 0.03^{a}	0.83 ± 0.04^{a}	$0.95 \pm 0.04^{b,c,f}$
Triglycerides (mmol/l)	1.2 ± 0.4	2.1 ± 1.1^{a}	1.3 ± 0.5	$3.0 \pm 0.9^{b,c,f}$
Cholesterol (mmol/l)	6.0 ± 1.2	5.7 ± 1.3	5.9 ± 1.1	6.2 ± 1.2^{e}
HDL cholesterol (mmol/l); men	1.38 ± 0.26	0.84 ± 0.14^{b}	1.37 ± 0.22	$0.88 \pm 0.12^{b,f}$
HDL cholesterol (mmol/l); women	1.71 ± 0.35	0.93 ± 0.14^{a}	1.59 ± 0.33^{a}	$0.92 \pm 0.12^{b,f}$
NEFA (μM)	850 ± 246	–	911 ± 384	1026 ± 427
Systolic blood pressure (mm Hg)	134 ± 18	131 ± 16	144 ± 19^{a}	$147 \pm 20^{a,c}$
Diastolic blood pressure (mm Hg)	79 ± 9	78 ± 12	79 ± 9	$84 \pm 10^{a,c,e}$
Fasting blood glucose (mmol/l)	4.9 ± 0.5	5.3 ± 0.7^{a}	8.8 ± 3.3^{b}	$9.4 \pm 2.8^{b,d}$
HbA$_{1c}$ (%)	5.3 ± 0.5	5.3 ± 0.5	7.6 ± 1.8^{b}	$7.7 \pm 1.6^{b,d}$
Fasting serum insulin (nmol/l)	6.8 ± 3.3	12.6 ± 9.5^{a}	12.9 ± 16.8^{a}	$19.3 \pm 13.9^{b,c,e}$
HOMA-index for insulin resistance	1.7 ± 0.9	3.6 ± 2.1^{a}	5.5 ± 6.7^{b}	$9.0 \pm 9.0^{b,c,e}$
Fasting C-peptide (nmol/l)	0.34 ± 0.16	0.59 ± 0.32^{a}	0.40 ± 0.26	$0.70 \pm 0.33^{b,e}$

Data are mean ± SD. NEFA was measured for 22 of the healthy control subjects, 64 of the nonobese Type II diabetic patients and 28 of the MSDR/Type II diabetic patients. No data on NEFA was available for MSDR/NGT patients.

^{a} $p < 0.05$, ^{b} $p < 1 \times 10^{-9}$ vs control subjects; ^{c} $p < 0.05$, ^{d} $p < 1 \times 10^{-9}$ vs MSDR/NGT and ^{e} $p < 0.05$, ^{f} $p < 1 \times 10^{-9}$ vs nonobese Type II diabetic patients

of this, MSDR clusters in families and healthy first-degree relatives of Type II diabetic patients have increased frequency of abdominal obesity, insulin resistance and dyslipidaemia [8]. In addition, many of them have a decreased metabolic rate [8]. Abdominal obesity in association with the characteristic dyslipidaemia and low basal metabolic rate points at disturbances in abdominal fat lipolysis.

Hormone-sensitive lipase (HSL) is the rate-limiting enzyme in the breakdown of triglycerides in adipose tissues. The enzyme is activated by catecholamines through cAMP-dependent phosphorylation, whereas insulin prevents this phosphorylation through increased hydrolysis of cAMP [9–11]. Lipolysis and lipid metabolism are disturbed in patients with Type II diabetes [12], MSDR [13, 14] and in subjects of normal weight with a family history of diabetes [15]. Therefore, genetic variants of the HSL gene could be related to abnormal lipid metabolism, abdominal obesity or Type II diabetes.

The HSL gene is located on chromosome 19q13.1–13.2 [16]. The adipocyte isoform of HSL is encoded by 9 exons [17]. Adipocyte HSL is composed of two major domains of which the N-terminal domain is largely encoded by exons 1–4 and the C-terminal catalytic domain by exons 5–9 [18].

To study whether alterations in the HSL gene contribute to the development of MSDR, we carried out association studies and an extended transmission disequilibrium test (ETDT) using a polymorphism in the HSL gene and screened the coding regions of the gene for mutations in diabetic and nondiabetic abdominally obese subjects with MSDR. As an additional functional marker for HSL catalytic capacity we used the in vitro maximum lipolytic rate of subcutaneous adipose cells [13, 14].

Subjects and methods

The study consisted of four parts. In the three first studies we analysed association and transmission disequilibrium between a polymorphism in the HSL gene (LIPE) and MSDR or a low lipolytic rate. In the fourth study we carried out mutation screening of the HSL gene in abdominally obese subjects with MSDR.

Subjects and study design. MSDR was defined by waist-to-hip ratio (WHR) > 1.0 (men) or > 0.9 (women) and HDL < 1.0 mmol/l (men) or < 1.1 mmol/l (women).

1) In the association study with MSDR as the phenotype, 78 Type II diabetic patients with abdominal obesity and low HDL cholesterol concentrations (MSDR/Type II diabetes), 157 nonobese Type II diabetic patients with normal HDL (nonobese Type II diabetes), and 37 abdominally obese subjects with normal glucose tolerance (NGT) and low HDL cholesterol concentrations (MSDR/NGT) were compared with 146 unrelated nonobese, healthy control spouses with NGT and normal HDL cholesterol concentrations and without family history of diabetes. Clinical characteristics of the three study groups are shown in Table 1. There was a family history of Type II diabetes in 27 of the 37 MSDR/NGT subjects. All subjects were unrelated and selected from the Botnia region in western Finland or from southern Sweden. All groups were matched for ethnicity and statistical tests were done to assure that allele frequencies did not differ between the different subgroups from the two regions.

Table 2. Clinical characteristics of the subjects analysed in the association study of the HSL gene and lipolytic activity in subcutaneous adipose tissue

	High lipolytic activity	Low lipolytic activity
n	71	71
Sex (males/females)	27/44	23/48
Age at visit (years)	38.9 ± 11.5	41.1 ± 13.0
BMI (kg/m^2)	31.5 ± 10.5	33.7 ± 8.6[a]
WHR	0.93 ± 0.08	0.95 ± 0.07[a]
Triglycerides (mmol/l)	1.8 ± 2.7	2.0 ± 1.1[a]
HDL cholesterol (mmol/l)	1.34 ± 0.37	1.20 ± 0.36[a]
Fasting blood glucose (mmol/l)	5.4 ± 1.2	5.5 ± 1.2
Fasting plasma insulin (mU/l)	13.8 ± 12.4	13.8 ± 8.2
Cell volume (pl)	634 ± 252	689 ± 183
Basal lipolysis (μmol/10^7 cells/2h)	1.79 ± 0.92	0.96 ± 0.52[b]
Isoprenaline-stimulated lipolysis (μmol/10^7 cells/2h)	8.67 ± 4.58	3.36 ± 1.06[c]

Data are mean ± SD. BMI in these groups ranged from 19.7–60.2 kg/m^2 (median 26.1 kg/m^2) in the group with high lipolytic rate, and from 20.5–52.6 kg/m^2 (median 31.8 kg/m^2) in the group with low lipolytic rate.

[a] $p < 0.05$, [b] $p < 1 \times 10^{-8}$, [c] $p < 1 \times 10^{-23}$ vs subjects with high lipolytic activity

2) In the extended transmission disequilibrium test, 42 unrelated subjects (23 men, 19 women) with both parents available for genetic analysis, were selected. Offspring were considered to have MSDR if their WHR exceeded the cut-off level for the highest WHR-quartile of a control group ($n = 405$, WHR ≥ 0.967 for men and ≥ 0.861 for women). All included offspring had NGT and a first-degree relative with Type II diabetes. Their age was 37.0 ± 9.3 years, WHR 1.01 ± 0.07 in men and 0.91 ± 0.07 in women, BMI 27.2 ± 4.6 kg/m^2, plasma triglycerides 1.5 ± 1.0 mmol/l, HDL 1.28 ± 0.33 mmol/l in men and 1.42 ± 0.35 mmol/l in women, while their homeostasis model assessment (HOMA)-index [19] for insulin resistance was 2.5 ± 2.1. Control allele frequencies were determined in 162 unrelated spouses with NGT, lipid metabolism and body constitution, and without family history of diabetes.

3) In the association study with lipolytic rate as the phenotype, 214 unrelated subjects from Sweden with a wide range of BMI (18–60 kg/m^2) were divided into tertiles of isoprenaline-stimulated lipolytic rate in abdominal subcutaneous adipose tissue, after correction for age and mean volume of subcutaneous fat-cells [13, 14]. For the analysis of allele frequency distribution, 71 subjects from the highest tertile were compared with 71 subjects from the lowest tertile (Table 2).

4) For mutation screening, 50 subjects with MSDR were chosen from the abdominally obese patients included in the association study. Of them, 20 subjects (11 men and 9 women, 17 Type II diabetic and 1 IGT, age 55.5 ± 14.2 years, WHR 1.00 ± 0.08, HDL 0.85 ± 0.23 mmol/l) were screened for all exons and the remaining 30 subjects (19 men and 11 women, 18 Type II diabetic and 9 IGT, age 58.0 ± 13.2 years, WHR 0.99 ± 0.07, HDL 0.92 ± 0.18 mmol/l) for exons 4, 6 and 8. In addition, 39 hypertriglyceridaemic subjects (23 men and 16 women, 27 Type II diabetic and 3 IGT, age 55.5 ± 14.7 years, plasma triglycerides 6.4 ± 1.4 mmol/l, WHR 0.89 ± 0.06) were screened for mutations, of whom 10 were screened for all exons and 29 for exons 4, 6 and 8.

Clinical investigations and laboratory assays. Waist circumference was measured with a soft tape on standing subjects midway between the lowest rib and the iliac crest and hip circumference over the widest part of the gluteal region [8, 12]. Mean blood pressure values were calculated from three recordings taken from sitting subjects 30 min after rest. Serum total cholesterol, HDL and triglyceride concentrations were measured on a Cobas Mira analyzer (Hoffman LaRoche, Basel, Switzerland). Serum non-esterified fatty acids (NEFA) were measured by microfluorometric assay [20] and serum insulin (Pharmacia, Uppsala, Sweden) and C-peptide [21] concentrations were measured with radioimmunoassay. Type II diabetes and IGT were diagnosed by WHO criteria [22]. The HOMA-index (fasting insulin times fasting plasma glucose divided by 22.5) [22] was used to estimate the degree of insulin resistance (reference value defined as 1.0 for a healthy subject with normal weight and age < 35 years).

Lipolysis assay. Subcutaneous adipose tissue was obtained by taking biopsies either during local anaesthesia or, for elective surgery, during general anaesthesia. The lipolytic measurements are comparable during both local and general anaesthesia [14]. Isolated adipocytes were prepared and incubated with increasing concentrations (10^{-13}–10^{-6}) of the non-selective β-adrenoreceptor agonist isoprenaline as described previously [13]. Glycerol was measured and used as a lipolytic index. Mean fat cell size and weight were determined and the number of adipocytes in each incubation were calculated [13]. The lipolytic rate at the maximum effective agonist concentration was expressed in relation to cell number.

Genotyping. Genomic DNA was extracted from peripheral blood lymphocytes using standard methods [23]. The LIPE marker [16] is a $(CA)_n$ dinucleotide repeat, located in intron 7 of the human HSL gene and highly polymorphic (heterozygosity index 0.79). It was genotyped with radioactive polymerase chain reaction (PCR) using γ-ATP end-labelled (Amersham Sweden AB, Solna, Sweden) forward primer (5′-CAAAACTGCACCTAATCTTCCC-3′) and unlabelled reverse primer (5′-GTAGGCTGTGTTTCCCCAGACT-3′). PCR was carried out as earlier described [16] but with the following changes: the reactions were done in a total volume of 15 μl with 16 mmol/l $(NH_4)_2SO_4$; 67 mmol/l Tris (pH 8.8); 0.01 % Tween 20; 0.13 mmol/l dNTP; 1 mmol/l $MgCl_2$; 3 % DMSO; 0.2 μmol/l of both primers and 0.5 U Taq polymerase (Perkin Elmer, Foster City, Calif., USA) using 21 ng of genomic DNA as template. The reactions were stopped with 95 % formamide buffer (1:1), denatured and applied onto a denaturing (5 % urea) 5 % polyacrylamide gel (acrylamide/bisacrylamide 19:1) and run at 50W for 3 h and 20 min.

