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Niina Siitonen

Candidate Gene Studies on Body Size, Type 2 Diabetes and Related Metabolic Traits

Genetics of ADRA2B, ADIPOQ, ADIPOR1 and ADIPOR2 in the DPS Study Population

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ABSTRACT:

Obesity is a major risk factor for type 2 diabetes (T2DM), and both conditions are increasing rapidly worldwide. Excess adiposity is largely explained by lifestyle related factors, but genetic factors also contribute to an individuals's susceptibility to gain weight or develop T2DM.

Two entities can be separated in the pathophysiology of T2DM: 1) the compromised response of target tissues to insulin, namely insulin resistance, and 2) the failure of pancreatic beta cells to respond to increased requirement for insulin secretion. The majority of currently known susceptibility genes for T2DM are involved in beta cell function, whereas excess adiposity is a major determinant of insulin resistance.

The aim of the present study was to examine the associations between common genetic variants in biologically plausible candidate genes and traits relating to obesity and T2DM in individuals with impaired glucose tolerance (IGT) who were participating in a randomised lifestyle intervention study, the Finnish Diabetes Prevention Study (DPS). The candidate genes were selected based on their known functions in metabolic pathways and the variants were selected based on previous literature and the haplotype structure of the human genome.

The functional insertion/deletion variant 12Glu9 within the *ADRA2B* gene, encoding α2B-adrenergic receptor, was associated with acute insulin secretion measured in a subpopulation of the DPS, and with the risk of converting from IGT to T2DM particularly in individuals with central obesity.

Adiponectin is a regulatory molecule secreted by adipose tissue with insulin-sensitising, anti-atherogenic and anti-inflammatory effects. Common single nucleotide polymorphisms (SNPs) within the *ADIPOQ* gene, encoding adiponectin, were associated with serum adiponectin levels, the risk of T2DM and obesity related traits. Common SNPs in the gene encoding adiponectin receptor 1, *ADIPOR1*, were associated with various measures of body size in both men and women. In addition, differences in insulin levels according to *ADIPOR1* SNPs were seen, particularly in men. Variants in the gene encoding adiponectin receptor 2, *ADIPOR2*, were associated with the risk of cardiovascular event, and this finding was supported by tissue and allele specific differences seen in the mRNA expression levels.

In summary, these studies suggest that genetic differences in susceptibility to obesity and its comorbidities exist in individuals with an increased risk of T2DM. An interactive effect was seen between *ADRA2B* and lifestyle, whereas the effects of the adiponectin pathway variants were not modified by lifestyle. The results of these studies partly support the findings of earlier candidate gene studies, but also reveal novel associations.

National Library of Medical Classification: QZ 50, WK 810, WK 820, GN 66
Medical Subject Headings: Diabetes Mellitus, Type 2; Genes; Polymorphism, Single Nucleotide; Obesity; Body Size; Insulin; Adipose Tissue, Adiponectin

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TIIVISTELMÄ

Lihavuus on yleistynyt maailmanlaajuisesti viime vuosikymmeninä. Yleistymistä selittävät muutokset ruokavaliossa ja fyysisessä aktiivisuudessa, mutta myös perinnöllisillä tekijöillä on merkitystä. Lihavuudessa rasvakudoksen toiminta on häiriintynyt ja riski sairastua tyypin 2 diabetekseen sekä sydän- ja verisuonitauteihin on suurentunut. Rasvakudos toimii energiavarastona ja osallistuu monien elimistön toimintojen säätelyyn erittämiensä adipokiinien välityksellä. Adiponektiini on adipokiini, jonka pitoisuus verenkierrossa on vähentynyt lihavuudessa ja tyypin 2 diabeteksessa. Sillä on sokeriaineenvaihdunnan kannalta edullisia sekä sydän- ja verisuonitaudeilta suojaavia vaikutuksia.

Tyypin 2 diabeteksen taustalla voidaan erottaa kaksi mekanismia, joiden häiriintyminen johtaa veren sokeritasojen nousuun: 1) insuliinin eritys haiman beta-soluista sekä 2) kohdekudosten herkkyys insuliinille on heikentynyt. Useimmat tähän mennessä tunnistetut tyypin 2 diabetekselle altistavat geenit liittyvät beta-solujen toimintaan, kun taas insuliiniherkkyys näyttäisi kytkeytyvän kiinteästi lihavuuteen.

Tämän työn tarkoituksena oli selvittää eräiden kandidaattigeenien merkitystä lihavuuden ja tyypin 2 diabeteksen taustalla yksilöillä, joilla on heikentynyt glukoosinsieto ja näin ollen suurentunut riski sairastua tyypin 2 diabetekseen.

ADRA2B-geenin toiminnallinen polymorfia, 12Glu9, oli yhteydessä ensivaiheen insuliinin eritykseen ja sitä kautta riskiin sairastua tyypin 2 diabetekseen. Glu9-alleelia kantavilla henkilöillä insuliinin eritys oli madaltunut ja tyypin 2 diabeteksen riski suurentunut. Elämäntapamuutokset näyttivät ehkäisevän riskialleelin kantajien sairastumista, kun taas vyötärölihavuus lisäsi entisestään riskialleelin kantajien sairastumisriskiä.

Adiponektiinia koodaavan *ADIPOQ*-geenin useilla varianteilla havaittiin yhteys lihavuuteen ja tyypin 2 diabetekseen liittyviin muuttujiin sekä seerumin adiponektiinipitoisuuteen. Adiponektiinireseptoria 1 koodaavan *ADIPOR1*-geenin sekvenssimuunnokset olivat yhteydessä kehon kokoon ja koostumukseen sekä insuliinitasoihin. Adiponektiinireseptoria 2 koodaavan *ADIPOR2*-geenin variaatiot vaikuttavat riskiin sairastua sydän- ja verisuonitauteihin. Lisäksi *ADIPOR2*-geenin ilmenemisessä havaittiin eroja yksitumaisissa valkosoluissa.

Nämä tulokset osoittavat, että geneettiset tekijät saattavat vaikuttaa yksilöllisiin eroihin alttiudessa lihoa ja sairastua lihavuuden liitännäissairauksiin.

Luokitus: QZ 50, WK 810, WK 820, GN 66
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Niina Siitonen

"I may not have gone where I intended to go,
but I think I have ended up where I needed to be."
Douglas Adams

List of the original publications

This dissertation is based on the following original publications:

I Siitonen N, Lindström J, Eriksson J, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Laakso M and Uusitupa M; Association between a deletion/insertion polymorphism in the alpha2B-adrenergic receptor gene and insulin secretion and Type 2 diabetes. The Finnish Diabetes Prevention Study. *Diabetologia. 2004; 47(8):1416-24.*

II Siitonen N, Pulkkinen L, Lindström J, Kolehmainen M, Eriksson JG, Venojärvi M, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J and Uusitupa M; Association of *ADIPOQ* gene variants with body weight, type 2 diabetes and serum adiponectin concentrations: the Finnish Diabetes Prevention Study. *BMC Med Genet. 2011; 12(1):5*.

III Siitonen N, Pulkkinen L, Mager U, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Laakso M and Uusitupa M; for the Finnish Diabetes Prevention Study Group.; Association of sequence variations in the gene encoding adiponectin receptor 1 (*ADIPOR1*) with body size and insulin levels. The Finnish Diabetes Prevention Study. *Diabetologia. 2006; 49(8):1795-1805.*

IV Siitonen N, Pulkkinen L, Lindström J, Kolehmainen M, Schwab U, Eriksson JG, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J and Uusitupa M; Association of *ADIPOR2* gene variants with cardiovascular disease and type 2 diabetes risk in individuals with impaired glucose tolerance: the Finnish Diabetes Prevention Study. *Cardiovasc Diabetol.* 2011 Sep 24; 10(1):83.

Contents

Abbreviations

ADIPOQ	adiponectin gene
ADIPOR1	adiponectin receptor 1 gene
ADIPOR2	adiponectin receptor 2 gene
ADRA2B	α2B-adrenergic receptor gene
AIR	acute insulin response
ANOVA	analysis of variance
APPL1	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1
AR	adrenergic receptor
AT	adipose tissue
bp	base pair
CAD	coronary artery disease
CEU	Utah residents with ancestry from Northern and Western Europe
CNS	central nervous system
CNV	copy number variation
CVD	cardiovascular disease
DI	disposition index
DPS	the Finnish Diabetes Prevention Study
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FA	fatty acid
FDR	false discovery rate
FFA	free fatty acid
FMD	flow-mediated dilatation
FSIGT	frequently sampled IVGTT
GLM	general linear model
GWAS	genome-wide association study
HDL	high-density lipoprotein
HOMA-IR	homeostasis model assessment of insulin resistance
HMW	high-molecular weight
HWE	Hardy–Weinberg equilibrium
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL	interleukin
IMT	intima-media thickness
indel	insertion/deletion polymorphism
IVGTT	intravenous glucose tolerance test
LD	linkage disequilibrium
LDL	low-density lipoprotein
MAF	minor allele frequency
MC4R	melanocortin 4 receptor
MODY	maturity-onset diabetes of the young
mya	million years ago
NO	nitric oxide
OGTT	oral glucose tolerance test

PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
S_I	insulin sensitivity index
SNP	single nucleotide polymorphisms
T2DM	type 2 diabetes
TNF	tumour necrosis factor
TG	triglyceride
UTR	untranslated region
WC	waist circumference
WHR	waist-to-hip ratio

1 Introduction

This series of studies examined the role of four candidate genes in obesity, type 2 diabetes (T2DM) and related phenotypes. The following literature review provides insight into the genetics of these complex phenotypes and also focuses on the functions of the candidate gene products.

Complex diseases and quantitative traits, such as T2DM and obesity, are caused by a combination of multiple environmental and genetic factors, and their interactions. Although recent changes in human diet and lifestyle explain the rapid increase in the prevalence of obesity and its co-morbidities, these traits also have a strong genetic component as suggested by their aggregation in families and different prevalence rates in different ethnic groups. Moreover, the heritability estimates are high, ranging from 40 to 70% for obesity and from 25 to 75% for T2DM.

The first draft of human genome was finished in 2001. A more complete picture of the genome and genetic variation among humans is emerging as more individuals are being sequenced and genotyped. Our genome contains approximately three billion base pairs and 20 000 protein coding genes. Genetic variation among humans contributes to phenotypic variation and also to susceptibility to complex diseases. The most prevalent category of genetic variants is single nucleotide polymorphisms (SNPs), which are widely used as tools in genetic association studies.

Various approaches can be used to study the genetic factors underlying complex traits. Recently, family-based approaches have been largely replaced by population based association studies performed most often in large case-control cohorts. By taking advantage of linkage disequilibrium (LD), the tendency of genetic markers to appear together more frequently than would be expected by chance, the majority of common genetic variation in a given locus or across the whole genome can be captured by selecting an appropriate set of SNPs for genotyping. The candidate gene approach is based on the knowledge of disease or trait biology, whereas genome-wide association studies performed in large study populations are hypothesis-free approaches that target common genetic variation across the genome. In the future, also other types of genetic variation, such as rare variants, copy number variants (CNVs) and epigenetic modifications will be of increasing interest.

In this work, variants in obesity and T2DM candidate genes were genotyped in the participants of the Finnish Diabetes Prevention Study (DPS). The candidate genes were selected based on previous research evidence demonstrating their potential role in molecular pathways underlying these traits. In addition to studying the effects of these gene variants on the phenotypes of interest, the aim of these studies was to examine the potential interactive effects between genetic and lifestyle factors.

2 *Review of literature*

2.1 THE HUMAN GENOME

The first draft of human genome sequence was completed in 2001 by the international collaboration, the Human Genome project (1), and a more complete version of the genomic sequence followed 2004 (2). The haploid human genome comprises approximately 3 billion base pairs (bp) and contains approximately 20 000 protein coding genes (1.5% of the genome), which is much lower number than previously expected (2, 3). Although the functions of the remaining parts of the genome are currently poorly understood (4), the diversity of human genome output is expanded greatly by multiple levels of gene expression regulation. A wide variety of regulatory sequences exist locating either within the genes they regulate or in other parts of the genome even great distances away (5). In addition, protein-DNA and RNA-DNA interactions, and epigenetic modifications of DNA control gene expression (6). Furthermore, the expression of the genome is also regulated by small RNA products (miRNA and siRNA), via alternative splicing, and at translational and post-translational levels (6).

2.1.1 Genetic variation

As a species, humans are genetically very homogeneous, and the genomes of different individuals differ only by 1-3% (3). In general, the genetic differences between individuals are referred to as variants, and a term polymorphism is used when the minor allele has a frequency greater than 1%. The two or more different forms of a variation are called alleles, and haplotype refers to a collection of alleles arranged linearly on a single chromosome that are inherited together (6). The inter-individual genetic variation, acting together with environmental influences, accounts for variability in physiological traits and susceptibility to various diseases.

A single genetic variant may be neutral without any detectable effect on phenotype (the observable characteristics of an individual), or it may result in altered expression, structure or function of the gene product. Evolution is a process in which the genetic make-up of populations is modified over time and over generations by natural selection. This gradual process favors individuals, and thus the genetic variants they carry, who are best adapted to their environments in terms of survival and reproduction.