Single-strand conformational polymorphism (SSCP). PCR was carried out with intronic primers for amplification of the coding HSL exons and the non-coding exon B located 1.5 kb upstream of the first coding exon (Table 3) [24]. For exons 1, 8 and 9, overlapping sets of 2 (exons 1 and 9) or 3 (exon 8) primers were used, as these exons were too large to be analysed in one fragment. A minimum distance of 25 bp between the intronic primer and exon border was used for all fragments. All the coding exons and the non-coding exon B were screened in 20 abdominally obese subjects with MSDR, and in 10 hypertriglyceridaemic subjects. In addition, 30 abdominally obese subjects with MSDR and 29 hypertriglyceridaemic subjects were screened for exons of putative functional importance: exon 6 encodes the serine of the catalytic triad and exon 8 encodes known phosphorylation sites [10, 18, 25]. In exon 4, a polymorphism (Arg309Cys) has been identified in a Japanese

Table 3. Sequences of primer-pairs and variable conditions for PCR-SSCP analysis of the HSL gene

Primer	Primer sequence	Fragment length (bp)	Annealing temperature (°C)	Enhancer of PCR-specificity
HSL B F	5′-TCTGTTTACAGCACGTGGTCC-3′	301	62	1.5 % formamide
HSL B R	5′-TGCTCCAGTGACTTCCCTCC-3′			
HSL 1a F	5′-AACAGGCCTCCCCACCTGCC-3′	314	63	5 % glycerol
HSL 1a R	5′-CGGGATTTGTGCAGGAGGTG-3′			
HSL 1b F	5′-TACCGCAGCCTAGTGCACAC-3′	347	63	5 % glycerol
HSL 1b R	5′-GCCTTCATTGTGGGGCCCAG-3′			
HSL 2 F	5′-CATCCCTCTCTTGAGCGGTG-3′	217	63	1.5 % formamide
HSL 2 R	5′-CCAGTGGGTCAGGCTGCTTG-3′			
HSL 3 F	5′-CAAGCAGCCTGACCCACTGG-3′	246	63	3 % DMSO
HSL 3 R	5′-CCTCAGATGATGCTCTGGGC-3′			
HSL 4 F	5′-ACCCCTGCAGGCAGACCTTC-3′	285	64	1.5 % formamide
HSL 4 R	5′-CCACGCTCCTCGGCTCTGTC-3′			
HSL 5 F	5′-AGCTCTCCCCAACCTCACAC-3′	348	63	1.5 % formamide
HSL 5 R	5′-GGCTCACCAAGGAGGGCGCA-3′			
HSL 6 F	5′-TCTGCCCCTGCCAGGTTGTC-3′	328	65	1.5 % formamide
HSL 6 R	5′-GCAGGAGTCAGACATCCATGCA-3′			
HSL 7 F	5′-GGAAAACAACTCAGCTACCCAC-3′	267	65	1.5 % formamide
HSL 7 R	5′-AGGCTGTCCCTCCTGCCCAC-3′			
HSL 8a F	5′-ACCAAACTAACGGAGCCAGG-3′	285	58	1.5 % formamide
HSL 8a R	5′-CAGCTCATTTTTGGCCTCAG-3′			
HSL 8b F	5′-GACACTTAGCCCCTCCACAC-3′	239	62	1.5 % formamide
HSL 8b R	5′-TGCTGTCGGGTGCCAGCAGC-3′			
HSL 8c F	5′-AGAGGCCTGGGCGTCCGTGC-3′	256	66	3 % DMSO
HSL 8c R	5′-AGGTGTACCGTGCCCGGTCC-3′			
HSL 9a F	5′-ACCCTCTCTCCACGTCCCTC-3′	237	64	1.5 % formamide
HSL 9a R	5′-ACGAGGCGGATGCGCTCCAC-3′			
HSL 9b F	5′-CGGCGCTGTGCCGCGAGAC-3′	283	63	3 % DMSO
HSL 9b R	5′-TGGCGAGGGTCTCAGCTTTC-3′			

The primers HSL BF and HSL BR amplify the 5′ non-coding exon of the HSL gene

population [26], and thus 89 subjects were screened seeking to identify subjects with this polymorphism.

PCR was carried out with initial denaturation (96 °C for 3 min) followed by 30 cycles of denaturation (96 °C for 30 s), annealing (30 s, see Table 3 for variable annealing temperatures) and extension (72 °C for 30 s), followed by final extension (72 °C for 10 min). The reactions were done in a total volume of 20 μl with 16 mmol/l $(NH_4)_2SO_4$; 67 mmol/l Tris (pH 8.8); 0.01 % Tween 20; 0.13 mmol/l dNTP; 1.5 mmol/l $MgCl_2$; 0.2 μmol/l of both primers; 0.5 μCi α-^{32}P-dCTP and 0.5 U Taq polymerase using 21 ng genomic DNA as template. Either 3 % DMSO, 1.5 % formamide or 5 % glycerol (Table 3) was used to increase PCR-sensitivity. The reactions were stopped with 95 % formamide buffer (1 : 1), denatured, cooled and electrophoresed on glycerol-free (35W for 3.5 h at 4 °C) and 5 % glycerol (8W for 12 h at room temperature), nondenaturing 5 % polyacrylamide gels (acrylamide/bisacrylamide 49:1). When differences in band pattern were observed, PCR-products were sequenced bidirectionally using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, Calif., USA) and analysed on an automated sequencer (ABI, model 373, Perkin Elmer). Heterozygous variants were confirmed by using the restriction fragment length polymorphism-technique (PCR-RFLP). Using this procedure, the estimated degree of mutation detection is approximately 95 % in our laboratory.

Statistical analysis. Differences in clinical characteristics were tested by Mann-Whitney nonparametric test statistics using the BMDP New System for Windows statistical package (Biomedical Data Processing, Los Angeles, Calif., USA). *P*-values of less than 0.05 were considered statistically significant. Allele frequency distribution was tested by χ^2-analysis, and alleles with expected frequencies of less than 5.0 were pooled to assure the validity of the χ^2-test. Extended TDT analysis was calculated using the ETDT software package 1.4 for multiallelic markers [27], whereby logistic regression was done to estimate parameters for transmitted and non-transmitted alleles from heterozygous parents. With these parameters, log likelihood was calculated for the allele-wise and genotype-wise model and deviation from the null-hypothesis tested by the McNemar χ^2-test.

Results

Association study of the HSL LIPE marker with MSDR as the phenotype. In these subjects we defined 14 alleles for the HSL LIPE marker (Table 4), ranging in size from 164 to 194 base pairs (bp). The allele frequency distribution of the HSL LIPE marker differed significantly between 235 Type II diabetic patients and 146 healthy control subjects ($\chi^2 = 20.6$, 6 degrees of freedom (d.f.), $p = 0.002$), between 78 MSDR/Type II diabetic patients and the control subjects ($\chi^2 = 16.8$, 6 d.f., $p = 0.010$) and between 157

Table 4. Distribution of the HSL LIPE alleles in the subjects analysed in the association study of the HSL gene and characteristics of MSDR

Alleles	Size (bp)	NGT		Type II diabetic	
		control subjects $n = 146$	MSDR $n = 37$	lean $n = 157$	MSDR $n = 78$
1	194	–	–	–	1 (0.6)
2	192	1 (0.3)	–	1 (0.3)	–
3	190	1 (0.3)	–	3 (1.0)	–
4	188	10 (3.4)	4 (5.4)	14 (4.5)	9 (5.8)
5	186	20 (6.8)	8 (10.8)	11 (3.5)	3 (1.9)
6	184	86 (29.5)	24 (32.4)	95 (30.2)	42 (26.9)
7	182	97 (33.2)	15 (20.2)	90 (28.7)	45 (28.8)
8	180	46 (15.7)	12 (16.2)	44 (14.0)	25 (16.0)
9	178	3 (1.0)	1 (1.3)	9 (2.9)	1 (0.6)
10	176	–	–	3 (1.0)	1 (0.6)
11	174	–	–	3 (1.0)	1 (0.6)
12	172	22 (7.5)	5 (6.8)	30 (9.6)	17 (10.9)
13	170	2 (0.7)	1 (1.3)	2 (0.6)	4 (2.6)
15	164	4 (1.4)	4 (5.4)	9 (2.9)	7 (4.5)
Total		292 (100)	74 (100)	314 (100)	156 (100)

Data are *n* (%). Alleles with expected frequencies < 5 were pooled into one group (1–3, 9–11, 13) for the χ^2-analysis

nonobese Type II diabetic patients and the control subjects ($\chi^2 = 15.1$, 6 d.f., $p = 0.020$). No difference was seen between MSDR/Type II diabetic and nonobese Type II diabetic patients ($\chi^2 = 2.2$, 6 d.f., $p = 0.9$). No significant difference in allele frequency distribution was observed between 37 MSDR/NGT and healthy control subjects ($\chi^2 = 7.4$, 5 d.f. and $p = 0.19$). The combined group of diabetic and nondiabetic individuals with MSDR, however, differed from control subjects ($\chi^2 = 15.1$, 6 d.f. and $p = 0.019$) but not from the pooled group of nonobese Type II diabetic and control subjects ($\chi^2 = 6.5$, 7 d.f., $p = 0.48$). To exclude ethnic distortion we also carried out the analysis in the Botnia population only. We observed significant differences in allele frequency distribution between all Type II diabetic patients and control subjects ($p = 0.007$), between MSDR/Type II diabetic patients and control subjects ($p = 0.010$) and a smaller difference between nonobese Type II diabetic patients and control subjects ($p = 0.06$).

Extended transmission disequilibrium test (ETDT) in abdominally obese offspring. We genotyped 42 abdominally obese offspring and their parents for the HSL LIPE marker. Of these offspring 39 had a diabetic parent and the remaining three had at least one diabetic sibling. There were eighteen homozygous parents who were thus not informative for transmission of alleles. Transmission was informative for sixty-six parental alleles, and in this set of families eleven different alleles of the HSL LIPE marker were transmitted. Results from both the allele-wise ($\chi^2 = 20.0$, 10 d.f., $p = 0.029$) and the genotype-wise ($\chi^2 = 35.1$, 23 d.f., $p = 0.052$) model indicated that certain alleles were preferentially transmitted to abdominally obese offspring in families with Type II diabetes. The result from the two models does not significantly differ ($\chi^2 = 15.1$, 13 d.f., $p = 0.30$). No individual allele explained the transmission disequilibrium, although allele 5 accounted for the greatest deviation from the expected transmission ($p = 0.14$, after correction for multiple comparisons).

Table 5. Distribution of the HSL LIPE alleles in the subjects analysed in the association study of the HSL gene and lipolytic activity in subcutaneous adipose tissue

Alleles	Size (bp)	High lipolysis $n = 71$	Low lipolysis $n = 71$
3	190	1 (0.7)	4 (2.8)
4	188	7 (4.9)	2 (1.4)
5	186	9 (6.3)	2 (1.4)
6	184	43 (30.3)	42 (29.6)
7	182	35 (25.6)	55 (38.7)
8	180	22 (15.5)	19 (13.3)
9	178	2 (1.4)	3 (2.1)
10	176	1 (0.7)	2 (1.4)
11	174	1 (0.7)	2 (1.4)
12	172	11 (7.7)	6 (4.2)
13	170	1 (0.7)	2 (1.4)
15	164	9 (6.3)	3 (2.1)
Total		142 (100)	142 (100)

Data are *n* (%). Alleles with expected frequencies < 5 were pooled into one group (1–5, 9–11, 13–15) for the χ^2-analysis

Association study of the HSL LIPE marker with low lipolytic rate as the phenotype. The difference in allele frequency distribution (Table 5) of the HSL LIPE marker between subjects with high ($n = 71$) and low ($n = 71$) lipolytic activity did not reach statistical significance ($\chi^2 = 8.6$, 4 d.f., $p = 0.072$).

SSCP mutation screening in abdominally obese patients with MSDR. We did not identify any missense mutations in the HSL gene in 20 abdominally obese patients with MSDR. In one subject, however, a silent variant was found in codon 756 (exon 9), changing a GCC (alanine) to a GCT (alanine). Furthermore, in another subject we identified a variant changing a G to T in nucleotide + 41 of the non-coding exon B. These variants were confirmed by PCR-RFLP using restriction enzymes *BcnI*, (for the codon 756 variant) and *MboI* (for the position + 41 variant). The codon 756 variant was detected in one of 60 subjects screened, and the position + 41 variant in one of 30 subjects screened. No additional mutations were identified in the 10 hypertriglyceridaemic subjects screened for all exons. Neither did we detect any mutations in the 30 abdominally obese subjects or in the 29 hypertriglyceridaemic subjects screened for exons 4, 6 and 8. A polymorphism described previously in exon 4 of the HSL gene (Arg309Cys), seen in 5.2 % of Japanese control subjects [26], could not be detected in 89 Scandinavian subjects.