Human genomic variation ranges from single base pair substitutions or point mutations to large, microscopically visible chromosomal rearrangements. The public Single Nucleotide Polymorphism Database (dbSNP build 132) contains more than 30 million human genetic variants, mostly SNPs and small insertions/deletions (indels), but also microsatellites (7)

Single nucleotide polymorphisms

SNPs are the most common type of genetic variation and occur throughout the genome approximately at every 500-1000 bases (8). SNPs are usually bi-allelic single nucleotide variants in the DNA sequence that differ between individuals or pairs of chromosomes of a single individual (9). SNPs may lie within protein coding sequences, non-coding regulatory sequences, or intergenic regions. Synonymous (or silent) SNP locates within the coding sequence, but does not alter the amino-acid sequence of the protein product. Non-synonymous SNPs cause alterations in the amino acid sequence and may be further categorised as missense SNPs (resulting in change of amino acid) or nonsense (resulting in premature stop-codon and truncated protein product). SNPs in non-coding regions may affect phenotype through alterations in regulation of gene expression or splicing. SNPs are easily assayed and widely used as markers by genetic and genomic approaches aiming to clarify the genetic background of complex diseases.

Other types of genetic variants

Small or large fragments of DNA may be removed (deletion), inserted (insertion), translocated to another location (translocation) or inversed (inversions). They range in size from one to thousands of base pairs and may be neutral or may affect phenotype with varying degrees. CNVs are large duplications, insertions, deletions and translocations of 1 kb to several million bp, while variants involving 100-1000 bp are referred to as indels (10). CNVs are turning out to be much more frequent than expected and are already implicated in certain complex diseases, such as Chrohn's disease, autism and schizophrenia (10, 11). Genomic variants are even larger-scale structural variants that have been implicated in syndromic anomalies and diseases, and are usually caused by de-novo chromosomal rearrangements unlike CNVs, which are most commonly inherited (10). Their role in population diversity and disease states is currently under extensive investigation. Micro- and minisatellites are highly polymorphic tandem repeats that vary among individuals and are utilised in genetic mapping studies (12).

Resources of human genetic variation

The International HapMap project was launched in 2002 with a goal to catalogue patterns of common genetic variation and LD in different human populations (13). LD describes a situation where alleles occur together more often than expected by chance alone, and may be exploited in planning genetic association studies. In phases I and II of HapMap, over 3.1 million common SNPs were genotyped in 270 individuals from four geographically distinct populations (14). In phase III, this public resource was expanded to include SNPs with lower allele frequency as well as CNVs, and common SNPs from seven additional populations (15).

The 1000 Genomes Project aims at identifying and genotyping all forms of human genomic variation by combining low-coverage whole-genome sequencing, array-based genotyping, and targeted high-coverage sequencing of all coding regions in 2500 individuals from five major human population groups (16). The pilot phase of the project described allele frequencies, locations and local haplotype structures for 15 million SNPs, 1 million short indels and 20 000 structural variants. Based on the results of the pilot study, each human genome is heterozygous for 50-100 variants implicated in inherited diseases according to the Human Gene Mutation Database, and approximately 250-300 genes per individual are affected by loss-of-function variants (16).

Epigenetics

Epigenetics refers to chemical changes of the DNA molecule that do not alter the primary sequence of the genome, but are heritable during cell division (17). The best-known examples of epigenetic modifications are the methylation of cytosines in cytosine-guanine di-nucleotides (CpG) and the modification of DNA packing proteins, histones, both of which may affect DNA transcription (17). Epigenetic modifications are affected by environmental and genetic factors, and the understanding of their role in the pathogenesis of complex diseases, most notably cancer, has advanced substantially over the past years (6, 17).

2.2 EVOLUTIONARY PERSPECTIVE TO OBESITY AND TYPE 2 DIABETES

2.2.1 Evolutionary perspective

During evolution, the human genome has been subjected to selective pressures relating to ecological conditions, such as changes in food supplies and climate. Part of our genome was modified even before the emergence of our genus, whereas parts of it have changed relatively recently. Understanding the genetic adaptations relating to diet and metabolism is crucial in order to understand current nutritional needs of humans and the underlying mechanisms of complex diseases associated with modern diets and lifestyle (18, 19).

Variety and flexibility are common characteristics of the diets of humans as well our primate relatives. The diets of wild chimpanzees, our closest extant relatives, are low in fat compared to diets of contemporary humans, and consist mainly of fruits, but also include leaves, nuts and insects, and occasionally minor amounts of meat (18, 20, 21). Captive chimpanzees with reduced levels of physical activity and diet rich in animal tissues and dairy, are susceptible to obesity and have increased risk of cardiovascular disease (CVD) (20, 22)

Our evolutionary lineage (hominin) diverged from that leading to common chimpanzees and bonobos approximately 6 million years ago (mya) (23). The major dietary shifts in our species' history include: adaptations to new food sources of woodlands and savannah (~4.4 mya), introduction of meat eating (~2.5 mya), inventing cooking (~1.5 mya-800 000 years ago), dispersion from Africa to highly variable habitats (~60 000 years ago), agricultural revolution (starting ~10 000 years ago) and industrial revolution (~200 years ago) (19, 21). All of these events go hand in hand with significant changes in anatomy, culture, social structures and technology, and are often connected with changes in global climate.

The energy expenditure level of early hominins was high and their plant-based diets included a wide range of food resources with sufficiently macro- and micronutrients, low fat content and high intake of fibre (21). By 2.5 mya hominins were becoming omnivorous with meat consumption providing higher caloric gains, but also increases in protein, iron and other important dietary components (20, 24). The introduction of cooking, on the other hand, increased the digestibility of both plant and animal derived foods (19, 25). Both meat eating and cooking, have been suggested to have been facilitating the increase in brain size and the coincidental reduction in gut size (19, 20, 24, 25). Moreover, since the brain is an energetically expensive organ, encephalisation was likely associated with increased body fatness to ensure sufficient energy stores especially during infancy, childhood, pregnancy and lactation (24, 26, 27). Relating to this, the asymmetry in reproductive cost between men and women may have resulted in differential adaptive strategies regarding fat storage, and may explain the current differences in adipose tissue (AT) distribution and metabolism (27).

The first anatomically modern human appeared approximately 200 000-150 000 years ago in eastern Africa (28). The migration out of Africa was accompanied by a significant population bottleneck. The size of the migrating founder population is estimated to have been approximately 1000 effective individuals, which together with the subsequent rapid population expansion, explains the low genetic diversity and high LD observed across all contemporary non-African populations (28). Modern humans (mostly) replaced the existing hominin species in Europe and Asia and colonised the entire globe by 25 000 years ago through large numbers of subsequent population bottlenecks of smaller amplitude (28). Since then, human populations have adapted to a wide variety of habitats with very different food sources, climates and ecology.

The diets of the Paleolithic hunter-gatherers varied greatly with latitude and season, and humans remained remarkably flexible eaters (26, 29). Generally, the amount of dietary carbohydrates varied inversely with meat intake, diets were high in protein, polyunsaturated fat, fiber, and most micronutrients, but low in saturated fat and sodium (18, 29). In addition, the energy expenditure was high compared to modern day settings. According to some estimates hunter-gatherer males typically spent 19.6-24.7 kcal/kg/day in physical activity whereas the corresponding value for the modern sedentary office worker is 4.4 kcal/kg/day (30).

2.2.2 Agricultural revolution and modernisation

Approximately 10 000 years ago agriculture, based on plant and animal domestication, began in various parts of the world and resulted in major changes in lifestyle and dietary patterns (26). In terms of nutrition, the breadth of food sources decreased dramatically with increased reliance on a few species of high-carbohydrate cereal crops and tubers (19, 21, 26). In addition, the supply of food fluctuated throughout the year and severe periodic famines were common. Analyses of skeletal materials suggest significantly reduced adult stature and a number of

nutrition related pathologies as a result of protein deficiencies and malnutrition in early agriculturalists (21, 26).

While early agriculture was labour intensive, industrial revolution and mechanisation has resulted in dramatic decrease in the energy cost of daily activities. Although dietary breadth has been at least partly restored and fluctuations in food availability reduced, modern diets have created another set of deleterious metabolic problems, including obesity and T2DM epidemics (21). The availability, a proxy for actual consumption, of fats, oils, meat, cheese, and frozen dairy products has increased in the USA over the past century (31). Increasing 10-year trends in total energy supply per capita has been found in most high-income countries and China from the mid-1980s to the mid-1990s (32). More specifically, consumption of highly processed and energy-dense foods and drinks has increased rapidly in higher-income countries during the past decades (33). In addition, general trends of occupational and transportation physical activity are decreasing and the proportion of sedentary individuals is increasing in many industrialised countries (34, 35).

2.2.3 The mismatch between genes and lifestyle

Changes in our lifestyle, induced by the agricultural revolution and especially those that have followed modernisation, have been extremely rapid. The pace of genetic adaptation generally occurs far more slowly even though a few relatively recent genetic adaptations have been described. The resulting mismatch between our genome and the modern environment is hypothesised to explain the increased prevalence of chronic degenerative diseases observed today. Few of the most important hypotheses concerning this mismatch are discussed here briefly.

The thrifty genotype hypothesis

According to the thrifty genotype hypothesis, proposed by Neel in 1962, the ability to store energy efficiently during feast periods gave a selective advantage to our hunter-gatherer ancestors during subsequent famine periods. Neel suggested that gene variants conferring efficient energy storage were favoured by natural selection, but are deleterious in modern environment where high caloric foods are continuously available predisposing their carriers to obesity and T2DM (36). Neel emphasised the role of quick insulin trigger after a meal in enhancing the glucose preservation. In contrast, according to the "not-so-trifty genotype" – hypothesis, muscle insulin resistance was considered evolutionarily beneficial in maintaining muscle mass during famine (37). In both models, temporary hyperinsulinemia and insulin resistance are considered to be survival mechanisms during fluctuations of energy availability, that only become pathogenic and persistent under conditions of surplus supply of energy (38).

Several aspects of Neel's original hypothesis have been challenged afterwards. It has been pointed out that famines were probably rare in pre-Neolithic populations as the hunter-gatherers exploited a wide variety of food sources and were able to change location in order to follow food resources (39, 40). Moreover, it has been suggested that famines were too rare and insufficient to act as a selective force on survival, and that random genetic drift, in combination with lack of selection, explains the genetic predisposition to obesity (41) or that the target of natural selection was not survival, but reproduction during regular famines of post-agricultural era (39). Others have emphasised the role of the large human brain in favouring enhanced energy storage. The energy requirements of the brain are largest in infancy, when brain metabolism accounts for >60% of resting metabolic rate, and early childhood. Early childhood also affect indirectly maternal energy metabolism through lactation (40, 42, 43).

The thrifty phenotype hypothesis

The "thrifty phenotype" hypothesis (or the developmental origins hypothesis), on the other hand, states that conditions during specific windows of early development can have

longstanding effects on metabolic pathways and physiology thereby influencing disease susceptibility later in life (44). The developmental origins hypothesis is supported by numerous epidemiological studies demonstrating a link between early development and the occurrence of diseases later in life (45). The mechanism linking prenatal conditions to later disease risk may involve epigenetic modifications (17).

Our genetic legacy

Metabolic thriftiness, genotypic or phenotypic, may be considered as an ability to reduce energy expenditure or to store energy rather than spend it (46). Bouchard has suggested five non-mutually exclusive categories for potential thrifty genes: 1) genes involved in regulation of metabolic rate or thermogenesis, 2) genes involved in appetite and satiety regulation, 3) genes predisposing to physical activity vs. inactivity, 4) genes regulating lipid oxidation and 5) genes involved in adipogenesis and lipid storage capacity (47).

As a species, humans represent general thriftiness compared with many other species, reflecting the metabolic adaptations early in our evolutionary history (46). We have lower muscle mass and higher adiposity compared with other primates and other mammals (43). This may be regarded as a thrifty trait, since the energy requirement of AT is significantly lower compared with that of muscle tissue (42). In addition, fat provides energy storage that buffers against environmental fluctuations in nutritional resources (42, 43). Human infants have higher adiposity compared with any other mammalian species and adiposity continues to increase during the first months declining thereafter in early childhood (43), and increasing again during adolescence when sexual dimorphism in adiposity emerges (46).

Population differences in susceptibility to obesity may be attributable to genetic adaptations to specific ecologic niches that have emerged since the last common ancestor (46). These differences reflect more localised selective pressures and affect different components of human metabolism (40). Negative selection acts to decrease population differences globally at amino acid altering mutations, whereas positive selection increases population differences through regional adaptations primarily at nonsynonymous and regulatory variants (48). For this reason, it is important to remember that alleles associated with complex diseases could also be geographically restricted highlighting the importance of analysing multiple populations with different demographic histories. In the case of complex diseases, the risk alleles are not necessarily new mutations, but rather ancestral alleles whose effects have become disadvantageous along with recent lifestyle changes (48).

2.3 COMPLEX DISEASES AND QUANTITATIVE TRAITS

Complex human diseases and quantitative traits are caused by multiple genetic, environmental and lifestyle related factors as well as their multifaceted interactions. They aggregate in families, indicating a genetic component, but do not follow clear Mendelian inheritance patterns, and are to a large extent influenced by non-genetic factors. Unlike in single gene disorders, the presence of a particular gene variant is neither necessary nor sufficient to cause a disease, but instead confers a modest alteration in disease susceptibility. The vast majority of all human diseases and quantitative phenotypic traits, including certain types of common cancers, T2DM, CVD, asthma, autoimmune diseases, obesity and height, have a complex aetiology. The contribution of genetic factors and the genetic architecture varies across complex phenotypes. The discussion here is mostly limited to obesity, T2DM and related traits.

Heritability

Heritability is an important parameter that estimates the proportion of the total phenotypic variation of a complex trait that can be attributed to genetic variation (49). Heritability can be

estimated by using twin or family studies, and it allows comparison of the relative importance of genetic and non-genetic factors. Heritability does not, however, describe the magnitude of each component and is always specific to a particular population and environment (50).

Genetic background of complex phenotypes

During the past few years genome-wide studies have been successful at identifying SNPs associated with common complex diseases and quantitative traits. The genetic variants characterised thus far are mostly common (minor allele frequency, MAF>5%) and thereby fit the traditional "common disease–common variant" –hypothesis, which assumes that common diseases are largely attributable to allelic variants present in 1-5% of the population (51). Individually such variants have only small effects on the disease risk, but collectively they may increase the risk substantially. Rather than affecting the structure or function of the gene product itself, these variants often have modest direct or indirect effects on gene expression (10-20%) or splicing (49, 52). On the other hand, the common variants currently identified explain only a small proportion, less than 10%, of genetic variance for most complex traits (53). This leads to the conclusion that either the heritability measures of complex traits are overestimated or different types of gene variants explain significant portion of the genetic component, and consequently, the focus is now gradually shifting from common SNPs to other types of genetic variants. Importantly, however, each complex disease and phenotype has its own unique genetic architecture, and thus diverse strategies may be necessary in finding the missing heritability in each case.

It has been hypothesised that a significant part of the unidentified genetic component of complex traits is accounted for by rare and diverse loss-of-function alleles, which in a homozygous state would have severe consequences (explaining the low population frequencies), but in heterozygous state would lead to a 50% loss in expression levels (49). The effects of these variants are too small to display clear inheritance patterns in family-based studies, but on the other hand, these variants are too rare to be captured by traditional association strategies (49). Efforts to systematically identify these variants are now emerging and the early results have already revealed that the low-frequency (MAF 0.5-5%) and rare (MAF<0.5%) variants by far outnumber the common variants in the genome, and are likely to contribute significantly to genetic component of many complex diseases (16). In addition to common and rare variants, epigenetic modifications, CNVs and gene-gene interactions may contribute significantly to the phenotypic variation and disease susceptibility in the population (4).

Characterising the genetic basis of each complex condition is further complicated by genetic heterogeneity and pleiotropy. Similar phenotypes may result from distinct combinations of individually rare variants. Moreover, separate variants within a given gene (allelic heterogeneity) or in different genes of same or related molecular pathways (locus heterogeneity) may lead to the same disorder. Finally, the same genetic variant may have pleiotropic effects on multiple phenotypic traits and may thus lead to different clinical manifestations in different individuals. It is thus likely that many complex human diseases represent a collection of aetiologically distinct conditions with different underlying genetic components. Identifying genetic variants underlying complex diseases will help in separating molecular subtypes from each other, which in turn has important implications for disease prevention and treatment.

2.4 METHODS TO STUDY GENETICS OF COMPLEX DISEASES

The rapid technological developments, declining costs and availability of larger sample sizes have induced major transformations in the field of disease gene discovery. Moreover, availability of public resources, such as the dbSNP (7), the HapMap project (15), and the 1000

genomes project (16) has proven invaluable for designing genetic association studies. Over the past decades, research has gradually progressed from family-based linkage studies via candidate gene approaches to genome-wide association studies (GWAS). In the future, the greatest challenges will involve setting global criteria for phenotype data and also managing and analysing enormous amounts of sequence data (3).

2.4.1 Family-based linkage studies

Genetic linkage refers to coinheritance of a genetic marker with a phenotypic trait in a family with multiple affected members (54). The basic idea is to enrich individuals with common genetic background and thus to increase statistical power to detect a genetic effect. Genetic markers are followed in a pedigree with the aim of finding markers that lie close to the unknown disease-causing variation. Linkage studies are powerful at locating even relatively rare variants with large effect sizes, but have limited power to detect variants with modest effect sizes. Other shortcomings of family studies include difficulty of collecting large number of families with sufficient numbers of affected individuals, and complexity of computational methods. Moreover, the chromosomal regions identified are large, often comprising even hundreds of genes, and identifying the disease associated genes and variants is challenging (55). In case of obesity and diabetes, family-based studies have been succesful in identifying variants responsible for extreme and early-onset forms that segregate in families, such as maturity onset diabetes of the young (MODY), mitochondrial diabetes with deafness, neonatal diabetes, and rare forms of severe childhood obesity (56).

2.4.2 Population based association studies

Genetic association is defined by a non-random occurrence of a genetic marker with a trait (54), and an association between genetic variant and phenotype is expected, when the variant has a functional effect or when it is in LD with a functional variant (57).

Compared with family-based studies, recruiting large numbers of unrelated subjects is often easier and genetic association studies usually result in more accurate localisation of the functional variant (58). On the other hand, population stratification, locus or allelic heterogeneity, and false-positive findings due to the large number of tests performed may lead to erroneous conclusions (58).

Modern large-scale population based studies have proven powerful at identifying gene variants with small to modest effect sizes, but until recently, the number of disease susceptibility loci that could be replicated in independent study populations was limited. The reasons for irreproducibility of results can be explained by several contributing factors: lack of statistical power, inappropriate selection of candidate loci, failure to capture variation across the whole gene region, low threshold for significance and over-interpretation of results (59).

Linkage disequilibrium and haplotype analysis

A key aspect in performing association studies is indirect association, which makes use of the LD patterns across the genome (60). If two loci are inherited together more often than would be expected by change, they are said to be in LD. The further apart the SNPs are located, the more likely they are to be separated by recombination, and consequently strong LD indicates that SNPs are likely to be inherited together. In addition to physical distance between the loci, LD is affected by cross-over rate and the number of generations since the mutation occurred or was introduced with younger populations demonstrating stronger LD on average (60). Two commonly used measures of LD are D′ and r^2. D′ is a unidirectional measure of LD (i.e., it is possible to predict the genotype of SNP2 from SNP1, but not the other way around), whereas r^2 is bidirectional measure of LD (i.e. it is the traditional correlation of SNP1 and SNP2) (60).

A subset of SNPs (tagSNPs) in a genomic region of interest or across the whole genome are selected for genotyping, and taking advantage of the known LD patterns, the untyped common SNPs are imputed from tagSNP genotypes (9, 61). Therefore, in genetic or genomic association

studies, the variants that associate with the phenotype are not necessarily the causative variants, but merely mark the genomic region harbouring the true functional variant. The power to detect the causal SNP by a tagSNP depends on the LD between the SNPs, allele frequencies and the association model.

The HapMap project has increased the understanding of LD patterns across the genome in different human populations and illustrates that by selecting maximally informative, non-redudant tagSNPs, genotyping of <500 000 SNPs may allow a nearly complete survey of all common genetic variability (62).

Association between genetic variants and a trait of interest may be analysed singly or by using haplotypes consisting of multiple variants. When study populations consist of unrelated individuals, haplotypes cannot be deduced directly but need to be inferred by statistical tools such as THESIAS (63). Analysis method based on haplotypes may be more efficient than separate analyses of individual markers in presence of multiple susceptibility alleles, particularly when LD between the variants is weak (64). In addition, in some situations haplotypes consisting of tagSNPs may more efficiently capture untyped common genetic variants in the region (65). On the other hand, even if haplotype analysis may be more informative than single SNPs, the power of haplotype analysis may be reduced by large number of haplotypes that needs to be studied.

Different study designs used in association studies

Most association studies are based on a case-control design, in which genotype frequencies of the variants are compared between cases, expressing the trait of interest, and controls, without the trait, to determine if any alleles are over-represented in either group. Compared with other types of study designs, case-control studies are often more affordable and easier to conduct, but may be prone to a number of biases mostly relating to the lack of comparability between cases and controls (55). More specifically, cases are typically sampled from clinical sources and may not be representative group as fatal, mild or silent cases are not included. On the other hand, the controls should be drawn from the same population and represent individuals who are truly free of the disease trait, but who are nevertheless at risk of developing the disease or trait.

In prospective studies, extensive baseline information on participants is gathered, these individuals are followed, and the incidence of a disease is assessed with the advantage that all participants are ascertained and followed up in the same way (55). While large case-control studies are suitable for the initial identification of susceptibility SNPs, prospective studies may be more useful in qualifying the true risk of known variables (66).

Candidate gene studies

The earliest forms of population-based association studies were candidate gene studies that focused on variants within a biologically plausible candidate gene(s). This approach limits the number of tests that needs to be performed, but is restricted to genes involved in known molecular pathways. Moreover, it excludes genomic regions outside gene loci, which may nevertheless have important regulatory functions (9).

In the earliest candidate gene studies only one or few variants, often known to be functional, were genotyped, whereas in later studies the tagSNP approach was used for variant selection in order to cover all common variation in the locus of interest. This approach helps to minimise the number of SNPs that need to be genotyped, but lowers the power compared to testing functional SNPs directly (57)

Numerous T2DM and obesity variants have been identified using this approach, but only a small fraction has been validated in replication studies. Examples of T2DM associated genes successfully identified through candidate gene studies include *PPARγ* and *KCNJ11*, which have been subsequently confirmed by GWAS (67-69).

Genome-wide association studies

In GWAS, a large number of variants across the whole genome are genotyped in a large group of individuals. By utilising the information on haplotype maps of human populations and careful selection of tagSNPs, GWAS can be designed to cover a large part of the common genetic variants in the whole genome (49, 54). GWASs are generally multistaged studies, where the top SNPs from discovery cohort are subsequently genotyped in a replication cohort (9). The SNPs that are successfully replicated are then meta-analysed in the combined discovery and replication cohort, and those that reach the level of genome-wide significance ($p<0.05 \times 10^{-8}$) will be studied further by other methods (9).

In general, the SNPs identified by GWASs are common (MAF>5%), have modest effect sizes, and are not highly differentiated across populations (52). However, GWASs do not easily identify rare risk alleles that exist in a given population (49). Another weakness of GWASs is that testing a large number of polymorphisms is required, which decreases the power to identify associations, meaning that a large number of cases are required to identify associated variants. Moreover, so far GWASs have been largely limited to populations of European descent (9).

Owing to GWASs, the number of validated associations between genetic variants and complex traits and diseases has increased dramatically during the past few years, and the list of associations is continuously updated in the National Human Genome Research Institute's catalogue of published GWASs (70). The majority of the currently known genes associating with T2DM have been identified through GWASs (54), which have implicated new pathways in the development of T2DM. An example is provided by a missense variant in *SLC30A8* gene which encodes a zinc transporter crucial for insulin packaging and secretion in beta cells (9, 71). Similarly, GWAS approach revealed the association between T2DM and variants in the *TCF7L2* gene (72), which was at the time not considered a candidate gene for T2DM, but has thereafter been shown to modulate beta cell function (73).

Molecular evolutionary methods

Evolutionary approaches, not requiring prior assumptions of the specific genes targeted by natural selection, may be used to identify genetic loci associated with complex diseases (74). Natural selection generates detectable patterns against the genome-wide background of neutrally evolving loci and investigating haplotype structures and allelic architecture can reveal signals of positive selection, such as reduced haplotype diversity (19, 74). Identifying the genetic adaptations relating to nutrition and metabolism may help in identification of risk alleles for modern diseases, such as obesity and T2DM (74).

Future directions

Since GWASs only capture the common variation of the genome, different approaches need to be developed in order to understand the role of other types of genetic variants in human phenotypic variation. The next generation technologies have reduced the costs and time requirements of sequencing, and systematic efforts to catalogue rare and structural sequence variants by exome and whole genome sequencing are already ongoing (15, 16). Moreover, epigenetic modifications controlling the potential of the genome to be transcribed may have a significant impact on complex human diseases (17). In the future, integrating epigenomic and genomic data may reveal genomic risk factors that are more powerful than those based on sequence variants alone (17). Finally approaches, such as transcriptomics, proteomics and metabolomics aim at integrating data from multiple levels of biological processes in order to clarify the interactions among gene variants and between genetic and environmental factors.

2.5 OBESITY

2.5.1 Definition and prevalence

Overweight and obesity are characterised as an excess accumulation of body fat. Body mass index (BMI) is the most commonly used index to classify overweight and obesity at population level, and is calculated as a weight in kilograms divided by squared height in meters (kg/m^2). Obesity is generally defined as BMI ≥ 30 and overweight as BMI≥25 (75). Other simple anthropometric measurements, such as waist circumference (WC) and waist-to-hip ratio (WHR), may be used to define obesity and also give a more precise picture of body fat distribution. For more accurate measurement of body composition more laborious and costly methods, such as bioelectrical impedance, dual energy X-ray absorptiometry, quantitative computed tomography, underwater weighing, magnetic resonance imaging or computed tomography, can be used (76).

The prevalence of obesity is increasing rapidly worldwide (77). According to WHO approximately 1.5 billion adults (aged over 20 years) were overweight. Of those more than 200 million men and nearly 300 million women were obese in 2008 (75). Moreover, almost 43 million children under the age of 5 years were overweight in 2010 (75). WHO further predicts that by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese (75). In Finland, the prevalence of obesity increased from 11.3% to 20.7% in men and from 17.9% to 24.1% in women aged ≥ 30 years between the two national surveys performed in 1978-1980 and 2000-2001 (78). However, more recent observations indicate that the prevalence of obesity may be, in fact, decreasing among 45-74 year old Finns (79).

Obesity is associated with an array of adverse metabolic conditions, such as insulin resistance, T2DM, hypertension, dyslipidaemia, atherosclerotic vascular disease, fatty liver disease and certain types of cancers (80). Successful long-term weight loss decreases the risk of obesity related co-morbidities (81), but has proven difficult to achieve and maintain (82).