Discussion

The allele frequency distribution of the HSL LIPE polymorphic marker showed a considerable difference between abdominally obese Type II diabetic patients with low HDL cholesterol and healthy control subjects. This was also true when we compared nonobese Type II diabetic patients without signs of MSDR and healthy control subjects, whereas no noticeable difference was observed between obese and nonobese Type II diabetic patients. Neither was there any important difference in the allele frequency distribution between nondiabetic subjects with features of MSDR and lean control subjects. This was also reported before [28], and it could thus mean that the HSL LIPE polymorphism increases susceptibility to Type II diabetes rather than to abdominal obesity or MSDR. The intrafamily association study, however, could provide some more insight into the phenotype associated with the HSL gene.

The ETDT study clearly showed distorted transmission of alleles to abdominally obese offspring in families with Type II diabetes. As abdominal obesity is a risk factor for Type II diabetes [8], it seems likely that the LIPE marker in the HSL gene is in linkage disequilibrium with a gene increasing susceptibility to abdominal obesity and thereby possibly to Type II diabetes. The positive TDT-result also imply that there is some degree of linkage, suggesting that this locus may be close.

Given the association between the HSL gene and Type II diabetes, an association is also probable with impaired stimulation of lipolysis. Patients with familial combined hyperlipidaemia [14] and nonobese women with polycystic ovary syndrome [29] have markedly impaired catecholamine-induced lipolysis which at least in part is due to impaired function of HSL. When comparing individuals from the lowest with those from the highest tertile of lipolysis we observed a slight difference in allele frequency distribution between the groups ($p = 0.07$). Given the number of alleles at this locus, however, the comparison between these extreme groups detected no noticeable difference. On the other hand, when comparing groups below and above the median of the lipolytic rate there was no difference at all between the groups ($p = 0.52$). It is therefore still possible that a biallelic marker in the HSL gene could show an association with a low lipolytic rate.

The coding regions of the HSL gene and the noncoding exon B were screened for mutations in abdominally obese patients with MSDR using the SSCP technique. Despite the positive association between MSDR and the HSL gene, no missense mutations were identified. A silent mutation (Ala^{756} GCC → GCT) and a non-coding mutation (exon B, bp 41 G → T) were found, but the prevalence of these mutations was very low.

Few previous studies have examined the role of the HSL gene in the development of obesity and MSDR. In a Japanese population, a polymorphism has been observed in the coding regions of the HSL gene [26], changing an arginine to a cysteine in codon 309 (exon 4). It was present in 5.2 % of the control population, and there was no difference in the allele frequency between Type II diabetic patients and healthy subjects. Serum triglyceride and HDL-cholesterol concentrations were similar in subjects with either arginine or cysteine in this position, while total serum cholesterol concentration were higher in Type II diabetic subjects heterozygous for Arg309-Cys [26]. The polymorphism in codon 309 could not be identified in our study group. In a more recent study, a polymorphic marker in intron 6 of the HSL gene was associated with both obesity and Type II diabetes in a French population [28].

In conclusion, our population and intrafamily association studies suggest that the LIPE marker in the HSL gene is in linkage disequilibrium with an allele and/or gene that increases susceptibility to abdominal obesity and thereby possibly to Type II diabetes.

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Note added in proof: Of the subjects analysed in the association study with MSDR as the phenotype, six of the 78 NIDDM/Type II diabetes patients (7.7 %) and seven of the 146 controls (4.8 %) had the C → T/codon 756 variant

($p = 0.38$). Additionally, one patient with MSDR/Type II diabetes carried the C → T/exon B variant, compared to none of the controls.

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III

Interaction between the Asn291Ser variant of the *LPL* gene and insulin resistance on dyslipidaemia in high risk individuals for Type 2 diabetes mellitus

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Abstract

Aims Lipoprotein lipase (LPL) is a major regulator of triglyceride clearance. A genetic variant of the *LPL* gene on chromosome 8p22, Asn291Ser, has previously been associated with dyslipidaemia and an increased frequency of cardiovascular disease as well as familial disorders of lipoprotein metabolism. The aim of this study was to test whether the phenotypic expression of the LPL Asn291Ser variant is dependent upon glucose tolerance and insulin resistance. Therefore, the Asn291Ser variant was examined in 192 patients with Type 2 diabetes, 278 subjects with normal glucose tolerance who are first degree relatives of patients with Type 2 diabetes and 226 healthy control spouses without family history of diabetes.

Methods The subjects were genotyped with an allele-specific mini-sequencing method. Insulin resistance was estimated using the homeostasis model assessment (HOMA) index.

Results The frequency of the Asn/Ser genotype was significantly increased in normoglycaemic subjects with hypertriglyceridaemia (> 1.7 mmol/l), and was associated with dyslipidaemia and increased systolic blood pressure. There was a significant interaction between Asn291Ser and insulin resistance in normoglycaemic subjects, indicating that dyslipidaemia is more severe in Asn/Ser carriers with reduced insulin sensitivity. The frequency of the Asn/Ser genotype was not increased in diabetic subjects with hypertriglyceridaemia, but was associated with increased systolic blood pressure.

Conclusions The Asn/Ser genotype of the *LPL* gene is associated with dyslipidaemia in normoglycaemic subjects, and the dyslipidaemic phenotype is more severe in insulin-resistant subjects. This association is not seen in diabetic subjects.

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Keywords dyslipidaemia, lipoprotein lipase, *LPL291*, Type 2 diabetes mellitus

Abbreviations ANOVA, analysis of variance; CI, confidence interval; CV, coefficient of variance; HDL, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LPL, lipoprotein lipase; NGT, normal glucose tolerance; OR, odds ratio; PCR, polymerase chain reaction; VLDL, very low density lipoprotein; WHO, World Health Organization

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Introduction

The key features of dyslipidaemia in Type 2 diabetes are high levels of plasma triglycerides and very low density lipoproteins (VLDL) and low levels of high density lipoproteins (HDL) [1]. Hypertriglyceridaemia is seen in 20–60% of Type 2 diabetic patients and two to three times more common than in non-diabetic populations [1]. Importantly, these lipid abnormalities are also observed in glucose-tolerant relatives of Type 2 diabetic patients [2–5] in whom they seem to cluster with other features of the metabolic syndrome, i.e. insulin resistance, abdominal obesity and hypertension.

A number of studies have shown that both genetic and environmental factors can modulate lipid levels in man [6]. Genes encoding for enzymes regulating key steps in the conversion of VLDL to low density lipoproteins (LDL) represent potential candidate genes that could modulate the expression of dyslipidaemia. Lipoprotein lipase (LPL) has a central role in both VLDL and HDL metabolism [7,8]. Low LPL activity has been associated with raised plasma triglyceride and low HDL cholesterol levels. In Type 2 diabetes, LPL activity is commonly subnormal and contributes to the elevation of serum triglycerides [9,10]. A prior report showed that post-heparin plasma LPL activity is reduced in insulin-resistant first degree relatives of patients with Type 2 diabetes [11]. Thus, genetic variants of the *LPL* gene could modulate the magnitude of dyslipidaemia in high-risk individuals for Type 2 diabetes. Several genetic defects of the *LPL* gene have been reported in exons 4, 5 or 6 of the *LPL* gene [12]; most of these are rare and cause severe dyslipidemia. However, a common asparagine to serine substitution at residue 291 (Asn291Ser, exon 6) has an allele frequency of 2–5% [13–15]. In most studies, the Asn291Ser variant has been associated with decreased HDL cholesterol and elevated triglyceride concentrations, although normal lipid values have also been reported in carriers of this variant [13–21]. The variability of the Asn291Ser genotype indicates that other genes or factors, such as the presence of insulin resistance or diabetes, may modulate the expression of the genotype.

To test the hypothesis that the phenotypic expression of the LPL Asn291Ser variant is dependent upon glucose tolerance and insulin resistance, the Asn291Ser variant was examined in 192 Type 2 diabetic patients, 278 subjects with normal glucose tolerance who are first degree relatives of patients with Type 2 diabetes and 226 healthy control spouses without family history of diabetes.

Subjects and methods

Subjects

Subjects were selected from the Botnia region of western Finland [22]. Allele frequency comparisons were made between 192 unrelated Type 2 diabetic patients, 278 unrelated subjects with normal glucose tolerance (NGT) who are first degree relatives of patients with Type 2 diabetes (NGT-rel) and 226 unrelated healthy control spouses without family history of diabetes. Clinical characteristics of these three groups are presented in Table 1. Each group was further divided by fasting plasma triglyceride levels and comparisons were made between individuals with normal (< 1.7 mmol/l; NorTg) and high (> 1.7 mmol/l; HiTg) triglyceride levels, based upon the recent proposal by the World Health Organization for the diagnosis of the metabolic syndrome [23]. All subjects with lipid-lowering treatment were excluded from the NorTg groups, although among the HiTg diabetic subjects one Asn/Ser carrier and four non-carriers were on lipid-lowering treatment. Informed consent was obtained from all subjects and investigations were performed in accordance with the Declaration of Helsinki.

Clinical investigations and laboratory assays

Clinical investigations and laboratory assays were performed as previously described [22]. Briefly, fasting plasma triglycerides

Table 1 Clinical characteristics of the study groups

Subjects (*n* = 696)	Control (*n* = 226)	NGT-rel (*n* = 278)	Type 2 DM (*n* = 192)
Age (years)	56 ± 10	55 ± 13	$63 \pm 12^{c,f}$
Triglycerides (mmol/l)	1.4 ± 0.8	1.8 ± 0.9^{b}	$2.1 \pm 1.2^{c,d}$
HDL cholesterol (mmol/l)	1.44 ± 0.37	1.33 ± 0.32^{a}	$1.25 \pm 0.35^{c,d}$
Cholesterol (mmol/l)	5.9 ± 1.2	6.2 ± 1.3^{a}	6.0 ± 1.1
BMI (kg/m^2)	25.8 ± 3.6	26.6 ± 3.4^{a}	$28.3 \pm 4.6^{c,e}$
Systolic blood pressure (mmHg)	113 ± 26	128 ± 26^{c}	$133 \pm 29^{c,d}$
Diastolic blood pressure (mmHg)	68 ± 15	76 ± 14^{b}	74 ± 18^{b}
Fasting blood glucose (mmol/l)	5.0 ± 0.5	5.1 ± 0.5	$8.4 \pm 2.5^{c,f}$
HOMA-index for insulin resistance*	2.09 ± 1.46	2.27 ± 1.37	$6.30 \pm 5.31^{c,f}$
Fasting C-peptide (nmol/l)	0.42 ± 0.20	0.50 ± 0.22^{a}	$0.63 \pm 0.36^{c,e}$
HbA$_{1c}$ (%)	5.6 ± 0.4	5.5 ± 0.5	$7.3 \pm 1.5^{c,f}$

Data is mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.001$, $^{c}P < 1 \times 10^{-6}$ vs. control subjects and $^{d}P < 0.05$, $^{e}P < 0.001$, $^{f}P < 1 \times 10^{-6}$ vs. NGT-rel subjects.
*HOMA index values are reported for Type 2 diabetic patients without insulin treatment (*n* = 147).

(coefficient of variance (CV) 2.2%), total cholesterol (CV 2.1%) and HDL-cholesterol (CV 4.6%) subfractions were precipitated and measured on a Cobas Mira analyser (Hoffman LaRoche, Basle, Switzerland). Apolipoprotein A-I (CV 3.5%) and A-II (CV 3.7%) were measured by turbido-immunometric methods using commercially available kits (Boehringer Mannheim, Mannheim, Germany), and apolipoprotein B (CV 4.4%) concentrations were measured by an immunochemical assay (Orion Diagnostica, Espoo, Finland). Fasting blood glucose (CV < 1%) was measured with a glucose oxidase method using a Beckman Glucose Analyser II (Beckman Instruments, Fullerton, CA). Serum insulin (interassay CV 5%; Pharmacia, Uppsala, Sweden) and C-peptide (interassay CV 9%) concentrations were measured with radioimmunoassay. Mean blood pressure was calculated from three sitting recordings after 30 min rest. The homeostasis model assessment (HOMA) index (fasting insulin times fasting plasma glucose divided by 22.5) [24,25] was used to estimate the degree of insulin resistance (reference value defined as 1.0 for healthy subjects with normal weight and younger than 35 years). Type 2 diabetes was diagnosed by 1985 WHO criteria [26].