2.5.2 Environment vs. genes in the development of obesity

The modifiable risk factors of obesity and weight gain are well recognised. Increased energy intake and decreased energy expenditure generally lead to storage of excess energy as fat, and the lifestyle changes that have occurred during the past decades largely explain the rapid increase in prevalence. Nevertheless, a strong genetic predisposition exists as suggested by the fact that some individuals seem to be more susceptible to weight gain than others in the current obesogenic environment (83). There are also racial differences in the prevalence of obesity that cannot be explained by environmental and lifestyle factors alone (84). Furthermore, the data from experimental twin studies support the notion that predisposition to obesity has a strong genetic component (85, 86). The heritability estimates for BMI range from 40 to 70% (87) and are comparable for other measures of adiposity (88, 89).

2.5.3 Genetics of obesity

The sporadic cases of monogenic obesity are caused by rare functional mutations in genes encoding appetite regulating proteins, such as leptin (*LEP*), leptin receptor (*LEPR*), melanocortin 4 receptor (*MC4R*), and pro-opiomelanocortin (*POMC*), highlighting the key role of neuronal regulation of overall adiposity (83, 90). These mutations often lead to a dysfunctional gene product, and severe early-onset phenotype (90). Although, common variants in several of these genes are also associated with common obesity (83, 91), its polygenic background is still fairly poorly understood.

An ever increasing number of common SNPs that associate with common obesity and related phenotypes have been identified through GWASs (70). A list of confirmed obesity associated loci identified through GWASs is presented in Table 1. Recently, a two-staged GWAS meta-analysis of up to 249, 796 individuals of European descent performed by the GIANT consortium (Genetic Investigation of ANthropometric Traits) confirmed 14 known loci and identified 18

novel loci associating with BMI (92). It has been estimated that altogether the common variants, including those already identified as well as those yet unidentified, only account for 6-10% of heritability of BMI (92). Although many of the genes identified by GWAS are implicated in neuronal functions (56), a substantial part of the variants are not located near genes involved in known obesity-related molecular pathways, suggesting that much of the biology of obesity remains unknown (92).

Whereas overall adiposity seems to be associated with variants implicated in hypothalamic functions, many of the variants associating with fat distribution lie close or within genes implicated in adipocyte development and function (56). In other words, fat distribution seems to be controlled by at least partly distinct genetic factors. Interestingly, many of the variants show markedly stronger effects in women compared with men (56, 93).

The fat mass and obesity-associated gene (*FTO*) is regarded the best established genetic risk factor for common obesity. It was identified in 2007 through GWAS (94) and the association with obesity has been replicated thoroughly in independent study populations (95). Interestingly, variation in the *MC4R* gene, associated with monogenic obesity, also seem to contribute less severe and more complex forms of obesity (91).

Table 1. Loci associating with obesity, BMI and body composition that have been identified through GWAS.

Nearest gene(s)	**Full name**	**Trait**	**Putative functions of the gene product**	**Reference**
BDNF	Brain-derived neurotrophic factor	BMI	Neuronal function, possible role in regulation of stress response and in the biology of mood disorders and eating behaviour	(92, 96)
FAIM2/ BCDIN3D	Fas apoptotic inhibitory molecule 2/ BCDIN3 domain containing	BMI, WC	Unknown	(92, 96, 97)
FTO	Fat mass and obesity associated	extreme and early-onset obesity, BMI, obesity-related traits	The exact function unknown, may be involved in nucleic acid demethylation, mRNA regulated by feeding and fasting in mice	(91, 92, 94, 96-105)
GNPDA2	Glucosamine-6-phosphate deaminase 2	BMI	Catalyses the reversible conversion of D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium, expressed at high levels in brain and hypothalamus	(92, 103)
KCTD15/ *CHST8*	Potassium channel tetramerisation domain containing 15/ carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8	BMI	KCTD15: Unknown, *CHST8:* sulfation of carbohydrates	(92, 96, 103)
LYPLAL1	lysophospholipase-like protein 1	WHR, adiposity	possibly acts as a TG lipase, reported to be up-regulated in subcutaneous AT of obese subjects	(93, 106)
MC4R	Melanocortin 4 receptor	BMI, extreme obesity, obesity, WC	Interacts with adrenocorticotropic and MSH hormones and is mediated by G proteins, associated monogenic obesity	(91, 92, 96, 97, 99, 102, 103, 107)

Table 1. continues

Table 1. continued

MTCH2/ *NDUFS3/ CUGBP1*	mitochondrial carrier 2/ NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)/ CUGBP, Elav-like family member 1	BMI	MTCH2: may function in cellular apoptosis, *NDUFS3*: mitochondrial function, *CUGBP1:* regulation of alternative splicing and may be involved in mRNA editing, and translation	(92, 103)
NEGR1	Neuronal growth regulator 1	BMI	expressed at high levels in brain and hypothalamus, involved in neuronal outgrowth, candidate CNV identified	(92, 96, 103)
NRXN3	neurexin 3	BMI, WC	nervous system cell adhesion molecule	(92, 97, 100)
SEC16B/ *RASAL2*	SEC16 homolog B (S. cerevisiae)/ RAS protein activator like 2	BMI	Involved in organisation of transitional ER sites and protein export, *RASAL2:* functions as activator of Ras superfamily of small GTPases	(92, 96)
SH2B1/ *APOB48R/ SULT1A2/ ATXN2L/ TUFM/ ATP2A1*	SH2B adaptor protein 1/ apolipoprotein B receptor/ sulfotransferase/ family, cytosolic, 1A, phenol-preferring, member 2/ ataxin 2-like/ Tu translation elongation factor, mitochondrial/ ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	BMI	*SH2B1*: implicated in leptin signaling, *APOB48R:* macrophage receptor that binds to the apolipoprotein B48 of dietary lipoproteins rich in TG, *SULT1A2:*encodes phenol sulfotransferase with thermostable enzyme activity, *ATXN2L:* ataxin type 2 related protein of unknown function, *TUFM:* participates in protein translation in mitochondria, *ATP2A1:* involved in muscular excitation and contraction	(92, 96, 103)
TFAP2B	Transcription factor AP-2 beta	BMI, WC	Transcription factor preferentially expressed in AT, over-expression leads to IS via enhanced glucose transport and increased lipid accumulation and down-regulates expression of adiponectin	(92, 93)
TMEM18	transmembrane protein 18	BMI	Possibly involved in cell movement, expressed in hypothalamus	(92, 96, 103)

AT, adipose tissue; BMI, Body mass index; CNV, copy number variant; ER, endoplasmic reticulum; MSH, melanocyte-stimulating hormone; WC, waist circumference, WHR, waist-to-hip ratio; IS insulin sensitivity

Adipose tissue

White AT functions as an energy storage buffering against fluctuations in energy supply in the long term (40). Energy is stored as TG during nutritional abundance and released as free fatty acids (FFAs) during nutritional scarcity. In order to store energy, appetite must increase beyond that required by energy expenditure, and indeed humans, as well as many other species, are able to gain weight rapidly when food is available, suggesting an evolutionary mechanism for seasonal storage of calories (40). Importantly, nowadays AT is also recognised to be an important endocrine organ having a central role in regulating various metabolic processes through secretion of bioactive regulatory molecules, adipokines (108).

Two types of white AT with distinct metabolic characteristics and adipokine secretion profiles can be distinguished in humans, namely intra-abdominal and subcutaneous AT (109). Intra-abdominal AT, which is also called visceral AT, is located in peritoneal cavity and is more active metabolically. It exists in all mammals and comprises 20% of fat in normal weight men but only 6% in women (110). High intra-abdominal fat is associated with central fat distribution and is an independent risk factor for metabolic syndrome and T2DM (110, 111). Subcutaneous AT is located beneath the skin. In addition smaller local adipocyte depots are associated with

various organs, such as the heart and kidneys (109). Subcutaneous AT located in lower body regions is unique to humans, especially women. It is less metabolically active and evolutionarily linked to the high costs of human reproduction (40, 112).

In addition to adipocytes, AT is composed of distinct cell types, such as adipocyte precursors, lymphocytes, macrophages, fibroblasts and stromal vascular cells (109). The cellular composition as well as the phenotypes of individual cells in AT may alter as a result of obesity (109). Most notably, the AT of obese is characterised by both adipocyte hypertrophy (increased cell size) and hyperplasia (increased cell number) (108). The number of adipocytes is set during childhood and adolescence which is why hypertrophy is thought to be the most important mechanism of increase in fat depots in adulthood (113). Adipocyte hypertrophy results in altered intracellular signalling and dysregulated adipokine production (108). Moreover, obesity is associated with increased macrophage infiltration to AT, and phenotypic switch of AT macrophages to more pro-inflammatory state (108, 114). Endothelial cell activation in inflamed AT results in production of cell adhesion molecules, chemokines and cytokines which further exacerbate inflammation (108). Increased evidence therefore indicates that obesity causes chronic low-grade inflammation, which contributes to systemic metabolic dysfunction and development of obesity linked disorders (109). Moreover, in obese individuals, increased release of FFAs leads to impaired beta cell function and induces insulin resistance by inhibiting insulin-stimulated glucose uptake in skeletal muscle, and by stimulating gluconeogenesis in liver (115).

Adipokines

The regulatory molecules produced and secreted by different cellular components of AT are collectively called adipokines. The most important members of this functionally diverse group of molecules are presented in Table 2. Adipokines play important roles in modulation of processes such as inflammation and energy metabolism, and a balanced production of different adipokines is crucial for maintaining systemic metabolic homeostasis (109). In obesity, the production of adipokines becomes dysregulated leading to locally and systemically altered immune responses, which are causally associated with obesity-related comorbidities (109).

2.6 TYPE 2 DIABETES (T2DM)

2.6.1 Definition and prevalence

Diabetes is a state of chronic elevation of blood glucose levels and is classified into several distinct categories. The underlying causes of hyperglycaemia are defects in insulin secretion, tissue responses to insulin, or both (117).

Type 1 diabetes is an autoimmune disease, in which insulin producing beta cells are destructed (117). T2DM is much more prevalent category, accounting for 90-95% of diabetic individuals, and is caused by combination of insulin resistance and inadequate compensatory insulin secretion by beta cells (117). In addition, several diabetes types with monogenic defects, usually in beta cell function, are known. These diseases are characterised by early onset hyperglycaemia and are hence referred to as maturity-onset diabetes of the young (MODY) (117).

Diabetes affects approximately 285 million people world-wide and according to latest estimates diabetes prevalence will be 439 million (approximately 7.7%) in 2030 (118). In Finland, more than 10% of the adult population are estimated to have T2DM and only half of the affected individuals recognise that they have this condition (119, 120).

Table 2. Key adipokines and their functions (108, 109).

Adipokine	**Function**	**Levels in obesity**
adiponectin	insulin sensitising, anti-atherogenic and anti-inflammatory	↓
AGT (angiotensinogen)	involved in hypertension and AT growth	↑
apelin	insulin sensitizing and anti-atherogenic	↑
ANGPTL2 (angiopoietin-like protein 2)	implicated in induction of insulin resistance, pro-inflammatory	↑
CCL2 (CC-chemokine ligand 2), MCP-1	acts as a receptor for several chemokines, potentially promotes glucose intolerance and insulin resistance, pro-inflammatory	↑
CXCL5 (CXC-chemokine ligand 5)	interferes with insulin signaling, pro-inflammatory	↑
IL-6	role in obesity-induced insulin resistance controversial, pro-inflammatory	↑
IL-18	complex role in metabolism, pro-inflammatory	↑
leptin	appetite control, pro-inflammatory	↑
lipocalin 2	implicated in induction of insulin resistance, pro-inflammatory	↑
PAI1 (plasminogen activator inhibitor 1)	physiological inhibitor of plasminogen activation	↑
NAMPT (nicotinamide phospho ribosyltransferase), visfatin	potential regulator of insulin secretion, pro-inflammatory	↑
RBP4 (retinol-binding protein 4)	retinol transporter, potentially contributes to systemic insulin resistance, pro-inflammatory	↑
resistin	promotion of insulin resistance in mice, pro-inflammatory	? (116)
SFRP5 (Secreted frizzled-related protein 5)	suppressor of pro-inflammatory Wnt signalling	↓
TNF (tumour necrosis factor)	pro-inflammatory, promotes insulin resistance by attenuating insulin signalling	↑

AT, adipose tissue; IL, interleukin

Currently, the diagnosis of T2DM is based on plasma glucose values in the fasting state or after an oral glucose tolerance test (OGTT) performed with 75-g glucose load according to the following diagnosis criteria: 1) any casual plasma glucose ≥11.1 mmol/l accompanied with symptoms of hyperglycaemia, 2) fasting plasma glucose ≥7.0 mmol/l, or 3) 2-h plasma glucose ≥11.1 mmol/l (117, 121). In the Finnish Diabetes Prevention Study (DPS), and hence in this study, however, the former WHO criteria (1985) for T2DM was applied with the higher diagnostic value of 7.8 mmol/l for fasting plasma glucose concentration (122). Since transition from normoglycaemia to T2DM is a gradual process, individuals with glucose levels above normal, but not meeting the criteria for diabetes, are considered pre-diabetic, and are defined according to the American Diabetes Association criteria as having impaired fasting glucose (IFG, fasting glucose levels 5.6-6.9. mmol/l) or impaired glucose tolerance (IGT, 2-h postload glucose 7.8-11.0 mmol/l) (123), whereas the European Diabetes Epidemiology Group sets the lower cut-off point for the definition of IFG to 6.1 mmol/l (124).

2.6.2 Risk factors

Overall, the risk factors of T2DM are well defined and include: age, BMI, abdominal obesity, lack of physical activity and family history of diabetes (117, 125, 126). In addition, a diet high in cereal fibre and polyunsaturated fat, and low in saturated fat, trans fat and glycaemic load associates with lower incidence of T2DM (127). Importantly, numerous well controlled intervention studies have demonstrated the efficacy of beneficial lifestyle changes in preventing or delaying development of T2DM in individuals with increased risk (81, 128-131).

2.6.3 Pathophysiology of T2DM

Carefully orchestrated insulin secretion by pancreatic beta cells in response to subtle changes in blood glucose levels is a prerequisite for maintaining normal glucose homeostasis. The relative importance of beta cell function and insulin resistance in the pathophysiology of T2DM has been debated extensively for few decades. It is now clear, however, that these two sides are interconnected by a tightly regulated feedback system, and both beta cell dysfunction and insulin resistance of the target tissues are already present at the early phases of T2DM (132).

Insulin resistance is a condition in which tissues exhibit a reduced response to insulin. The main functions of insulin include glucose uptake and storage in skeletal muscle, inhibition of lipolysis in AT, and inhibition of endogenous glucose production in liver. The binding of insulin to its receptor on the target cell membrane triggers a cascade of signalling events leading ultimately to the translocation of the main insulin responsive glucose transporter, GLUT4, from intracellular vesicles to cell membrane (133). Obesity, especially intra-abdominal fat accumulation, is a major determinant of insulin resistance (134), disrupting the insulin signalling pathway at multiple levels through alterations in the levels and activities of signalling molecules and transcription factors (135). In addition, factors such as age, exercise, diet and genetics influence insulin sensitivity (132).

The relationship between insulin secretion and insulin sensitivity is best described as a hyperbolic function (136), and beta cell action should thus always be interpreted in the context of concomitant insulin sensitivity (132). The main regulator of insulin secretion is blood glucose. Glucose enters beta cell via a transporter molecule and results in increased ATP/ADP ratio and closure of ATP-sensitive potassium channels. This leads to depolarisation of the cell membrane and opening of voltage sensitive calcium channels. Eventually, the influx of calcium triggers exocytosis of insulin granules. This, however, is a simplified view and in reality insulin secretion is a complex process affected by multiple factors, such as the quality, quantity and the administration route of the secretagogue, gastrointestinal hormones, prevailing glucose concentration and the degree of insulin sensitivity (132). Moreover, two separate entities, namely reduction in beta cell mass and function, seem to be involved in impaired insulin secretion and development of hyperglycaemia (137).

2.6.4 Measurement of insulin sensitivity and secretion

The gold standard for measurement of insulin sensitivity is considered to be the euglycemic hyperinsulinemic clamp technique (138). However, this method is too laborious and time consuming to be used in large study populations, and does not evaluate insulin secretion. Intravenous glucose tolerance test (IVGTT) with frequent sampling in the beginning (FSIGT) allows measurement of insulin and glucose during the dynamic phase immediately following glucose injection. The main advantage of this method is that beta cells are stimulated with known glucose dose without confounding effects of incretins and gastrointestinal hormones (139). In addition, reasonably accurate estimates of beta cell function and insulin resistance can be obtained by using the OGTT, which is simple and more suitable for various clinical settings (140).

2.6.5 The cardiometabolic syndrome

Metabolic syndrome refers to a clustering of metabolic disorders, such as hypertension, dyslipidemia, central obesity, insulin resistance, and hyperglycaemia, and it is associated with an increased risk of CVD and T2DM (10, 141). Several definitions of the metabolic syndrome have been suggested (142), but its value as a CVD risk marker relative to individual risk factors or other risk scores has also been questioned (143, 144). Insulin resistance is the trait that connects all the components of the metabolic syndrome, and is causally involved in the development of CVD (133). Moreover, as discussed above, obesity is associated with systemic low-grade inflammation and dysregulated production of adipokines, both of which contribute significantly to development of CVD (109).

The molecular link between insulin resistance and increased atherosclerosis occurs through dysfunctional insulin signalling. The signaling cascade, leading to stimulation of glucose transport via GLUT4, is impaired in skeletal muscle of diabetic and obese individuals leading not only to decreased glucose uptake (133, 145), but also deficient production of nitric oxide (NO), which in turn is associated with endothelial dysfunction (146, 147). Moreover, insulin functions as a growth-factor promoting cell proliferation and differentiation via mitogen-activated protein kinase pathway. This pathway remains sensitive to insulin, is excessively stimulated by compensatory hyperinsulinemia, and may contribute to accelerated atherosclerosis through vascular smooth muscle cell proliferation, and production of inflammatory cytokines (133, 148).

2.6.6 Genetics of T2DM

Although environmental factors play significant role in aetiology of T2DM, strong genetic component is suggested by high concordance rate in monozygotic twins compared with dizygotic twins, increased disease risk for individuals with family history of the disease, and large variation of prevalence observed among ethnic groups living in similar environments (149-151). The lifetime risk of T2DM is approximately 40% for offspring of at least one affected parent (152) and the relative risk was reported to be 2.24 in first-degree, 1.36 in second-degree, and 1.14 in third degree relatives of T2DM patients (153). The highest prevalence rates of T2DM are seen in Pima Indians of Arizona and lowest in populations with European ancestors (154, 155).

The heritability estimates of T2DM range from 26 to 75% (149, 150, 156). Evidence from twin studies suggests that both insulin sensitivity and insulin secretion have significant genetic component in nondiabetic individuals, but the former is more influenced by environmental factors (89). The heritability estimate for insulin secretion and insulin resistance are generally reported to range from 50 to 70%, and from 26 to 40%, respectively (89, 150, 157), and differential genetic architecture for these traits is suggested by meta-analyses (158).

MODY

MODY represents a group of monogenic diabetes disorders occurring in young adult life. Studies identifying the genes for MODY have been successful and at least seven different subtypes of MODY have been identified with distinct genetic, metabolic and clinical features (159). The majority of MODY genes are expressed in beta cells and are involved in beta cell development and glucose sensing (160).

T2DM

The genetic basis of common T2DM is polygenic, heterogeneous and still largely unknown. Currently, over forty confirmed susceptibility variants for T2DM have been identified, but these variants explain less than 10% of the heritability of T2DM (53, 161). These variants are distributed across the whole genome and mitochondrial DNA, and the majority of them are located within or near genes that are involved in beta cell development or function (161). Genes related to insulin resistance and related phenotypes are clearly underrepresented, and it has been suggested that T2DM emerges as a result of environmentally triggered insulin resistance in individuals whose genetically challenged beta cells fail to respond to increasing insulin demands (160). A list of the most replicated loci associated with T2DM is presented in Table 3.

Common variation in genes associated with different MODY types have been studied for association with T2DM with the rationale being that variants with less radical effects could be associated with a milder disease phenotype and also be more common because of less stringent negative selection pressure (160). The candidate gene approach has yielded positive, albeit modest, associations with T2DM for many MODY genes (160). Interestingly, *HNF1B*, one of the MODY genes also named *TCF2*, was identified by a recent GWAS for T2DM (162). The MODY2

gene *GCK*, encoding glucokinase, has been identified by four GWASs for fasting glucose and glycated haemoglobin levels (158, 163-165).

Mutations in the gene encoding wolframin (*WFS1*) cause Wolfram syndrome, which is a severe autosomal syndromic form of diabetes (160). Two GWASs have reported association of common *WFS1* SNPs with T2DM (162, 166). The association between *KCNJ11* gene, encoding islet ATP-sensitive potassium channel subunit Kir6.2, and T2DM was originally identified by candidate gene approach (167, 168), and subsequently confirmed by GWAS approach (69, 169-172).

Table 3. The best confirmed loci for T2DM

Nearest gene(s)	Full name	Putative function of the gene product/ connection with T2DM	Reference
C2CD4A, C2CD4B	C2 calcium-dependent domain containing 4A, 4B	nuclear factors with a role in regulating genes that control cellular architecture	(158, 177, 178)
CDC123, CAMK1D	cell division cycle 123 homolog/ calcium, calmodulin-dependent protein kinase ID	unclear, possibly cell cycle dysregulation	(170, 178)
CDKAL1	CDK5 regulatory subunit associated protein 1-like 1	unclear, possibly involved in first phase insulin secretion	(69, 162, 166, 170-172, 179-183)
CDKN2A, CDKN2B	cyclin-dependent kinase inhibitor 2A, 2B	possibly involved in cell cycle regulation, beta cell mass	(69, 162, 170-172, 180, 184)
HHEX	hematopoietically expressed homeobox	transcription factor that may play a role in hematopoietic differentiation	(69, 71, 162, 170-172, 178, 180)
HNF1A	hepatocyte nuclear factor 1 homeobox A	MODY3 gene	(162, 184)
IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2	functions by binding to the 5' UTR of the insulin-like growth factor 2 (IGF2) mRNA and regulating IGF2 translation	(69, 162, 170-172, 180, 181, 184)
JAZF1	juxtaposed with another zinc finger gene 1	unclear, transcriptional repressor of NR2C2	(162, 170)
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11	insulin secretion	(69, 169-171)
KCNQ1	subunit of voltage-gated potassium channel	beta cell function	(162, 180, 181, 184-186)
PPARG	peroxisome proliferator-activated receptor gamma	nuclear receptor involved in adipocyte differentation	(69, 162, 170-172)
PTPRD	protein tyrosine phosphatase, receptor type, D	unknown	(185, 187)
SLC30A8	islet-specific zink membrane transporter ZnT8	involved in insulin synthesis and secretion	(69, 71, 158, 162, 166, 169, 171, 172, 180, 183)
TCF7L2	transcription factor 7-like 2	beta cell development and function, plays a key role in the Wnt signaling pathway	(69, 71, 162, 166, 169-172, 172, 180, 182, 183, 188)
TSPAN8/LGR5	tetraspanin 8/ leucine-rich repeat containing G protein-coupled receptor 5	unknown	(162, 170)

MODY, maturity onset diabetes of the young; UTR, untranslated region

The strongest effect on T2DM risk described thus far is by the *TCF7L2* gene. This gene was identified through linkage analysis followed by fine mapping, and the association has been subsequently replicated in several studies and meta-analyses (173, 174). A single copy of the *TCF7L2* risk allele confers an approximately 40% increased risk, and two copies (carried by approximately 10% of individuals with European or African ancestors) an approximately 80%

increased risk (160). The exact role of *TCF7L2* in T2DM susceptibility is not fully understood, but functional studies suggest that it may be involved in beta cell function (160, 175, 176).
The *PPARG* gene encodes the peroxisome proliferator-activated receptor γ and is a rare example of T2DM susceptibility genes affecting insulin sensitivity. The Pro12Ala polymorphism of this gene was originally identified and replicated consistently successfully by using candidate gene approach (67), and later on confirmed by GWAS approach (69, 162, 170-172).

The recent discoveries in the field of T2DM genetics have already provided valuable insights into basic biological pathways underlying the disease, but have not yet led to clinical utility in risk prediction. Several studies have examined whether genetic factors improve the accuracy of predicting future T2DM beyond traditional risk factors, and most have concluded that the improvement in risk prediction is statistically significant, but clinically irrelevant (189, 190). Lyssenko *et al.* (189) found that 11 genetic variants previously associated with T2DM, improved slightly, but significantly, the prediction of T2DM compared with a model including only clinical risk factors. Interestingly the predictive value of genetic factors increased with longer duration of follow-up suggesting that the assessment of genetic risk factors may be more meaningful earlier in life (189). In the Finnish DPS participants lifestyle intervention reduced the T2DM incidence, whereas family history of T2DM or 19 previously identified risk SNPs did not have significant predictive value (191).

2.7 CANDIDATE GENES IN THE PRESENT STUDY

2.7.1 Adiponectin

Adiponectin (also named Acrp30, AdipoQ, apM1, and GBP28) was described in the mid-1990s by four independent research groups (192-195). Adiponectin is the most abundant adipokine in circulation with concentration 0.05% (5-30μg/ml) of total serum protein (193). It is a secretory protein mainly produced by mature adipocytes (192), but is also detected in brown adipocytes, bone marrow, myocytes, cardiomyocytes, osteoblasts, salivary gland epithelial cells, placenta and wide range of fetal tissues (196-200).

Human adiponectin comprises 244 amino acids, has a C-terminal globular domain and N-terminal collagen-like domain, and resembles structurally the complement 1q and TNF family (193). Adiponectin is produced as a 32 kDa monomer, which is sequentially assembled into trimers, hexamers and high-molecular weight (HMW) multimers (12–18 mers) (193, 201). The HMW form is suggested to be biologically the most important isoform (202, 203), but its levels are highly correlated with total plasma adiponectin levels (204). Post-translational modifications, including hydroxylation, glycosylation and disulfide bond formation, are prerequisite for multimer formation and secretion of adiponectin (205). In addition, the proteolytic cleavage of the full-length protein generates globular form of adiponectin which circulates in low levels, but has been shown to be biologically active (206). The different adiponectin forms are likely to have at least partly distinct and possibly tissue-specific roles (201, 207).

In 2003, two adiponectin receptors, ADIPOR1 and ADIPOR2, were characterised (208). These two receptors are structurally conserved, with 67% amino acid sequence identity, and highly conserved between species (208). They have seven transmembrane domains, but unlike G-protein coupled receptors, they possess an intracellular N-terminus and extracellular C-terminus (208). Interestingly, the HMW adiponectin has been suggested to act as a ligand for T-cadherin (209), but the biological relevance of this receptor is currently poorly understood.

Adiponectin signalling pathway

Adiponectin gene expression is up-regulated by multiple transcription factors involved in adipogenesis, such as PPARγ, FoxO1, C/EBPα, SREBP, and down-regulated by hypoxia, inflammation, endoplasmic reticulum (ER) stress, transcription repressors such as NFATs and

CREB, and pro-inflammatory factors such as TNFα, IL-6 and IL-18 (205). Several ER associated proteins, such as Ero1-Lα, ERp44 and DsbA-L, play important roles in regulation of adiponectin multimerisation and secretion (205, 210).