Genotyping

Subjects were genotyped for the LPL Asn291Ser variant with an allele-specific mini-sequencing method as previously described [21], using the upstream primer 5′-ATC TTG GTG TCT CTT TTT TAC CC-3′ and a biotinylated downstream primer 5′-AGT CTT CAG GTA CAT TTT GCT GCT-3′ to amplify this region by polymerase chain reaction (PCR). The biotinylated PCR fragment was linked to a streptavidin-coated well, incubated with detection primer (5′-CAA TCT GGG CTA TGA GAT CA-3′) and [^{3}H]dGTP, and a liquid scintillation counter was used to detect allelic variants with an A at the second position of codon 291.

Statistical analyses

Descriptive clinical data were compared with the Student's *t*-test (mean ± SD) and differences in clinical characteristics of the different Asn291Ser genotype carriers were tested by the Mann–Whitney non-parametric test, using the BMDP New System for Windows statistical package (Biomedical Data Processing, Los Angeles). Data from non-parametric analyses was expressed as median (interquartile range) unless otherwise stated. A *P*-value < 0.05 was considered statistically significant. Bonferroni correction for multiple comparisons was calculated when specified, although the corrected *P*-value is too conservative when variables are not independent. Frequencies were compared with Pearson χ^2-analysis, and Fisher's exact test (two-tailed) when expected numbers were too low (< 5) to ensure the validity of the χ^2. Comparative risk was estimated with the odds ratio (OR) and 95% confidence intervals ($CI_{95\%}$). Genetic interaction with physiological parameters was calculated by two-way analysis of variance (ANOVA).

Results

Frequency of the Asn/Ser in subjects with normal glucose tolerance, Type 2 diabetes and hypertriglyceridaemia

The serine allele of the LPL Asn291Ser variant was present in 13 of 384 (3.4%) chromosomes from Type 2 diabetic patients, in 26 of 556 (4.7%) chromosomes from NGT-rel subjects and in 13 of 452 (2.9%) chromosomes from control subjects. All identified carriers of the serine allele were heterozygous for the variant, and the corresponding Asn/Ser genotype frequencies were 6.8% in Type 2 diabetic patients, 9.4% in NGT-rel subjects and 5.8% in control subjects (Table 2). Allele and genotype frequencies were in Hardy–Weinberg equilibrium in all three groups studied. Neither Type 2 diabetic patients nor NGT-rel subjects had increased frequency of the Asn/Ser genotype compared to control subjects.

When the groups were divided by fasting plasma triglyceride levels, genotype frequencies differed significantly between non-diabetic groups with high (HiTg) and normal (NorTg) triglyceride levels (Table 2). The frequency of the Asn/Ser genotype was higher among HiTg control subjects (11.5%) than among NorTg control subjects (3.6%) ($P = 0.047$), and the Asn/Ser frequency was higher among HiTg NGT-rel subjects (13.5%) than among NorTg NGT-rel subjects (5.5%) ($P = 0.022$). In addition, the frequency of the Asn/Ser genotype was increased in NGT-rel subjects as compared to NorTg control subjects (9.4 vs. 3.6%,

Table 2 Genotype frequencies of the Asn291Ser variant of the lipoprotein lipase gene

			Plasma triglyceride level			
			Normal (NorTg)		High (HiTg)	
All Subjects	Asn/Asn	Asn/Ser	Asn/Asn	Asn/Ser	Asn/Asn	Asn/Ser
Control	213 (94.2)	13 (5.8)	159 (96.4)	6 (3.6)	54 (88.5)	7 (11.5)*
NGT-rel	252 (90.6)	26 (9.4)	137 (94.5)	8 (5.5)	115 (86.5)	18 (13.5)*,**
Type 2 diabetic	179 (93.2)	13 (6.8)	80 (93.0)	6 (7.0)	99 (93.4)	7 (6.6)

Data are *n* (%). *$P < 0.05$ vs. NorTg control subjects, **$P < 0.05$ vs. NorTg NGT-rel subjects.

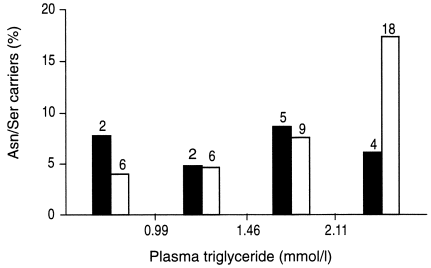

Figure 1 Frequency (%) of Asn/Ser genotype carriers in diabetic (filled bars) and normoglycaemic (open bars) subjects according to plasma triglyceride quartiles (cut-off levels were based on the pooled group of normoglycaemic and diabetic subjects and are shown on the *x*-axis). The number of Asn/Ser genotype carriers in each quartile is given above the bar.

$P = 0.02$). There was no difference between HiTg NGT-rel subjects and HiTg control subjects (13.5 vs. 11.5%), or between HiTg Type 2 diabetic patients and NorTg diabetic patients.

There were more normoglycemic Asn/Ser genotype carriers in the highest than in the lowest triglyceride quartile ($P < 0.001$), while this difference was not seen among the diabetic subjects (Fig. 1). Dividing subjects into quartiles of age did not show any distortion from the expected distribution (data not shown). These results were very similar when diabetic and normoglycaemic subjects were divided into quartiles separately, as well as when plasma triglycerides were adjusted for age.

Estimation of risk for hypertriglyceridaemia in Asn/Ser carriers

In normoglycaemic subjects (NGT-rel and control subjects), the odds of having hypertriglyceridaemia, defined as plasma triglycerides > 1.7 mmol/l, was 3.3 times greater among the Asn/Ser genotype carriers compared to non-carriers ($P < 0.001$, $CI_{95\%}$ 1.7–6.8). In diabetic subjects, the odds of having hypertriglyceridaemia were not increased in Asn/Ser genotype carriers compared to non-carriers (OR = 0.9, $CI_{95\%}$ 0.3–2.9).

Phenotypic characteristics of Asn/Ser genotype carriers among subjects with normal glucose tolerance and Type 2 diabetes

As shown in Table 3, normoglycaemic carriers of the Asn/Ser genotype (NGT-rel and control subjects) had increased concentrations of fasting plasma triglycerides ($P = 0.00009$) and reduced concentrations of HDL-cholesterol ($P = 0.0009$) and apolipoprotein AI ($P = 0.05$), compared with non-carriers. The reduction of HDL-cholesterol concentration was accounted for by a reduction of both the HDL2 ($P = 0.01$) and the HDL3 ($P = 0.03$) subfractions. In addition, carriers of the Asn/Ser genotype had significantly higher systolic blood pressure ($P = 0.01$) than non-carriers. There were no significant differences in body mass index (BMI) or HOMA index between normoglycaemic Asn/Ser genotype carriers and non-carriers. In female normoglycaemic carriers of the Asn/Ser genotype, triglyceride concentrations ($P = 0.0004$) were increased and HDL cholesterol concentrations reduced ($P = 0.003$) compared to female non-carriers (Table 2). In male normoglycaemic Asn/Ser genotype carriers, triglyceride concentrations ($P = 0.04$) and systolic blood pressure ($P = 0.05$) were increased compared to male non-carriers (Table 3).

In Type 2 diabetic patients there were no differences in plasma triglycerides, HDL-cholesterol or apolipoprotein AI concentrations between Asn/Ser genotype carriers and non-carriers (Table 4). However, Type 2 diabetic carriers of the Asn/Ser genotype had increased systolic ($P = 0.004$) and diastolic ($P = 0.03$) blood pressure compared to diabetic non-carriers (Table 4). Male, but not female, diabetic carriers of the Asn/Ser genotype had significantly increased systolic blood pressure ($P = 0.01$). Excluding subjects on lipid-lowering treatment did not change the results in diabetic subjects.

Genetic interaction between the Asn/Ser genotype and features of the metabolic syndrome

Interaction between genotype and physiological parameters was investigated by comparing fasting plasma triglyceride levels or systolic blood pressure between carriers and non-carriers of the Asn/Ser genotype within and between each quartile of BMI and HOMA index for insulin resistance using a two-way ANOVA. In normoglycaemic subjects, a significant interaction between the Asn/Ser genotype and HOMA index for insulin resistance was seen on triglyceride levels ($P = 0.05$, Fig. 2) but not systolic blood pressure (data not shown). No significant interaction was seen between the Asn/Ser genotype and BMI (Fig. 2). In Type 2 diabetic subjects, there was no significant interaction between the Asn/Ser genotype and BMI on triglyceride levels or systolic blood pressure (data not shown). There were too few carriers without insulin treatment in each quartile to allow such analysis for HOMA index in the diabetic subjects.

Discussion

In the Botnia population, the Asn/Ser genotype was present in 5.8% of control subjects, which is consistent with other populations [15]. The frequency of the Asn/Ser genotype was, however, similar between subjects with Type 2

Table 3 Comparisons between normoglycaemic subjects with Asn/Asn and Asn/Ser genotypes of the LPL Asn291Ser variant

	Asn/Asn (*n* = 465)	Asn/Ser (*n* = 39)
Sex (women/men)	227/238	16/23
Age (years)	54 (46–65)	58 (46–67)
Triglycerides (mmol/l)	1.3 (0.9–1.9)	2.1 (1.3–2.5)***,§
women	1.7 (0.9–1.8)	2.1 (1.5–2.5)**
men	1.3 (1.0–2.0)	2.1 (1.0–2.9)*
Cholesterol (mmol/l)	6.0 (5.2–6.9)	6.0 (5.4–7.2)
HDL cholesterol (mmol/l)	1.3 (1.1–1.6)	1.2 (1.0–1.4)**,§
women	1.5 (1.3–1.8)	1.2 (1.1–1.4)*
men	1.2 (1.1–1.4)	1.1 (1.0–1.3)
HDL2 cholesterol (mmol/l)	0.5 (0.3–0.7)	0.4 (0.3–0.5)*
HDL3 cholesterol (mmol/l)	0.9 (0.8–1.0)	0.8 (0.7–0.9)*
Apolipoprotein A-I (mg/l)	137 (125–154)	130 (122–144)*
Apolipoprotein A-II (mg/l)	38 (34–42)	36 (32–40)
Apolipoprotein B (mg/l)	97 (83–114)	96 (84–114)
Systolic blood pressure (mmHg)	122 (101–137)	134 (119–142)*
women	122 (105–139)	134 (120–143)
men	122 (100–135)	133 (117–142)*
Diastolic blood pressure (mmHg)	74 (60–83)	80 (71–83)
BMI (kg/m^2)	26 (24–28)	26 (24–27)
Waist-to-hip ratio	0.89 (0.82–0.96)	0.93 (0.84–0.98)
HOMA index for insulin resistance	1.9 (1.3–2.6)	1.9 (1.4–2.9)
Anti-hypertensive treatment	47/39 (18)	6/2 (20)

Data are median (interquartile range) or women/men (%).*$P < 0.05$, **$P < 0.001$ and ***$P < 0.0001$ vs. Asn/Asn carriers.

§The difference is significant after Bonferroni correction for multiple comparisons.