ADIPOR1 and ADIPOR2 serve as receptors for both globular and full-length forms of adiponectin, and mediate the increased AMP-activated protein kinase (AMPK), PPARα ligand activity, the fatty acid (FA) oxidation, and glucose uptake by adiponectin (208). Certain functional differences between ADIPOR1 and ADIPOR2 signalling pathways have been detected. ADIPOR1 has a high affinity for the globular adiponectin and low-affinity for the full-length adiponectin, while ADIPOR2 has intermediate affinity for both globular and full-length adiponectin (208). Moreover, ADIPOR1 signalling leads to inhibition of gluconeogenesis and increased FFA oxidation through activation of the AMPK, whereas ADIPOR2 is involved in FFA oxidation and inhibition of oxidative stress and inflammation through PPARα activation (211).

The downstream signalling of adiponectin is not fully understood. In mammalian cells, the cytoplasmic N-terminal domain of ADIPOR1 interacts with APPL1 (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1) and this interaction is stimulated by adiponectin (212). APPL1 mediates the metabolic functions of adiponectin, but also functions in the insulin signalling pathway (213). Since the downregulation of T-cadherin leads to enhanced ADIPOR1/2 signalling, it has been proposed to function as adiponectin co-receptor either competing for adiponectin binding or interfering with the downstream signaling (209).

Adiponectin in different physiological conditions

The circadian variability (214) and the long-term within-person variability (215) of circulating adiponectin are low, but several genetic and non-genetic factors influence circulating adiponectin levels.

Sexual dimorphism has been described for total and HMW adiponectin with women having higher concentrations than men, independent of body mass and fat distribution (201, 216). The explanation for gender differences is currently lacking, but it is possible that differences in estrogen and androgen levels are involved (216). Circulating levels of adiponectin are high at birth and fall gradually during the first decade of life (217, 218). In healthy adults, however, age correlates positively with adiponectin levels (216).

Circulating adiponectin levels correlate negatively with BMI (219), most notably with central fat distribution (202, 216), and weight loss results in increase in adiponectin levels (220). Moreover, circulating adiponectin levels correlate more strongly with insulin resistance than with obesity (221), and although circulating adiponectin levels are generally decreased in obesity (219), metabolically healthy obese individuals have similar adiponectin levels to those observed in normal weight individuals (222). In addition, hypoadiponectinemia is observed in conditions, such as insulin resistance and T2DM (221, 223), dyslipidemia (224), and CVD (225). In contrast, high adiponectin levels are observed in type 1 diabetes (226) and anorexia nervosa (227). The causal relationship between adiponectin levels and insulin resistance, T2DM and CVD in human is currently unclear. A recent meta-analysis of 13 prospective studies demonstrated that high adiponectin levels are associated with decreased risk of T2DM (228), whereas for CVD the results are ambiguous (229).

Factors affecting adiponectin levels in humans

Several dietary factors have been reported to affect adiponectin levels. High glycaemic load was associated with low adiponectin concentrations in diabetic (230) and non-diabetic (231) men, and adherence to a Mediterrean-type diet was associated with higher adiponectin levels in diabetic women (232). In a recent isocaloric cross-over feeding study, a diet rich in monounsaturated fat led to a higher increase of both HMW and total adiponectin than diets rich in carbohydrates or protein (233). In addition, several studies have consistently demonstrated

that moderate alcohol usage is associated with higher circulating adiponectin (231, 232). Acute exercise or endurance training do not seem to result in significant changes in circulating adiponectin levels when loss of AT and increase of lean mass is accounted for, and it seems more likely that some of the insulin sensitising effects of exercise are mediated by tissue-specific alterations in expression levels of adiponectin receptors (234). Finally, some existing drugs, such as statins, angiotensinogen converting enzyme inhibitors, thiazolidinediones and sulfonylureas, have been shown to increase and others, such as β-adrenergic agonists, to decrease adiponectin concentrations in humans (210).

Adiponectin levels are also largely determined by genetic factors, only part of which has been identified thus far. The heritability estimates for circulating total and HMW adiponectin are generally high ranging from 30 to 70 % (203, 235-238). Association between circulating adiponectin levels and variants in the gene encoding adiponectin, *ADIPOQ*, have been reported by several candidate gene studies (203, 235, 238-241), and confirmed recently by GWASs (237, 242-244). In addition, few other genetic loci associating with circulating adiponectin levels have been identified by GWASs. Wu *et al.* (242) reported that variation *ADIPOQ* and two other gene loci, *CDH13* and *KNG1*, explained approximately 8 % of variability in adiponectin levels in Filipino mothers and offspring. Interestingly, association of *CDH13* variation with adiponectin levels has also been observed in GWASs performed on European (237) and Korean (245) populations. *CDH13*, located on chromosome 16, encodes T-cadherin, which is a receptor for HMW forms of adiponectin (209).

Expression of Adiponectin Receptors in Humans

In humans, the genes for both adiponectin receptors (*ADIPOR1* and *ADIPOR2*) are expressed in wide range of tissues including liver, pancreatic beta cells, skeletal muscle, AT, central nervous system, macrophages and atherosclerotic lesions (246-252). The exact roles of *ADIPOR1* and *ADIPOR2* are not currently fully understood and may be diverse and even opposing in some tissues. Human studies regarding the link between expression levels of adiponectin receptors in various tissues and physiological conditions have been performed mainly at mRNA level and the results have been inconsistent.

The AT mRNA expression levels of either or both receptors are reported to be decreased in obese and/or insulin resistant individuals in most (251, 253, 254), but not all (255) studies, and increase as a result of weight loss and physical activity (246, 251) .

The expression levels of adiponectin receptors are higher in muscle than in AT (204). Civitarese *et al.* (248) reported that *ADIPOR1/2* expression in skeletal muscle correlated positively with insulin sensitivity and was lower in individuals with family history of T2DM than in those without family history, whereas others found no difference in muscle *ADIPOR1/2* expression between T2DM patients and lean controls (256), or reported increased expression of both receptors in insulin resistance and T2DM (257).

In the liver, the mRNA expression of both receptors was higher in obese insulin resistant individuals than in obese insulin sensitive subjects (258). Studies investigating adiponectin receptor expression in steatosis and non-alcoholic steatohepatitis have produced contradictory results as some have reported up-regulated (252, 258, 259) and others down-regulated (260, 261) expression levels.

The expression of both receptors was increased in lymphocytes of anorectic patients compared to obese individuals (262). In monocytes of obese and T2DM patients the expression of *ADIPOR1/2* was elevated at mRNA level, but decreased at protein level in comparison with normal weight controls (263). Similarly, suggesting separate transcriptional and translational regulation, *ADIPOR1* and *ADIPOR2* expression was decreased in monocytes of obese subjects with coronary artery disease (CAD) compared with obese controls at protein level, but no difference was observed at the mRNA level (264).

Adiponectin pathway in mice

In mice, injection of recombinant full-length adiponectin ameliorates insulin resistance (265, 266) by suppressing hepatic gluconeogenesis (267). Moreover, a transgenic mouse model with 3-fold elevated endogenous adiponectin levels displayed improved hepatic insulin sensitivity and increased lipid clearance (268). Interestingly, leptin-deficient (*ob*/*ob*) mice over-expressing adiponectin were morbidly obese, indicating even further expansion of AT compared with *ob*/*ob* mice, but displayed a normal metabolic profile (269). Although the physiological significance of the globular adiponectin domain is unknown, acute administration of this cleavage product in mice increased FA oxidation by muscle, and daily administration of low dose resulted in weight loss (206). Another study demonstrated that chronic over-expression of globular adiponectin protected *ApoE*-deficient mice from atherosclerosis and ameliorated insulin resistance in *ob*/*ob* mice (270).

Different strains of adiponectin-deficient mice demonstrate phenotypic variation with different degrees of insulin resistance, which is likely explained by differences in genetic background. Kubota *et al.* (271) reported that adiponectin KO-mice are insulin resistant and show increased neointimal formation, whereas others found that adiponectin KO-mice have relatively normal insulin sensitivity on standard diet, but are susceptible to diet-induced insulin resistance (272, 273). Unexpectedly, Ma *et al.* (274) found that beta oxidation was increased in muscle and liver of adiponectin-deficient mice and these mice had similar insulin sensitivity than their wild-type counterparts.

In mice, *AdipoR1* is expressed ubiquitously, most abundantly in skeletal muscle, whereas *AdipoR2* is predominantly expressed in liver (208). The expression levels seem to be negatively regulated by insulin in its target tissues. In insulin resistant *ob*/*ob* mice, the expression levels were decreased in muscle and AT, whereas insulin-deficiency was associated with increased, and insulin replenishment with decreased, expression levels of both receptors (275). Adenovirus mediated expression of *AdipoR1* and *AdipoR2* in liver improved obesity-related insulin resistance and T2DM in leptin-receptor-deficient (*db*/*db*) mice through AMPK and PPARα pathways, respectively (211). The expression of *AdipoR1* resulted in significant increase in AMPK activation and decreased expression of genes encoding enzymes involved in gluconeogenesis and lipogenesis, whereas expression of *AdipoR2* increased PPARα and its target genes in liver of *db*/*db* mice (211).

Mice deficient for both *AdipoR1* and *AdipoR2* are more glucose intolerant and insulin resistant than adiponectin-deficient mice, suggesting that the functions of the adiponectin receptors extend beyond the role of adiponectin itself (211). Unexpectedly, mice deficient for either *AdipoR1* or *AdipoR2* exhibited remarkably opposite phenotypes (276). Yamauchi *et al.* (211) reported that mice deficient for either adiponectin receptor had normal body weight, but were insulin resistant. However, in another study, *AdipoR1*-deficiency led to increased adiposity, through decreased energy expenditure, and decreased glucose tolerance (276). On the other hand, *AdipoR2*-deficient mice in this study were lean and resistant to high-fat diet induced weight gain, had lower liver TG, and showed improved glucose tolerance (276). Long-term high-fat feeding, however, seems to predispose *AdipoR2*-deficient mice to T2DM due to failure of beta cells to compensate for the moderate insulin resistance (277).

Metabolic effects of adiponectin in its target tissues

In addition to the well-documented insulin-sensitising and anti-inflammatory effects, adiponectin has diverse effects on numerous other physiological functions in different target tissues, such as protective role in carcinogenesis (278), and beneficial effects on reproduction and fertility (279). The key metabolic actions of adiponectin in target tissues are illustrated in Figure 1.

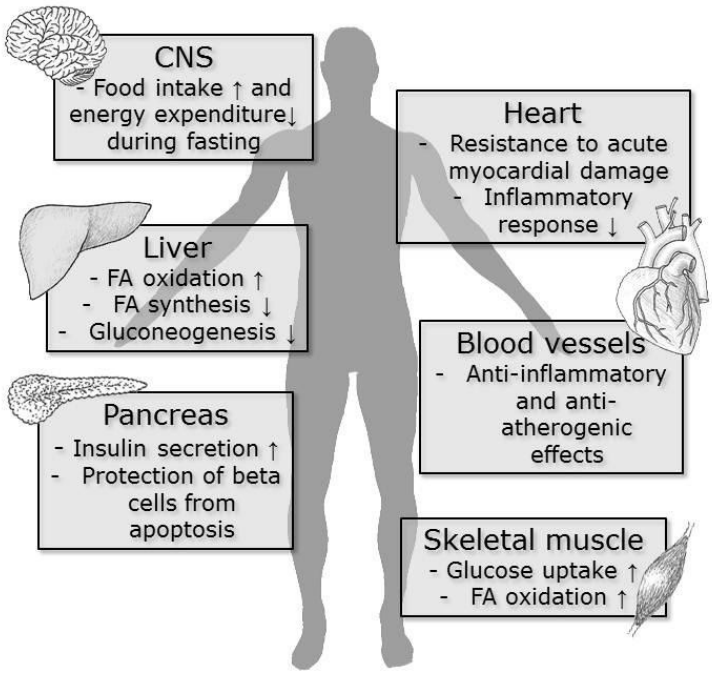

Figure 1. The metabolic and antiatherogenic effects of adiponectin in key target tissues. FA, fatty acid; CNS, central nervous system.

Adiponectin improves insulin-mediated glucose uptake by skeletal muscle and suppresses hepatic glucose output (208, 267, 280). It also promotes FA oxidation and thus reduces the TG content of skeletal muscle and liver, and thus ameliorates insulin resistance (266, 270). In pancreatic beta cells, adiponectin stimulates insulin secretion (281) and may protect beta cells from cytokine- and FA-induced apoptosis (282). Moreover, adiponectin accumulates in the injured vascular wall (283), inhibits monocyte adhesion to endothelial cells (284), suppresses the smooth muscle cell proliferation (285), decreases monocyte to foam cell transformation (283), and stimulates directly the NO production by endothelial cells (286). The direct cardioprotective effects of adiponectin include regulation of cardiac injury through modulation of anti-inflammatory and anti-apoptotic pathways, and inhibition of hypertrophic remodeling (287). Finally, adiponectin promotes clearance of apoptotic cells by macrophages, thereby restraining inflammatory reactions (98).

In addition to the peripheral actions, adiponectin is also involved in central regulation of energy homeostasis (207). While the HMW form seems to associate closely with peripheral insulin sensitivity, the trimeric and hexameric forms play major role in hypothalamus were they act through ADIPOR1 and AMPK activation in modulating food intake and energy expenditure (207).

Since adiponectin has well established insulin-sensitising, anti-inflammatory and anti-atherogenic effects at least in animal models, the genes encoding adiponectin and its receptors are attractive and widely investigated candidate genes for complex human traits ranging from obesity and T2DM to cardiovascular diseases and common cancers.

ADIPOQ

Adiponectin is encoded by the *ADIPOQ* gene (previously named *AMP1* or *ACDC*) at chromosomal locus 3q27. Genome-wide scans have linked this chromosomal region to susceptibility to T2DM and metabolic syndrome (288-290). An enormous number of candidate gene studies have examined the association of common genetic variations in *ADIPOQ* with a plethora of metabolic traits and various diseases. Most studies have been performed in underpowered case-control cohorts and have produced to some extent contradictory and inconsistent results. Nevertheless, findings overall suggest that variants in the *ADIPOQ* locus may contribute to the phenotypic variation in metabolic traits, circulating adiponectin levels and predisposition to T2DM. Only few studies with prospective design have analysed the association of *ADIPOQ* variants with the risk of T2DM and these studies are summarised in Table 4.

Table 4. The key ADIPOQ candidate gene studies with a prospective study design.

Study design	**Study population**	**No. of SNPs**	**Results**	**Ref**
3-year prospective study in subjects from the Insulin Resistance Syndrome (DESIR) cohort	~4, 500 French Caucasian	4	3-year risk of becoming hyperglycemic was affected by rs17300539 and rs2241766	(291)
3.3-year multicenter study (STOP-NIDDM) comparing the effect of acarbose with placebo on T2DM prevention,	770 middle-aged overweight subjects	2	rs2241766 and rs1501299 predicted conversion from IGT to T2DM	(292)
12-weeks clinical intervention study with a caloric reduction of 300kcal/day	294 nondiabetic/ overweight–obese Koreans	3	rs1501299 was associated with different responses of circulating adiponectin and insulin resistance to mild weight loss	(293)
5-year prospective study	262 Chinese subjects with IGT	10	rs2241766 was associated with persistent hyperglycaemia at 5 years	(66)
OGTT at baseline and after three years	550 subjects with increased risk of type 2 diabetes,	2	rs17300539 and rs266729 haplotypes were associated with serum adiponectin levels, T2DM risk and glucose tolerance	(294)
15-year follow-up	Healthy Caucasian men (n=3, 012)	4	rs2241766 was associated with T2DM risk, while two four-SNP haplotypes and rs1501299 increased T2DM risk in interaction with obesity	(295)
three clinical examinations over a 7-year period	837 French-Caucasian subjects	2	rs17300539 and rs266729 haplotype were associated with WHR changes and adiponectin levels	(296)
28-year follow-up	Participants in the Framingham Offspring Study (n = 2543)	22	rs17300539, rs822387 and rs6773957: adiponectin levels rs17366743: diabetes incidence and mean fasting glucose over 28 years of follow-up No associations with other adiposity and metabolic phenotypes	(241)
12-week dietary and physical activity intervention	Individuals with IFG or newly diagnosed T2DM (n=363)	3	SNPs rs266729 and rs1501299 modified the changes in serum adiponectin and HOMA-IR in response to intervention	(297)

OGTT, oral glucose tolerance test ; IGT, impaired glucose tolerance; IFG, impaired fasting glucose, WHR, waist-to-hip ratio, HOMA-IR, homeostasis model assessment of insulin resistance

The most extensively studied of the common *ADIPOQ* variants are rs2241766 (often denoted as +45), a synonymous SNP located in exon 2, and rs1501299 (often denoted as +276), located in intron 2. These SNPs have been associated with circulating adiponectin levels (203, 235, 240, 241, 298, 299), T2DM or related quantitative traits (66, 291-293, 297, 298, 300-302), and obesity (66, 298, 303-306) in various populations. Overall, however, the results vary and have not been replicated in some populations (235, 307).

In addition, many association studies have focused on SNPs located in the *ADIPOQ* promoter region, particularly on SNPs rs266729 (often denoted as -11374 or -11377), and rs17300539 (often denoted as -11391), which are frequently associated with adiposity (305, 305, 308), T2DM (235, 309-311), insulin sensitivity (312), and serum adiponectin concentrations (235, 240, 241, 299, 308, 309, 313). More recently, the association of *ADIPOQ* locus with circulating adiponectin levels has been confirmed by GWASs (237, 242-244), but the association with other metabolic traits remains inconclusive.

Few association studies have focused on rare *ADIPOQ* variants, which may directly affect its ability to form multimers, and some of these variants have been associated with circulating adiponectin levels and T2DM (201, 314). Recently, Bowden *et al.* (315) used combined family-based and association approach with exome sequencing to identify a rare *ADIPOQ* variant (G45R) that accounted for 17% of the variance in plasma adiponectin levels in the Hispanic-American population, but was not observed in African-American or European-American populations.

ADIPOR1 and ADIPOR2

Human *ADIPOR1* and *ADIPOR2* genes are located at chromosomal loci 1p36.13-q41 and 12p13.33, respectively (208). To date, the role of *ADIPOR1* and *ADIPOR2* sequence variations in metabolic traits related to obesity T2DM and cardiovascular risk factors have been the focus of several candidate gene association studies that have produced variable and inconclusive results summarised in Table 5.

2.7.2 Alpha2B- adrenergic receptor

Altogether three human α2-adrenergic receptor (AR) subtypes (A-C) have been identified that belong to the family of G-protein coupled receptors (336). α2-ARs mediate a wide variety of the physiological actions of the catecholamines, adrenaline and noradrenalin, including regulation of sympathetic tone, blood pressure, lipolysis and insulin secretion (337, 338). Often more than one subtype is involved in regulation of a particular physiological function with both opposing and parallel actions (339). All three subtypes are widely distributed in human tissues, including beta cells (340), where noradrenalin acts through α2-ARs to inhibit glucose stimulated insulin secretion (341).

ADRA2B gene

The *ADRA2B* gene, located in chromosome 2, encodes the α2B-AR (336). A common genetic variant (12Glu9) leading to the deletion/insertion of three glutamic acid residues (301-303) in a stretch of 16 acidic amino acids, located in the third intracellular loop, has a pronounced impact on receptor phosphorylation, and as a result impairs the agonist-promoted desensitation *in vitro* (342, 343) and *in vivo* (344).

Genetic association studies have linked the 12Glu9 variant with various traits, such as long-term body weight change (345), reduced basal metabolic rate in obese individuals (342), hypertension or blood pressure (346, 347) risk of sudden cardiac death and acute myocardial infarction (348), acute coronary events (349), silent myocardial ischemia (284), impaired endothelial function (350), autonomic nervous function (351), sympathetic nervous response during sustained handgrip exercise (352), emotional and traumatic memory (353, 354), and amygdala activity during processing of emotional events (355).

Interestingly, many studies have found gene-lifestyle-interactions regarding the 12Glu9 variant. Men homozygous for the Glu9 allele were susceptible to hypertension, but only in stressful work environment (356). In the DPS population, physical activity decreased the risk of T2DM more in carriers of the Glu12 allele, whereas favourable dietary changes associated with greater risk reduction in Glu9 homozygotes (357). Ten-week strength training resulted in significant decrease in intramuscular fat in Glu9 carriers, but not in individuals with Glu12/12 genotype (358). On the other hand, individuals with the Glu12/12 genotype benefited from a 12-month resistance training more than Glu9 carriers, and sedentary individuals with the Glu12/12 genotype were particularly vulnerable to adverse changes in body composition (359). Li *et al.* (360) reported that individuals homozygous for the Glu9 allele, particularly those less physically active, had a more favourable anthropometric phenotype than individuals homozygous for the Glu12 allele.

Table 5. Candidate gene studies focusing on the role of ADIPOR1 and ADIPOR2 variants in metabolic traits.

Study population	**No. of SNPs**			
	ADIPOR1	2	Main results	Ref
990 black and 977 white women	19	27	no association with serum adiponectin concentration or BMI	(316)
CAD cases (n=40) and controls (n=28)	-	8	rs767870 was associated with CAD, IMT, FMD	(317)
451 subjects with metabolic syndrome and an additional replication population (n=1754 cases and controls)	3	3	*ADIPOR1* rs10920533 was associated nominally with WC and HOMA-IR in interaction with plasma saturated FAs	(318)
302 Finns and two replication cohorts (600 Swedish men, 3000 Finns)	1	2	*ADIPOR2* rs767870 were associated with liver fat content and its surrogate markers in three cohorts	(319)
441 probands and siblings, and 262 parents	7	13	*ADIPOR1* rs7539542 and *ADIPOR2* rs12826079 were nominally associated with IS in whites; *ADIPOR1* rs1342387 and rs7539542 were nominally associated with IS in African-Americans	(312)
Korean T2DM patients (n=757) and controls (n=644)	7	4	*ADIPOR1* rs7572865 was associated with HOMA-IR and transcriptional activity *ADIPOR2* -63442G>C and rs1044471 were associated with WC, no association with T2DM	(320)
622 non-diabetic subjects from the Quebec Family Study	2	2	*ADIPOR1* 3882T>C was associated with glucose and insulin metabolism variables; no association with circulating adiponectin	(302)
567 Brazilian individuals of European (n=443) or African (n=124) ancestry	2	-	no association with T2DM or metabolic/anthropometric risk factors	(321)
Russians T2D patients (n=129) and non-diabetic controls (n=117)	1	2	*ADIPOR2* rs11061971 and rs16928751 were associated with T2DM	(322)
cross-sectional population based study examining the prevalence of cardiovascular risk factors (n=700)	2	3	*ADIPOR2* rs1029629 and rs1044471 were associated liver function in diabetics and circulating adiponectin in nondiabetics	(323)
French-Canadians participating in the Québec Family Study (n=759)	2	2	no association with BMI; *ADIPOR1* rs1539355 and *ADIPOR2* rs2058112 were associated with RQ, *ADIPOR1* rs1539355 in interaction with *ADIPOQ* SNP was associated with overall and abdominal adiposity	(306)
prospective, nested case-control study with 714 T2DM cases and 1120 controls from Nurses' Health Study cohort	6	16	*ADIPOR1* rs1139646 and haplotype were associated marginally with T2DM	(324)

Table 5 continues

Table 5 continued

population-based T2DM case–control (n=2, 127) and population-based metabolic quantitative trait study (n=1, 721)	9	15	no association with insulin and glucose metabolism, T2DM or BMI	(325)
African-American adolescents (n=483)	7	-	*ADIPOR1* rs1342387 and haplotypes associated with HOMA-IR in the non-lean subset	(326)
case-control study (n= 594) with Chinese people		1	*ADIPOR2* rs12342 associated with T2DM, FPG, fasting TG, and BMI	(327)
20 German subjects with metabolic syndrome		3	*ADIPOR2* rs16928751, +870C/A (Ile290Ile) and rs9805042 were associated with serum adiponectin levels and fasting TG	(328)
2 populations of CAD-positive and -negative subjects with T2DM (n = 411 and n = 533)	6	-	3' haplotype was associated with CAD, lower mRNA levels in mononuclear cells and AT	(329)
Mexican-Americans (N=439) from the San Antonio Family Diabetes Study	6	24	*ADIPOR1* rs7539542 was associated with adiposity 14 *ADIPOR2* SNPs associated with fasting TG	(330)
T2DM case-control study of 1498 Caucasian subjects and two replication cohorts (altogether n=2876)	5	12	*ADIPOR2* rs767870, rs35854772 and rs2286380 were associated with T2DM	(331)
502 nondiabetic Caucasians	1		*ADIPOR1* rs6666089 was associated with IS dependent on adiposity	(185)
502 nondiabetic Caucasians	6	7	*ADIPOR1* rs6666089 and -1927 were associated with IS and liver fat	(332)
Amish subjects with T2DM (n = 137), IGT (n = 139), and NGT (n = 342)	5	14	*ADIPOR1* rs2275737 and rs1342387/+5843 associated with T2DM, but not with with BMI; 5 *ADIPOR2* SNPs associated with T2DM	(333)
192 diabetic and 192 non-diabetic Japanese subjects	14	24	no association with T2DM	(334)
Northern Europeans and African-Americans with (n=192 and n=269, respectively) and without T2DM (n=192 and n=136, respectively)	22	-	no association with T2DM, IS or insulin secretion	(335)

CAD, coronary artery disease; FA, fatty acid; FMD, flow mediated dilatation; FPG, fasting plasma glucose; HOMA-IR, homeostasis model assessment of insulin resistance; IMT, intima-media thickness; IS, insulin sensitivity; RQ, respiratory quotient; TG, triglycerides; WC, waist circumference.

3 Aims of the Study

The purpose of the present study was to investigate whether sequence variants of certain candidate genes, selected based on their role in metabolism, contribute to traits related to body size, T2DM and CVD in the Finnish Diabetes Prevention Study (DPS) population. The specific aims of the present study were:

1. To examine the association of the 12Glu9 polymorphism in the gene encoding *ADRAB2* with T2DM risk and related phenotypes.
2. To determine whether genetic variations in the *ADIPOQ* gene are associated with serum levels of adiponectin, T2DM risk and body size.
3. To investigate the association of genetic variations in the *ADIPOR1* gene with body size and the risk of T2DM.
4. To study the association between *ADIPOR2* genetic variants and CVD and related risk factors.