Table 4 Comparisons between Type 2 diabetic subjects with Asn/Asn and Asn/Ser genotypes of the LPL Asn291Ser variant

	Asn/Asn (*n* = 179)	Asn/Ser (*n* = 13)
Sex (women/men)	92/87	7/6
Age (years)	66 (57–70)	67 (60–72)
Duration of diabetes (years)	5 (1–11)	6 (2–10)
Triglycerides (mmol/l)	1.8 (1.2–2.8)	1.8 (1.5–2.7)
HDL cholesterol (mmol/l)	1.2 (1.0–1.4)	1.2 (1.0–1.7)
Systolic blood pressure (mmHg)	138 (110–152)	151 (138–172)*
women	139 (111–155)	151 (134–170)
men	134 (107–149)	155 (142–179)*
Diastolic blood pressure (mmHg)	78 (60–85)	81 (77–93)*
women	76 (59–83)	80 (78–88)
men	79 (61–89)	89 (75–104)
BMI (kg/m^2)	28 (25–31)	26 (23–29)
Waist-to-hip ratio	0.93 (0.88–0.98)	0.95 (0.83–0.99)
HOMA-index for insulin resistance†	4.7 (2.9–7.5)	3.5 (2.2–3.9)
Insulin treatment§	18/17 (25)	1/4 (45)
Hypoglycemic agents	17/21 (27)	–
Diet	28/27 (39)	5/1 (54)
Anti-hypertensive treatment	53/40 (52)	3/3 (46)

Data are median (interquartile range) or women/men (%).*$P < 0.05$ vs. Asn/Asn carriers.
No differences were significant after Bonferroni correction.
†HOMA-index was analysed for Type 2 diabetic patients without insulin treatment.
§Insulin alone or in combination with hypoglycemic agents.
Eleven (85%) of the diabetic Asn/Ser genotype carriers and 140 (78%) of the non-carriers had recordings on treatment for diabetes, whereas treatment for one female and one male carrier and six female and six male non-carriers was unknown (9%).

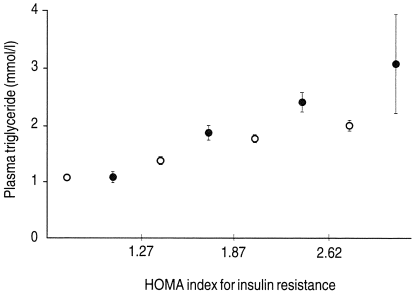

Figure 2 The interaction between the Asn/Ser genotype and insulin resistance on dyslipidaemia in normoglycaemic subjects. Filled symbols represent carriers of the Asn/Ser genotype, and open symbols non-carriers. Subjects were divided into quartiles of HOMA index (cut-off levels were based on all normoglycaemic subjects and are shown on the x-axis) and the interaction was analysed by two-way ANOVA. With increasing insulin resistance, triglyceride concentrations increased more in Asn/Ser genotype carriers than in non-carriers ($P = 0.05$).

diabetes, glucose-tolerant first degree relatives of patients with Type 2 diabetes and control spouses. The Asn/Ser genotype was most frequent in hypertriglyceridaemic subjects with normal glucose tolerance, approximately 2–3 times more common than in the general population, but it was not significantly increased in hypertriglyceridaemic subjects with Type 2 diabetes. It is possible that diabetic Asn/Ser genotype carriers could be under-represented as the result of an increased mortality, although this seems unlikely as carriers and non-carriers were of similar age in all groups. It is more likely that the effect of the Asn/Ser genotype is too small to be detected in diabetic patients, in whom several diabetes-specific factors can modify plasma triglyceride levels.

In normoglycaemic subjects the Asn/Ser genotype was associated with both dyslipidaemia and increased systolic blood pressure, whereas in diabetic subjects only an association with increased blood pressure was observed. As previously described [15], the phenotypic expression of the Asn/Ser genotype was dependent upon sex. In the diabetic patients, loss of glucose control could have attenuated differences in triglyceride levels. In control spouses from the Botnia region ($n = 405$), a fasting plasma triglyceride level of 1.7 mmol/l represents the upper quartile, whereas in diabetic subjects from the same region ($n = 841$) the corresponding value is 2.4 mmol/l. Therefore, each group was divided into quartiles according to plasma triglyceride levels and the presence of Asn/Ser genotype carriers in each group was examined. In the normoglycaemic group, there was a significant increase of Asn/Ser carriers with increasing plasma triglycerides, whereas this could not be seen among the diabetic subjects. Thus, the *LPL291* Asn/Ser genotype is not associated with dyslipidaemia in Type 2 diabetic subjects.

Few previous studies have investigated blood pressure in relation to the *LPL* gene. The present study found that the Asn/Ser genotype is associated with elevated blood pressure in both normoglycaemic and diabetic subjects. A recent study reported that three polymorphic markers at the *LPL* locus on chromosome 8p22 were linked to systolic blood pressure [27]. It is possible that the *LPL291* Asn/Ser genotype, or a variant in linkage disequilibrium with the serine allele, could be related to the development of increased blood pressure.

In general, there seems to be a large variability of the Asn291Ser phenotype indicating that other genes or factors, such as the presence of diabetes, obesity or insulin resistance may modulate the expression of this genotype. It was found that dyslipidaemia is more severe in insulin-resistant non-diabetic carriers of the Asn/Ser genotype than in insulin sensitive carriers, indicating that the phenotype is dependent upon insulin sensitivity. This interaction might be the result of the insulin resistance of cells important for lipoprotein metabolism [28], or to other genetic factors influencing insulin resistance. However, once hyperglycaemia is present, the influence of the Asn/Ser genotype on lipid levels vanishes.

In vitro studies of the Asn291Ser variant have shown that adipocytes from heterozygous carriers display 37% reduced LPL catalytic activity per nanogram protein [13], and that the serine allele may produce an enzyme that is less stable in its dimeric form [13,29]. In diabetic subjects, no association between the Asn/Ser genotype and dyslipidaemia could be found. This could be explained by an overproduction of VLDL particles in diabetic dyslipidaemia that overrides the influence of the Asn291Ser variant on LPL activity [30,31], or by the fact that most diabetic subjects are hyperinsulinaemic which might counteract reduced LPL activity.

In conclusion, it was found that the Asn291Ser variant of the *LPL* gene is associated with dyslipidaemia in normoglycaemic subjects, and that the dyslipidaemic phenotype is more severe in insulin-resistant subjects. This association is not seen in diabetic subjects.

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IV

The common PPARγ Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes

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Genetic association studies are viewed as problematic and plagued by irreproducibility[1]. Many associations have been reported for type 2 diabetes[2–17], but none have been confirmed in multiple samples and with comprehensive controls. We evaluated 16 published genetic associations to type 2 diabetes and related sub-phenotypes using a family-based design to control for population stratification, and replication samples to increase power. We were able to confirm only one association, that of the common Pro12Ala polymorphism in peroxisome proliferator-activated receptor-γ (PPARγ) with type 2 diabetes. By analysing over 3,000 individuals, we found a modest (1.25-fold) but significant (P=0.002) increase in diabetes risk associated with the more common proline allele (~85% frequency). Moreover, our results resolve a controversy about common variation in PPARγ. An initial study found a threefold effect[12], but four of five subsequent publications[18–22] failed to confirm the association. All six studies are consistent with the odds ratio we describe. The data implicate inherited variation in PPARγ in the pathogenesis of type 2 diabetes. Because the risk allele occurs at such high frequency, its modest effect translates into a large population attributable risk—influencing as much as 25% of type 2 diabetes in the general population.

Many factors may contribute to variable association results: multiple hypothesis testing, publication bias, ethnic stratification, population-specific linkage disequilibrium between markers and causal variants, inadequate statistical power, and gene-gene and gene-environment interactions. To evaluate reported associations to type 2 diabetes and test newly discovered alleles, we designed a study to address as many of these confounders as possible.

We used a multi-layered design (Table 1). Associations were first tested in 333 Scandinavian parent-offspring trios with type 2 diabetes or abnormal glucose homeostasis using transmission disequilibrium testing (TDT; ref. 23) to eliminate false positives due to population stratification. Alleles showing nominal association in the initial sample (P<0.05 in the direction of the original report) were tested for replication in three additional samples: 1,130 individuals from Scandinavian sibships discordant for type 2 diabetes, 481 case-control pairs from Scandinavia and 127 case-control pairs from the Saguenay-Lac-Saint-Jean region of Quebec, Canada. The use of sibships further addresses stratification (by sib-TDT (ref. 24) or related methods), whereas the case-control sample assesses population risk.

At least 16 common single-nucleotide polymorphisms (SNPs) have been associated with type 2 diabetes or related sub-phenotypes[2–17]. Notably, family-based controls were not used (except in a single report[16]). We genotyped the 16 variants in the initial 333 parent-offspring trios (Table 2). Three variants were rare or absent (<1% allele frequency). Our data cannot exclude a biological effect of these variants, but such low frequency rules out a significant impact on diabetes risk in our population. Of the remaining 13 variants, 11 showed either non-significant deviation from 50:50 transmission from heterozygous parents or a trend in the opposite direction from the original report. Because some of these variants were originally associated with quantitative phenotypes rather than diabetes itself, we genotyped an independent sample of 379 parent-offspring trios in which the offspring had normal glucose tolerance; using the TDTQ5 method[25], we found no association with the relevant phenotype (Table 2). Of course, such data do not rule out small effects; the statistical power

Table 1 • Characteristics of study populations

	Sex (male/ female)	Age (y)	Age at onset of diabetes (y)	BMI (kg/m^2)	HbA$_{1c}$ (%)	Fasting plasma glucose (mmol/l)	Plasma glucose at 2h OGTT (mmol/l)
TDT trios (Scandinavia)							
DM/IGT/IFG	176/157	39±9	37±9	27±5	5.9±1.8	7.2±2.6	8.5±2.9
NGT	187/192	31±10	–	24±5	5.1±0.5	5.2±0.5	5.6±1.1
Sibships (Scandinavia)							
DM/severe IGT sibling	283/329	65±10	56±11	29±5	7.3±1.7	9.4±3.5	14.2±5.5
NGT sibling	216/302	62±10	–	26±3	5.4±0.6	5.4±0.4	6.0±1.0
Case control (Scandinavia)							
DM/severe IGT subjects	252/229	61±10	54±11	28±5	7.5±1.8	9.7±3.2	15.3±5.5
NGT subjects	252/229	60±10	–	27±4	5.4±0.5	5.5±0.6	6.2±1.5
Case control (SLSJ)							
DM subjects	70/57	53±8	n/d	29±5	6.5±1.9	6.4±1.8	12.8±2.1
NGT subjects	70/57	52±8		29±4	5.1±0.6	5.1±0.6	6.1±1.1

Data are presented as mean ±s.d. Plasma glucose was measured at baseline (fasting) and 2 h after an oral glucose tolerance test (OGTT). DM, type 2 diabetes; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; NGT, normal glucose tolerance; severe IGT, 10.0 mmol/l >120 min blood glucose >8.5 mmol/l; SLSJ, Saguenay-Lac-St-Jean. n/d, not determined.

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obtained is represented in the upper limits of the confidence intervals (Table 2).

Table 2 • Association results of 16 published variants in 333 DM/IGT/IFG trios

Gene	Variant	Reported effect	T	U	Ratio	95% CI	*P* value	TDTQ5
ABCC8	C/T (exon 22)	higher risk DM[9]	26	12	2.17	1.18–4.71	0.012	
PPARG	Pro12Ala	lower risk DM[12]	81	104	0.78	0.59–1.05	0.045	
IRS1	Gly972Arg	higher risk DM[8]	30	26	1.15	0.71–2.03	0.30	
ADRB2	Gln27Glu	higher risk DM[14]	96	124	0.77	0.60–1.01	NS	
INS	–23A/T (HphI)	higher risk DM[16]	104	115	0.90	0.70–1.18	NS	
		paternal transmissions only	39	45	0.76	0.57–1.36	NS	
IRS1	Ala512Pro	higher risk DM[2]	14	21	0.67	0.36–1.37	NS	
KCNJ11	Glu23Lys	higher risk DM[13]	138	154	0.90	0.73–1.15	NS	
ABCC8	T/C (intron 24)	higher risk DM[9]	127	139	0.91	0.73–1.18	NS	
TNF	–238A/G	higher HOMA[17]	14	13	1.08	0.35–2.48	0.42	NS
ADRB3	Trp64Arg	higher 2 h insulin[3]	43	43	1.00	0.67–1.56	0.50	0.20
PON2	Ala148Gly	higher fasting glucose[10]*	122	133	0.92	0.72–1.18	NS	0.20
FABP2	Ala54Thr	higher fasting insulin[4]	112	120	0.93	0.73–1.21	NS	NS
GYS1	Met416Val	higher fasting insulin[11]	16	20	0.80	0.44–1.62	NS	NS
GCGR	Gly40Ser	higher risk DM[5]	1	4		too rare to analyse		
INSR	Val985Met	higher risk DM[15]	2	6		too rare to analyse		
IAPP	Ser20Gly	higher risk DM[7]	0	0		absent from our population		

T, Number of variant alleles transmitted from heterozygous parents of diabetes/impaired glucose homeostasis offspring; U, number of variant alleles not transmitted; Ratio, transmission ratio; 95% CI, 95% confidence intervals around the transmission ratio distortion; *P* value, one-tailed nominal *P* values are reported for trends in the same direction as the original report; NS, not significant, data trended in the opposite direction from the original report; TDTQ5, for variants originally reported to be associated with a quantitative trait, the nominal one-tailed *P* value is shown using TDTQ5 method[25] to analyse the relevant trait in 379 nondiabetic trios; DM, type 2 diabetes mellitus; OGTT, oral glucose tolerance test; HOMA (a measure of insulin sensitivity) is calculated as (fasting insulin)*(fasting glucose)/22.5 (ref. 17). *The *PON2* variant was associated with higher fasting glucose in diabetic subjects only.

Two variants showed nominally significant replication in the initial sample: a missense variant in *PPARG* (Pro12Ala) and a silent C/T polymorphism in exon 22 of *ABCC8* (Table 2). *PPARG* encodes PPARγ, a nuclear hormone receptor that regulates adipogenesis[26] and is a target for thiazoladinediones, medications used to treat diabetes[27]. The less frequent alanine allele (16%) was associated with decreased diabetes risk (81:104, odds ratio 0.78, $P<0.045$ one-tailed). *ABCC8* encodes the drug target for sulphonylurea antidiabetic medications[28]. The less frequent 'T' allele (3%) was associated with increased diabetes risk (26:12, odds ratio 2.2, $P<0.012$ one-tailed; Table 2). For both variants, transmission ratios were not significantly different from 50:50 in non-diabetic trios, ruling out transmission ration distortion (data not shown).

We attempted to further replicate these results in additional samples. The *ABCC8* association was not confirmed, as the trend in the additional samples was in the opposite direction from our initial results (sib-TDT Z-score=0.84; Scandinavian case genotypes were CC:375, CT:24, TT:1 and control genotypes were CC:363, CT:35, TT:2). These data suggest that the initial result might represent a statistical fluctuation, not unexpected when testing 13 hypotheses. By contrast, we confirmed the *PPARG* association (Table 3): the three follow-up samples demonstrated similar odds ratios (0.71, 0.74 and 0.88), with a pooled *P* value of 0.012. Combining our initial and replication samples, the *P* value is 0.002. Because our analysis was prompted by a previously reported association, and given the biological role of PPARγ, we believe these data constitute strong replication.

We then estimated the genotype relative risk (GRR) and population attributable risk of PPARγ Pro12Ala. The transmission ratio (TDT) and genotype counts (case-control) of the proline allele are consistent with codominant, additive, multiplicative or recessive models. Under any of these scenarios, the GRR of the proline allele is estimated as approximately 1.25. Because this risk allele is so common (frequency=83–87% in our samples), a GRR of 1.25 corresponds to a population attributable risk of approximately 25%. That is, these data indicate that if the population were monomorphic for the protective alanine allele, the prevalence of type 2 diabetes would be 25% lower.

We also examined whether Pro12Ala is associated with other phenotypes by applying the TDTQ5 method[25] to the 712 offspring in the 2 trio populations. No significant alteration in body mass index (BMI) or metabolic profile was observed (Table 4). We also compared the phenotypes of 206 sibling pairs concordant for diabetic status but discordant for *PPARG* genotype, and found no significant association to BMI (data not shown).

Our results resolve a controversy concerning the association between PPARγ Pro12Ala and type 2 diabetes. An initial report indicated that the alanine allele reduced diabetes risk by 75% (ref. 12). Of five[18–22] subsequent studies, four[18,20–22] failed to detect a statistically significant association, leading the authors to dismiss a role for Pro12Ala in diabetes risk; however, all published data are consistent with the modest effect that we have described (Fig. 1), although most individual studies lack sufficient sample size to reliably detect the association. In our study, a combination of samples was required to achieve adequate power. Combining all published studies yields an estimated risk ratio for the alanine allele of 0.79 ($P=0.00007$). Furthermore, our two family-based samples rule out population stratification as the cause of these results.

Table 3 • Replication of the PPARγ Pro12Ala association with diabetes risk in three independent samples

	Trios* (Scandinavia)	Sibships (Scandinavia)	Case-control (Scandinavia)	Case-control (SLSJ)	
Ala alleles transmitted	81				
Ala alleles not transmitted	104				
Ala alleles in DM sibs (DAT)		37			
Ala alleles in NGT sibs (DAT)		50			
z score (Sib TDT)		1.47			
Ala allele frequency in cases			14.6%	9.4%	
Ala allele frequency in controls			16.8%	13.5%	
Genotype frequencies, cases			336:117:9	105:20:2	
Genotype frequencies, controls			324:121:17	98:24:5	
Estimated risk ratio	0.78	0.74	0.88	0.71	
P value	0.045	0.071	0.10	0.08	
P value, three replication samples					0.012
P value, all data from this study					0.002

See Table 1 for descriptions of populations. DAT, discordant allele test. *Trio data is from Table 2, presented here for comparison. Genotype frequencies are presented as proline homozygotes:heterozygotes:alanine homozygotes. All *P* values are one-tailed in the direction of Deeb *et al.*[12]

Although PPARγ Pro12Ala is reproducibly associated with type 2 diabetes, this polymorphism may not be the aetiologic variant, but rather in linkage disequilibrium with it. The case for Pro12Ala being causative is strengthened by functional differences between the proline-containing and alanine-containing protein products[12,29] and by the lack of additional missense variants found in our systematic screening of 70 diabetic individuals (data not shown). Nonetheless, the causal variant may be some distance away (for example, in adjacent regulatory regions or conceivably in a nearby gene). To address this issue, we are identifying and testing haplotype associations in and around the gene.

We suggest that these data have several general implications. First, the low rate of replication (1/13 associations) indicates the importance of family-based controls and large, tiered samples to decrease false-positive reports[1]. Second, the previous difficulty demonstrating association of Pro12Ala with diabetes highlights the converse problem: that studies with modest sample sizes can fail to detect true associations. Third, although much can be learned from rare genetic variants (such as seven patients carrying rare amino acid changes in PPARγ; refs 30,31), PPARγ Pro12Ala highlights the potential importance of common alleles of weak effect. Pro12Ala (or possibly a variant in linkage disequilibrium with it) has a modest impact on individuals, but a dramatic effect on the human population: the risk allele is carried by billions of people, with a correspondingly high population attributable risk (25%). Critically, despite the population impact of common risk alleles, their contribution will be impossible to discover by linkage analysis: in a case such as Pro12Ala, the risk allele will typically be transmitted from both parents, requiring a genome scan of roughly 3 million sib pairs to obtain a lod score of 3. Thus, the genetic dissection of common diseases will surely involve association studies performed on large population samples.

Table 4 • Analysis of PPARγ Pro12Ala and metabolic phenotypes

Trait	Trio Population	Trait value (mean±s.d.) Pro/Pro	Pro/Ala	Ala/Ala	TDT Q5 *P* value
body mass index	DM/IGT/IFG	27.6±5.2	27.6±5.1	24.3±5.3	0.12
(kg/M^2)	NGT	24.8±4.4	25.8±4.7	28.3±7.6	0.78
systolic BP	DM/IGT/IFG	129±16	131±19	120±17	0.06
(mm Hg)	NGT	120 ±13	122±16	129±2	0.65
diastolic BP	DM/IGT/IFG	79±10	81±12	72±11	0.005
(mm Hg)	NGT	74±11	75±13	77±4	0.98
cholesterol	DM/IGT/IFG	5.4±1.1	5.5±1.0	5.2±1.1	0.13
(mM)	NGT	5.0±0.9	5.0±1.0	5.0±0.6	0.52
HDL	DM/IGT/IFG	1.2±0.3	1.2±0.3	1.4±0.4	0.02
(mM)	NGT	1.4±0.4	1.4±0.3	1.3±0.5	0.65
triglycerides	DM/IGT/IFG	1.9±1.5	1.6±0.8	1.6±1.4	0.85
(mM)	NGT	1.1±0.6	1.1±0.6	1.0±0.4	0.62
fasting glucose	DM/IGT/IFG	7.3± 2.7	7.2±2.3	5.5±0.6	0.19
(mM)	NGT	5.3±0.5	5.3±0.5	5.5±0.1	0.52
glucose 120 OGTT	DM/IGT/IFG	8.8±3.1	8.3±3.1	8.2±1.0	0.82
(mM)	NGT	5.5±1.2	5.8±1.0	6.2±1.2	0.97
fasting insulin	DM/IGT/IFG	10.7±6.9	14.0±17.0	6.8±2.8	0.16
(mU/L)	NGT	7.1±3.9	7.8±3.6	5.9±1.9	0.46
insulin 120 OGTT	DM/IGT/IFG	56.7±44.0	58.4±42.3	42.4±24.2	0.86
(mU/l)	NGT	33.9±27.0	34.6±23.0	21.0±13.0	0.44
HOMA	DM/IGT/IFG	3.7±3.6	5.3±11.8	1.7±0.8	0.12
	NGT	1.7±1.0	1.8±0.8	1.4±0.4	0.59

Metabolic phenotypes were evaluated in parent-offspring trios with both abnormal glucose homeostasis (DM/IGT/IFG, n=333) and normal glucose tolerance (NGT, n=379), in an oral glucose tolerance test (OGTT). For comparison purposes, the mean trait value and standard deviation for each genotypic class is presented. Note that the number of Ala/Ala homozygotes is very small, and thus the accuracy of trait measurements in this group is limited. HOMA, a measure of insulin sensitivity, is calculated as in Table 2. The reported *P* values are calculated using the family-based TDTQ5 (ref. 25). None of the *P* values are significant after correction for the number of hypotheses tested.

Methods

Patient populations. The clinical characteristics of the subjects included in the study are shown (Table 1). We selected Scandinavian families from three distinct family collections in Sweden and Finland: the Botnia collection, consisting of type 2 diabetic individuals from the Botnia region in western Finland[32]; the Helsinki Collection, including 290 multiplex families from northern and eastern Finland; and the Malmö collection, including 275 multiplex families from southern Sweden (A. Parker *et al.*, manuscript submitted). All subjects gave informed consent and the study was been approved by each of the three local ethics committees. The subjects underwent extensive phenotyping including oral glucose tolerance tests (OGTT), with venous measurements of blood glucose, serum insulin and C-peptide following ingestion of 75 g glucose. The WHO98 definitions of type 2 diabetes, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) were used[33]. To avoid confounding with type 1 diabetes, patients with age of onset under 18 years, GAD-ab ≥5 reference index units[32] and/or measured c-peptide levels ≤0.2 were excluded. Families with segregating mutations known to cause MODY diabetes were excluded.

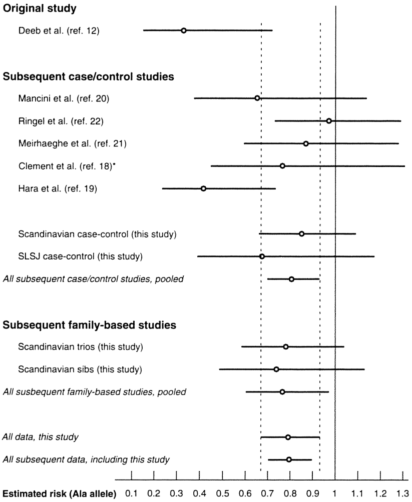

Fig. 1 Estimated risk (with 95% confidence intervals) for PPARγ Pro12Ala. For each study, the circle represents the estimated risk for the alanine allele and the line indicates the extent of the 95% confidence interval around this estimate. The dashed lines indicate the 95% confidence interval for pooled data in this study. Data are shown for odds ratios based on allele counts (for case-control studies) and the transmission ratios in trios or allele counts in phenotypically discordant sibs (for the family-based studies). Under a multiplicative model, these are all estimators of genotype relative risk. *Clement *et al.*[18] also studied severely obese individuals (BMI>40). As 56% of this group had abnormal glucose homeostasis at initial evaluation, and the likelihood of conversion to diabetes in the non-diabetic 'control' individuals was high, data from this group were not included. Inclusion of this group does not significantly affect either the pooled odds ratios or 95% confidence intervals. SLSJ, Saguenay-Lac-St-Jean.

Trio populations. Because transmission disequilibrium testing requires two living parents, a parent-offspring trio population is biased towards patients of young age. When studied, such individuals may not yet manifest the complete disease phenotype for a late-onset disorder such as type 2 diabetes. For this reason, we chose to include patients with IGT and IFG in our trio population. Epidemiological studies have shown that these disorders often constitute a 'pre-diabetic' state: between 25 and 50% of all subjects with IGT convert to type 2 diabetes within 10 years[34], and patients with IFG convert to type 2 diabetes at a similar rate[35]. Because our patients with IFG or IGT are young (average age 30) and are selected from families with multiple diabetic members, their abnormal glucose homeostasis serves as a strong predictor of eventual development of diabetes. We identified 333 such trios: 99 offspring had IFG, 108 had IGT and 126 had type 2 diabetes. To investigate phenotypes in a non-diabetic population, we studied a non-overlapping sample of 379 parent-offspring trios; all offspring were NGT. The offspring have a waist/hip ratio from either the upper quintile or the lowest decile as defined in a healthy control population.

Replication samples. To evaluate findings with a nominal P value <0.05 in the initial trio population, we also selected two independent replication samples from the same geographic areas. After excluding all subjects with age at onset of type 2 diabetes ≤30 y, we identified 481 of cases with type 2 diabetes or severe IGT (10.0 mmol/l $>$ 120 min blood glucose ≥ 8.5 mmol/l). Because there was no requirement for parental DNA in these samples, older subjects and a more stringent definition of affectation status were used. In our experience, patients with 'severe IGT' have a very high rate (25%) of conversion to overt type 2 diabetes within a three-year follow-up period (P. Almgren and L.G., unpublished data). We then assembled 481 age-, gender- and geographically matched normal glucose tolerance (NGT) controls. Specifically, affected individuals were matched to controls from the same geographic regions (Botnian cases were matched with controls from Botnia, and so on). In addition, 612 diabetic and 518 NGT sibs from 367 sibships discordant for diabetes or severe IGT were selected. The type 2 diabetic siblings had ages of onset ≥30 y and the non-diabetic (NGT) siblings were all older than 45. The same criteria for excluding MODY and type 1 diabetes were used for these samples. In addition, we tested a case-control sample from a geographically distinct population. These samples were unrelated adults (above 18 y) recruited from the region of Saguenay-Lac-St-Jean (Northeastern Quebec) and of French Canadian descent. Patients newly diagnosed with type 2 diabetes (using WHO98 criteria following a 75 g oral glucose load) were included, and each patient was age- and sex-matched with an individual from the same population having a normal glucose tolerance.

Genotyping. Genotyping was performed by either single-base extension with fluorescence resonance energy transfer (SBE-FRET; ref. 36) or single-base extension with fluorescence polarization (SBE-FP; ref. 37), using modified protocols (S.B. *et al.*, manuscript in preparation). PCR primers, SBE primers and PCR conditions are available on request. A mix (5 µl; containing 1 U shrimp alkaline phosphatase (Roche), 2 U exonuclease I (Epicentre), 150 mM Tris HCl, pH 8, and 15 mM $MgCl_2$) was added to 10 µl PCR product in a 384-well black polypropylene plate (MJ Research) and incubated at 37 °C for 45 min and 80 °C for 15 min. SBE reaction mixture (5 µl) was added (containing 0.5 U Thermosequenase DNA polymerase (Amersham), 200 mM Tris HCl, pH 9.5, 8 mM $MgCl_2$, 1.6 µM SBE primer and 0.2 µM each of ROX- and TAMRA-labelled ddNTPs specific to the alleles being assayed). Reactions were denatured for 2 min at 92 °C, followed by 50 cycles of 92 °C for 10 s and 50 °C for 30 s. Plates were read on an Analyst Fluorescence Plate-reader (LJL Biosystems); polarized fluorescence signals (mP values) for ROX and TAMRA were used to assign genotypes. For SBE-FRET, the SBE mix contained 5′-FAM-labelled SBE primer (0.4 µM) and ROX- and TAMRA-labelled ddNTPs (0.4 µM each). Six cycles of SBE (96 °C×15 s, 50 °C×30 s, 60 °C×30 s) were performed in an ABI 7700 (Perkin Elmer), reading during the extension phase. The difference in fluorescence between cycle 6 and cycle 1 was calculated for ROX and TAMRA after matrix correction for spectral overlap and these differences used to assign genotypes. In both methods, genotypes were assigned by clustering data from 96 to 960 individuals and assignments were reviewed by at least two individuals.

Statistical analysis. All phenotype data are expressed as mean±s.d. Transmission ratios from heterozygous parents in the trio population were calculated for transmission disequilibrium testing (TDT; ref. 23). The discordant sib sample was evaluated using the sib TDT comparing allele frequencies between affected and unaffected siblings[24]. To estimate the relative risk, we also performed a discordant allele test (DAT) with a randomly chosen affected sib and the oldest unaffected sib from each sib-ship[38]. Because only one sib-pair was used from each sibship, this is also a 'pure' test of association. χ^2 distributions were used to calculate P values for the TDT and DAT, and 95% confidence intervals were calculated using a binomial distribution. For the case-control samples, standard allelic odds ratios were calculated, and P values for differences in allele frequencies between cases and controls were determined by using a χ^2 distribution with one degree of freedom. For replication tests, one-tailed P values were calculated based on the direction of effect described in the original report. To pool P values, we calculated the expected mean and variance of the number of risk alleles found in cases given the number of samples analysed. These were combined to generate an overall mean and variance, and 2-tailed P values were calculated. This approach also allows combination of case-control studies with TDT and sib-TDT data, similar to the approach described[26]. To evaluate the effect of alleles on quantitative phenotypes in the trio populations, we used the TDTQ5 method[25]. TDTQ5 is a family-based test used to analyse the effect of offspring genotype on phenotype; by first regressing against parental genotype, stratification is avoided. Quantitative phenotypes were separately normalized for age for each gender before analysis; for all traits reported with TDTQ5, the phenotype distributions were found to be consistent with normality. Population attributable risk was calculated in standard fashion as $PAR=(X-1)/X$, where $X=(1-f)^2 + 2f(1-f)\gamma + f^2\gamma^2$, γ is the estimated GRR (in this case 1.25), f is the frequency of the risk alelle (in this case ~0.85) and a multiplicative model is assumed. As noted in the text, similar values of PAR are obtained assuming an additive or codominant model.

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V

Preliminarily accepted for publication in Diabetes

Association Between A Variant In The Phosphodiesterase 3B Gene And Hyperinsulinemia In Genotype-Discordant Sibling Pairs

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Subjects with type 2 diabetes and their first degree relatives show elevated postprandial FFA levels. Output of FFA from adipose tissue is downregulated by insulin via activation of phosphodiesterase 3B (PDE3B). PDE3B is also expressed in pancreatic β-cells and could be involved in postprandial regulation of insulin secretion. The aim of this study was to investigate if variants in the *PDE3B* gene increase susceptibility to type 2 diabetes or prediabetic phenotypes. Mutation screening of the gene in 41 subjects identified no novel variants in addition to a known variant in exon 4 (G1389A). Phenotypic characteristics were com-pared in 266 sibling pairs discordant for this variant. Two hours after an oral glucose tolerance test insulin concentrations were ele-vated in GG/GA vs AA siblings (57±4 vs 44±2 mU/l, p=0.0015*)*. Quantitative trans-mission disequilibrium test (TDT) in 157 parent offspring trios with abnormal glucose tolerance did not indicate association with two hour insulin levels. Haplotype TDT including other variants near the PDE3B locus revealed unequal transmission of several PDE3B/G1389A haplotypes to offspring with abnormal glucose tolerance. Analysis of sibling pairs discordant for genotype combinations in PDE3B and the sulphonylurea receptor gene further strenghtened the result (p=0.000035), suggesting interaction between these two loci. In conclusion, data from siblings discordant for the G1389A variant in the *PDE3B* gene suggest that variability in or near the *PDE3B* gene may contribute to elevated insulin levels.

Type 2 diabetes (non-insulin dependent, NIDDM) is a multifactorial and most likely polygenic disease defined by chronic elevation of plasma glucose (1). Several more or less monogenic forms of type 2 diabetes have been identified (2), representing about 5% of subjects with diabetes. Genetic factors that contribute to the disease in a polygenic fashion are expected to be more frequent. Such factors could influence insulin sensitivity or the capacity to compensate for increased insulin demands by increasing insulin secretion (3).

Phosphodiesterases hydrolyse cAMP, which is a key second messenger in intracellular signalling of hormones (4). Phosphodiesterase 3B (PDE3B) is specifically expressed in adipocytes, liver and pancreatic β-cells (5, 6). In the adipocyte, PDE3B is activated postprandially in response to insulin to

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decrease the output of free fatty acids (FFA) *in vivo* (7). Subjects with type 2 diabetes have an impaired ability to downregulate FFA output in response to insulin (8), resulting in increased serum levels of FFA which in turn could promote peripheral insulin resistance. In the hepatocyte PDE3B could influence endogenous glucose production, and in the β-cell PDE3B could modulate the regulation of insulin secretion (9-11). The presence of PDE3B in tissues important for glucose homeostasis makes PDE3B an interesting candidate gene for type 2 diabetes.

We screened the *PDE3B* gene for novel variants and examined if such variants are associated with type 2 diabetes and abnormal glucose tolerance or prediabetic phenotypes like insulin resistance or impaired β-cell function. To avoid confounding by ethnic admixture, we used family-based approaches to study association in genotype-discordant siblings and parent-offspring trios.

To identify new variants within the gene, we screened the entire coding region and flanking intronic segments in 40 subjects (including 20 subjects with type 2 diabetes) and one healthy control spouse with no known family history of diabetes by single strand conformational polymorphism technique (SSCP). One novel variant was identified in one diabetic subject, shifting in intron 6 T→G, 8 bp upstream of exon 7.

A known common biallelic variant in exon 4 (G1389A) (5) was selected for phenotypic analysis in genotype-discordant sibling pairs. The frequency of the 1389G allele was 59% (387 chromosomes of 652) among 326 control spouses with normal glucose tolerance. The frequency of AA, AG and GG genotypes were 15% (49 subjects), 51% (167 subjects) and 34% (110 subjects), respectively. Allele and genotype frequencies were in Hardy-Weinberg equilibrium ($\chi^2 = 1.2$, p=0.27).

From a cohort of 1840 siblings we identified 266 sibling pairs discordant for G1389A. The siblings had similar fasting plasma levels of lipids and FFA, and similar body composition (Table 1). Insulin resistance, determined by the homeostasis model assessment (HOMA-IR) index, fasting serum insulin and FFA levels two hours after an oral glucose tolerance test (OGTT) did not statistically differ between the siblings (Table 1).

The levels of serum insulin two hours after OGTT were higher in GG/AG siblings than in AA siblings (57±4 vs 44±2 mU/l, p=0.0015), particularly after correcting for plasma glucose levels (Table 1). This difference was seen in 168 non-diabetic pairs (57±12 vs 43±7 mU/l, p=0.00057) but not in 19 diabetic pairs without insulin-treatment (48±10 vs 52±13, p=0.72). The effect was less pronounced in 97 non-diabetic siblings matched for degree of glucose tolerance (both siblings had normal or impaired (IGT) glucose tolerance, p=0.031), indicating that this variant could be associated with or interact with IGT.

To try to replicate the findings in another family-based material, 157 parent-offspring trios (404 subjects from 126 nuclear families) were genotyped for the G1389A-variant and analyzed for phenotypic variation using the quantitative transmission disequilibrium test (QTDT). Sixty-four transmissions were informative in this locus but not correlated with insulin or insulin/glucose two hours after OGTT in the parental and offspring generations (χ^2=0.1, p=0.79).

The *PDE3B* gene is located on chromosome 11p15.1 (6), close to the sulphonylurea receptor (ABCC8) gene (Figure 1). Variants in this chromosomal region (11p15) have been associated with hyperinsulinemia and type 2 diabetes as summarized by Vionnet *et al.* (12) and Löbbert *et al.* (6). Mexican Americans homozygous for the 3819A allele of the ABCC8 gene are characterized by hyperinsulinemia (13). To exclude that the effect seen in our study was due to the concomitant presence of the G3819A variant in the *ABCC8* gene, we also stratified for the G3819A genotype. The association between *PDE3B*/G1389A and insulin levels two hours after OGTT was preserved in 112 sibling pairs concordant for *ABCC8*/G3819A (Table 2, p=0.048, one-sided test). Analysis of non-diabetic siblings discordant for both variants showed that 27 siblings AA for *PDE3B*/1389 had lower insulin levels in spite of also carrying the *ABCC8*/3819A allele previously associated with higher insulin levels (Table 2, 31±4 vs 80±15, p=0.000035). Analysis of non-diabetic siblings discordant for both variants in the opposite direction showed that 35 siblings that were GG for *ABCC8*/3819 and AA for *PDE3B*/1389 did not statistically differ with respect to insulin levels compared to their discordant siblings (Table 2, 47±9 vs 60±17, p=0.33). This implies that in our Scandinavian population the *ABCC8*/3819G allele interacts with *PDE3B*/1389G to increase serum insulin levels, either representing a physiological interaction or linkage disequilibrium with a third genetic factor.

To test if the *PDE3B*/G1389A variant is associated with type 2 diabetes or abnormal glucose tolerance we used the transmission disequilibrium test (TDT). A alleles were slightly overtransmitted to unrelated offspring with type 2 diabetes or abnormal glucose tolerance (113 transmitted and 86 untransmitted A alleles of totally 392 transmissions, $\chi^2=3.7$, p=0.056).

We also looked at the interaction between the *PDE3B*/G1389A locus and other variants in this chromosomal region (Figure 1; *KCNJ11*/Leu270Val (14), *KCNJ11*/Ile337Val (14), *ABCC8*/Ser1370Ala (15), *ABCC8*/G3819A (15), *ABCC8*/exon 22 C/T (15), *ABCC8*/exon 24 T/C (15), *INS*/A-23T (16) by following the transmission of haplotypes. *PDE3B*/1389A was overtransmitted together with *KCNJ11*/270Leu (p=0.012) and *INS*/-23A (p=0.028) to 108 offspring with abnormal glucose tolerance (Table 3). Vice versa, *PDE3B*/1389G was undertransmitted together with *KCNJ11*/270Val (p=0.0056), *KCNJ11*/337Ile (0.024), *ABCC8*/1370Ala (p=0.0094), *ABCC8*/exon 22/C (p=0.029) and *INS*/-23T (p=0.012) to these offspring. No other combination of examined haplotypes showed skewed transmission to these offspring. When the phenotype was changed to include also offspring with type 2 diabetes there was a skewed co-transmission of alleles independently of *PDE3B*/G1389A. This suggests that *PDE3B*/G1389A may be an important contributor to chromosome 11p15 haplotypes associated with abnormal glucose tolerance in this Scandinavian population.

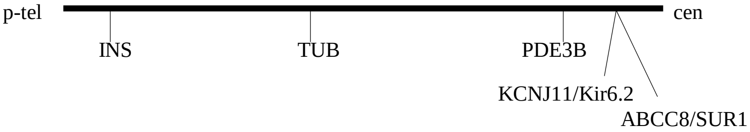

FIG 1. Organization of potential diabetes-related genes on the short arm of chromosome 11. Distances have been approximated from sequence drafts in the nucleotide database (NT_009062, NT_024163, NT_009306 and NT_009307) and information available at www.ncbi.nlm.nih.gov/locuslink. The distance between the genes are approximately 6 Mbp between INS and TUB, 8 Mbp between TUB and PDE3B and 1-2 Mbp between PDE3B and KCNJ11/Kir6.2. INS is the insulin gene. TUB is the human locus syntenic to the mouse tubby locus. PDE3B is the phosphodiesterase 3B gene. KCNJ11/Kir6.2 is the invardly-rectyfying potassium channel J11 gene. ABCCC8/SUR1 is the ATP-binding cassette C8/sulphonylurea receptor gene.

To our knowledge this is the first study examining a potential role for the *PDE3B* gene in the pathogenesis of abnormal glucose tolerance and type 2 diabetes, suggesting that variability in or near the PDE3B gene may contribute to elevated insulin levels. The findings further strengthen previous data that genetic variability in this region of chromosome 11 may be important in the pathogenesis of abnormal glucose tolerance and type 2 diabetes.

STUDY DESIGN AND METHODS

The study subjects were selected from Finland (including the Botnia region) and Southern Sweden and extensively phenotyped as previously described (17). All subjects have given informed consent, and the local ethic committees have approved the study. Diagnosis of diabetes, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) was determined by WHO criteria from 1998 (1) or by a previous diagnosis of diabetes and treatment with oral agents and/or insulin. Genomic DNA was extracted from peripheral blood lymphocytes using standard methods as previously described (18).

Genotyping samples for discordant sib-pair and TDT analyses. Sibling pairs were matched for age, gender and diabetes status, which identified 1840 siblings from 769 sibships. The siblings were genotyped for *PDE3B*/G1389A, and 266 sibling pairs (age 52±17 years, age difference 0.2±9.7 years, 117 male pairs, 149 female pairs and 77 pairs with type 2 diabetes) were discordant (one sibling GG or GA and the other AA). Healthy control spouses with normal glucose tolerance (150 men, 176 women, age 58±12 years, BMI 26±4 kg/m^2) were genotyped for G1389A for estimation of allele- and genotype frequencies. For the quantitative transmission disequilibrium test (QTDT) 157 parent-offspring trios (404 subjects from 126 nuclear families) independent of the sample of discordant siblings were genotyped for the G1389A-variant. All offspring had IGT or IFG (age 37±9 years). Identity-by-descent (IBD) status was estimated from genotype information from seven other variants in this region of chromosome 11p15 genotyped by single base extension: *KCNJ11*/Leu270Val, *KCNJ11*/Ile337Val, *ABCC8*/Ser1370Ala, *ABCC8*/G3819A, *ABCC8*/exon 22 C/T, *ABCC8*/exon 24 T/C, *INS*/A-23T (Hph1) (19). For the transmission disequilibrium test (TDT) one offspring with diabetes and/or IGT/IFG per family was randomly selected from a cohort of 196 parent-offspring trios with one or more offspring. This identified 114 trios with one diabetic offspring (age 43±11 years), 108 trios with one offspring that had IGT/IFG (age 37±8 years) and 196 trios with one offspring that had diabetes or IGT/IFG (age 40±10 years). Totally 222 trios (including 614 subjects from 196 nuclear families) were genotyped for the G1389A-variant. Exon 4 of the *PDE3B* gene was amplified by the polymerase chain reaction (PCR) and G1389A genotyped with HaeIII (New England Biolabs; G allele cut to 165 and 88 bp) [(5)]. A segment of *ABCC8* (*SUR1*) exon 9 (also referred to as exon 31 in the literature) was PCR-amplified using a mismatch reverse primer and G3819A genotyped with NlaIV (New England Biolabs; G allele cut to 125 and 25 bp) (13) (15). Oligonucleotide primers and PCR-conditions for amplification of these segments and the entire coding region of *PDE3B* are available in the appendix section (www.diabetes.org).

Single-strand conformational polymorphism (SSCP) mutation screening. Forty-one subjects were selected for the screening: Ten sibling pairs discordant for the variant with a large difference in insulin levels 2-hours after OGTT (sibling carrying GG or GA 107±17 vs sibling carrying AA 49±3 mU/l, age 55±16 years, 10 male, 10 female, all non-diabetic), twenty subjects with type 2 diabetes with high FFA-levels after an oral glucose load (fasting FFA 486±163 mmol/l and FFA 2 hours after OGTT 527±636 mmol/l, age 56±13 years, age at onset of diabetes 50±10 years, BMI 31±4 kg/m^2) and one healthy control spouse (male, 44 years, fasting FFA 741 mmol/l and FFA 2 hours after OGTT 165 mmol/l). Intronic primers amplifying the 16 exons in 19 fragments were used as previously described (5) with some modifications (see appendix at www.diabetes.org). The PCR reactions were performed as for the genotyping, with the addition of 0.5 μCi α-^{32}P-dCTP. The amplified products were separated with PAGE and samples deviating in band pattern were sequenced bidirectionally as previously described (18). Using this procedure, the estimated degree of mutation detection is around 95% in our laboratory.

Statistical analyses. Phenotypic differences between genotype discordant sibling pairs were compared using a simulation-based permutation test as previously described (20). The observed sum of differences (OSD) is the sum of all differences between sibling pairs. The OSD is compared to the total distribution of 10^6 permutations of the data set to determine the level of significance. The quantitative transmission disequilibrium test (QTDT) (21) was analyzed using the variance components model and with age, gender and BMI as covariates. TDT was performed using Genehunter 2.1 (22) implementing the TDT2 function to analyze haplotype data. The homeostasis model assessment (HOMA-IR) index was calculated as fasting insulin times fasting plasma glucose divided by 22,5 (23). Descriptive data are shown as mean ± standard deviation (SD) and comparative data as mean ± standard error of the mean (SEM). P-values of <0.05 were considered statistically significant.

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Appendix 1. Primers and conditions for PCR-amplification of the coding region of the *PDE3B* gene and exon 9 of the *ABCC8* (*SUR1*) gene.

Segment	Forward (sense) primer	Reverse (antisense) primer	Size (bp)	Temp (°C)
PDE3Bex1A	TACGAGCGGGGTGTGCTGAG	TCTCGGGTTCCGCTCCCAGC	343	64 [*, ‡]
PDE3Bex1B	CTCTCGCTGGGCGACCTGGC	CCGCGGACCCTGCGTCGCC	316	66 [*, †]
PDE3Bex1C	ACTTCTTGGTGTGGCAGTGG	ACTTCTTGGTGTGGCAGTGG	343	60 [*]
PDE3Bex1D	TGGCCCTGGGGTTGGATCAC	GCTCGCGTCCCAGACCTCGC	239	64 [*]
PDE3Bex2	GCTGAATGTCTTACATATAC	CTTTAAGTGTCAAAAGTTGC	210	52
PDE3Bex3	ATGCCATGTACAAATGAATG	CTATATAGAATTTAAAGGAGCAG	376	52 [*]
PDE3Bex4	ATATATAGCATATTAAAGAGTGAC	ATTGACAGAAACAAAGTAGATG	253	50
PDE3Bex5	TTAAATTGAGAACCATGAGG	ACACTACTATACTTTGAG	244	52
PDE3Bex6	CTACACACATAAATACATTC	AGGACTTAACTGTAATCAAC	324	50
PDE3Bex7	AACTTGTACCTCATTTACCG	GTAAGAAATTTCTCTTAAACTC	171	52
PDE3Bex8	AAGCTGTACATATAGGGCAC	CTGGGCAAAAGTAATGAAACC	287	56
PDE3Bex9	AATCCTAAGTGTTCTCATTG	CCATTACATATTAGATTTTGC	302	52
PDE3Bex10	AGCTACAAATAGAAATTCTG	GATGATAAAGAGTTTCAAAACC	232	50
PDE3Bex11	GTTGTGTGTTAAACCTAACAG	CTTGATTTACTATTCCTGATC	210	54
PDE3Bex12	CTCCATAGCAAATTGTGAG	AGGGATACACAGCATGACTG	334	50
PDE3Bex13	ATCTTTGAGGTGTCTGCCTC	CAGTAACACTGACAATAAAG	301	50
PDE3Bex14	TGCCTTCAGAACCTAAAATG	GTCTTCTCATGACAGCTC	255	54
PDE3Bex15	TCAGTGGTGGTTCTTTCACC	TGGAATACTCAACCTATAAC	361	50
PDE3Bex16	GCTTAGATACCTAGAATAGG	TGGGCACAACCCTCTGGGC	300	54
ABCC8ex9	GTAGAACAGGGTCCTGTGGC	AGGCCCACCAGGCCAGCAGAGAGGTC	150	62 [‡]

Primers are 5' to 3'. Size (bp) is the size of each PCR amplicon in basepairs and Temp (°C) is the annealing temperature. The segment amplified is annotated by exon number (*PDE3B* exons 1-16 or *ABCC8* exon 9, also referred to as exon 31 in the literature). *PDE3B* exon 1 was amplified with four overlapping segments (A, B, C and D). *ABCC8* exon 9 was amplified with a mismatch (underlined) in the reverse primer. PCR reactions were performed in a total volume of 20 µl with Pharmacia standard PCR-buffer or [*]16 mmol/l $(NH_4)_2SO_4$, 67 mmol/l Tris (pH 8.8) and 0.01% Tween 20; 1.5% formamide or [†]3% DMSO; 0.13 mM dNTP; 1.5 mM $MgCl_2$; 0.2 µM of both primers and 0.5 U Taq polymerase (Perkin Elmer, Foster City, CA) using 25 ng genomic DNA as template. PCR was performed with initial denaturation at 96°C for 3 min followed by 30 or [‡]35 cycles of denaturation (94°C for 30 s), annealing and extension (72°C for 30 s) and final extension at 72°C for 10 min.