4 Subjects and Methods

4.1 STUDY POPULATIONS AND STUDY DESIGNS

4.1.1 The Finnish Diabetes Prevention Study (*Studies I-IV*)

The DPS is a randomised and controlled longitudinal lifestyle intervention study carried out in five study clinics in Finland (Helsinki, Tampere, Turku, Kuopio and Oulu) during 1993-2000 (129). The main aim of the study was to assess the effect of lifestyle modification on T2DM risk in overweight, middle-aged individuals with IGT. Glucose tolerance status was defined according to the WHO 1985 criteria (122): IGT was defined as fasting plasma glucose < 7.8 mmol/l and a 2-h plasma glucose 7.8–11.0 mmol/l (OGTT, glucose load 75 g). The inclusion criteria of study participants were: BMI>25, age 40-64 years, and IGT based on the mean value of to OGTTs (361). The exclusion criteria were previous diagnosis of diabetes, vigorous exercise, glucose lowering treatments, and certain chronic diseases and metabolic conditions which could affect 6-year survival or interfere with glucose metabolism (361). Altogether 522 participants were randomly allocated either into a control group (n=257) or into a diet and exercise intervention group (n=265) with randomisation stratified according to study clinic, sex and the mean value of two hour plasma glucose concentration in OGTT.

Medical history was recorded and anthropometric and laboratory measurements were taken at baseline and at each annual follow-up visit. The subjects in the intervention group received individually tailored diet and exercise counselling (129, 362). The main goals of the intervention were: 1) 5-10% reduction in body weight, 2) reduction in the intake of total fat to ≤ 30% and 3) of saturated fat to ≤ 10% of daily energy intake, 4) increase of the intake of dietary fibre to at least 15 g per 1000 kcal, and 5) moderate-to-vigorous exercise at least 30 minutes per day. The subjects in the control group received general information on the benefits of healthy diet, physical activity and weight reduction. All subjects underwent an OGTT at each annual visit and in the cases of new diagnosis of T2DM the OGTT was repeated within a week to confirm the diagnosis (361). The median length of the active intervention period was four years, ranging from 1 to 6 years. DNA samples were available from altogether 507 study participants.

Cardiovascular mortality and morbidity data were collected after median follow-up of 10.2 years (range 1-13 years) from the national Hospital Discharge Register and Causes of Death Register using the national personal identification number (363). The end-points during follow-up were total mortality, and incident cardiovascular events (fatal and non-fatal), including acute coronary events, coronary heart disease, stroke and hypertensive disease.

Significant differences between the groups were not observed in any baseline clinical characteristics (361). The baseline characteristics of DPS participants whose DNA sample was available are shown in Table 6. The study protocol was approved by Ethics Committee of the National Public Health Institute in Helsinki, Finland and written informed consent was received from all participants.

4.1.2 The Genetics of obesity and insulin resistance study (*Study IV*)

Altogether, 75 overweight or obese (BMI 28-40 kg/m^2) men and women (aged 40-70 years) were recruited to the Genetics of obesity and insulin resistance study (Genobin). The subjects had impaired fasting glycemia (IFG: fasting plasma glucose concentration 5.6-7.0 mmol/l) or IGT, and fulfilled at least two criteria of metabolic syndrome according to the Adult Treatment Panel III Criteria (364) as modified by the American Heart Association (365): 1) WC >102 cm for men

and >88 cm for women, 2) fasting serum TG ≥1.7 mmol/l, 3) fasting serum HDL cholesterol <1.0 mmol/l for men and <1.3 for women; blood pressure ≥130/80 mmHg. The baseline characteristics of the Genobin participants are presented in Table 6. The Ethics Committee of the District Hospital Region of Northern Savo approved the study plan. All participants volunteered for the study and gave their written informed consent.

Table 6. Baseline characteristics of the DPS and Genobin study participants.

	DPS	Genobin
N (male/female)	160/324	29/27
Age (y)	55.19±7.03 (484)	59.43±6.82 (56)
Weight (kg)	86.26±14.17 (484)	92.77±13.99 (56)
BMI (kg/m^2)	31.23±4.51 (484)	32.72±2.86 (56)
Waist circumference (cm)	101.22±11.04 (482)	108.88±8.87 (56)
Fasting plasma glucose (mmol/L)	6.13±0.75 (484)	6.44±0.49 (56)
2-h plasma glucose (mmol/L)	8.88±1.49 (484)	7.42±2.15 (56)
Fasting serum insulin (mU/L)	14.73±7.46 (439)	11.96±7.29 (56)
2-h serum insulin (mU/L)	95.57±65.83 (436)	83.38±72.92 (56)
Serum total cholesterol (mmol/L)	5.61±0.93 (483)	5.16±0.98 (56)
Serum HDL cholesterol (mmol/L)	1.21±0.29 (483)	1.23±0.22 (56)
Serum LDL cholesterol (mmol/L)	3.62±0.84 (481)	3.37±0.91 (56)
Serum triglycerides (mmol/l)	1.73±0.78 (483)	1.66±0.84 (56)
Diastolic blood pressure (mmHg)	85.68±9.54 (479)	88.80±10.00 (56)
Systolic blod pressure (mmHg)	137.94±17.43 (479)	136.27±13.97 (56)

Data are mean±SD

4.2 METHODS

4.2.1 Anthropometric measurements (*Studies I-IV*)

Detailed methodologies for all measurements performed in the DPS have been described earlier (366). Weight and height were measured in light clothing, and BMI was calculated as kg/m^2. WC was measured midway between the lowest rib and iliac crest, and hip circumference over the great trochanters in a standing position. Sagittal and horizontal diameters were measured in supine position on a hard surface as a distance from the surface to the highest point of abdomen (sagittal) and the maximal width of the abdomen (horizontal) at the level of iliac crest. The three-year weight change (*Study I*) was calculated as the difference between baseline weight and weight at the three-year examination or last available measurement.

4.2.2 Biochemical measurements

Two hour OGTT

In the entire DPS population, a two hour OGTT was performed after a 12 hour fast to determine glucose tolerance status at baseline and annually. Samples for glucose and insulin determinations were drawn before, and 30 and 120 minutes after a 75 g glucose dose. Plasma glucose was measured locally by standard methods, and the measurements were standardised by central laboratory in Helsinki (129). Serum insulin was determined with radioimmunoassay (Pharmacia, Uppsala, Sweden).

Blood lipid measurements

Serum total cholesterol, HDL cholesterol and TG were analysed by using an enzymatic method (CHOD-PAP, Boehringer Mannheim, Germany). The Friedewald formula was used to calculate LDL cholesterol concentration (367).

Frequently sampled intravenous glucose tolerance test (Study I)

In the Kuopio study clinic, an insulin-modified FSIGT was performed at baseline (368). In total 83 DPS participants with available genetic data underwent the FSIGT. After drawing the fasting sample, a dose of 300 mg/kg of glucose was administered, as a 50% solution over 1.5 minutes, through a catheter inserted into the antecubital vein following infusion of 0.9% NaCl solution. After 20 minutes, a 0.03 U/kg bolus of insulin vas injected. Venous blood samples were collected before the glucose load (-5 min and 0 min) and 23 times after the glucose load. Plasma glucose concentration was analysed by a glucose oxidase method (Glucose Auto & Stat, Model GA-11, Daiichi, Kyoto, Japan) and insulin by radioimmunoassay method (Phadaseph Isulin RIA 100; Pharmacia Diagnostica, Uppsala, Sweden). The insulin sensitivity index (S_I) was calculated using the MINMOD program (369). The acute insulin response (AIR) was calculated as the area under the curve above the baseline level from 0 to 10 min after the glucose load. The disposition index (DI) was calculated as a product of AIR and S_I (136).

Serum adiponectin measurements (Study II)

Fasting serum adiponectin levels were measured using an enzyme-linked immunosorbent assay (B-Bridge International, Inc., CA, USA), on whole plasma samples stored at –80°C. The intra-assay and inter-assay coefficients of variation were 5.5–7.9% and 6.5%, respectively. Samples for adiponectin measurements were only available from subset of participants from three study clinics (n=243 at baseline, and n=209 at 4-year examination). In altogether 190 subjects both baseline and year four serum adiponectin concentrations were measured.

4.2.3 SNP Selection and genotyping

In *Study I*, the *ADRA2B* 12Glu9 variant was selected as a candidate polymorphism for T2DM based on previous findings and functional effects of the variant. In *Studies II* and *IV*, the tagSNPs with minimum minor allele frequency of 5% were selected based on the genotype data of the Hapmap CEU (Utah residents with ancestry from Northern and Western Europe) population (13) by using the Tagger algorithm (370). In *Study II*, the *ADIPOQ* SNPs rs2241766 and rs1501299, were selected based on previous literature and were forced into the tagSNP selection. In *Study II*, the ten SNPs selected for genotyping covered 73% of the common variation within *ADIPOQ* region with $r^2 \geq 0.8$. In *Study IV,* the eight *ADIPOR2* SNPs were based solely on the HapMap data to capture 63% of common variants within the *ADIP*OR2 locus with $r^2 \geq 0.8$. In *Study III*, the seven SNPs were selected both on basis of previous studies and the HapMap data (13).

Genomic DNA was isolated from peripheral blood leucocytes by the Puregene® DNA purification kit (Gentra Systems, Inc. Minneapolis, USA). The genotyping methods used in this work vary reflecting the technical developments in the field. In *Study I*, the *ADRA2B* 12Glu9 variant was detected by a combination of PCR and fragment length analysis by gel electrophoresis. The *ADIPOQ* SNPs rs2241766 and rs1501299 (*Study II*) were genotyped using PCR followed by SNaPshot ddNTP Primer Extension Kit technique (ABI Prism; Applied Biosystems) as described earlier (292). *ADIPOQ* rs17366568 (*Study II*), *ADIPOR1* SNPs rs2275738 and rs2275738 (*Study II*) were genotyped using PCR-RFLP method by using *MseI*, *MboII* and *MSlI* restriction endonucleases, respectively. Other SNPs were genotyped with TaqMan Allelic Discrimination assays according to manufacturer's instructions by using the ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA). For a subset of randomly selected samples (6.3%) genotyping was repeated in order to calculate success rate.

The total number of DPS participants included in each association study varies to some extent due to availability of DNA samples at each time point and the permission given by the participants for use of their samples in genetic analyses: n=506 in *Study I*, n=507 in *Study II* and *Study III*, and n=484 in *Study IV* .

4.2.4 Gene expression analysis (*Study IV*)

Collection of peripheral blood mononuclear cells (PBMCs) and AT samples

In the Genobin study, PBMCs were isolated according to the manufacturer's instructions from anticoagulated peripheral blood by using Lymphoprep reagent (Axis-Shield, Oslo, Norway). AT biopsies were taken by aspiration with a syringe from the subcutaneous abdominal AT under local anesthesia (Lidocaine 10mg/ml without epinephrine). The AT samples for the RNA extraction were treated with RNA later according to manufacturer's instructions (Ambion, Austin, TX, USA) and stored at -80 ℃. Altogether, 56 baseline PBMC and AT samples with genotype data were available for subsequent mRNA expression analyses.

RNA extraction and quantitative real-time PCR

Total RNA from AT samples was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA, and Qiagen, Valencia, CA, USA) following further purification with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA from PBMS samples was isolated with RNeasy Mini Kit according to the manufacturer's instructions. The integrity of RNA sample was assessed using gel electrophoresis and the RNA concentration and the A_{260}/A_{280} ratio were measured using NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) with acceptable ratio being 1.9-2.1. RNA was reverse transcribed into cDNA by using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time PCR was performed as triplets with ready-made assays based on TaqMan® chemistry and analysed with ABI Prism 7500 SDS software (Applied biosystems). Each reaction comprised 6 ng cDNA, 1X Assay Mix, and 1X Taqman Universal PCR Master Mix (Applied biosystems). On each plate, a standard curve with five concentrations (0.5, 1.5, 6, 18, and 36 ng/µl) and calibrator (6 ng/µl) were included to determine the relative quantity of cDNA in each sample by using the methods described in ABI Prism User Bulletin no. 2. The quantities on each plate were first corrected by the calibrator and the relative amount per plate was corrected with the corresponding values of endogenous control. The expression of *glyceraldehydes-3-phosphate dehydrogenase* and *cyclophilin A1* were used as an endogenous controls for normalising gene expression in PBMCs and AT, respectively.

4.2.5 Statistical analyses

The pairwise LD between SNPs was evaluated with Haploview software (371). In *Study III*, the haplotype analyses were performed with THESIAS programme version 3 (372). The compliance of genotype frequencies with Hardy–Weinberg equilibrium (HWE) was tested with the chi-square test.

Otherwise, the statistical analyses were performed with SPSS for Windows (SPSS Inc, Chicago, IL, USA) version 11.0.1. (*Studies I* and *III*) or 14.0 (*Studies II* and *IV*). Data are presented as mean ± SD or as median (interquartile range), unless otherwise indicated. Normality of variable distributions was tested with the Kolmogorov-Smirnov test with Lilliefors correction or by plotting the residuals of each statistical test. Appropriate variable transformations were used to improve normality when necessary. Homogeneity of variances was tested using Levene's test.

In *Study I*, the relationship between insulin secretion and insulin sensitivity for each genotype was explored by fitting an exponential curve and calculating coefficients of determination. In all studies, the distribution of genotypes between the study groups was examined by chi-square test. Genotype differences in continuous baseline variables were analysed with the general linear model (GLM) univariate analysis of variance (ANOVA) with appropriate adjustments. Kruskal-Wallis test was used instead of ANOVA for variables that failed to meet normality assumptions. Longitudinal data was examined by using GLM for repeated measurements (*Study III*) or linear mixed models analysis (*Study II*) with appropriate

covariates. The significance of differences in the three-year diabetes incidence between genotypes was analysed by the chi-square test (*Studies I* and *III*). Logistic regression (*Study I*) or Cox regression (*Studies II-IV*) analysis with appropriate covariates were used to assess whether genotypes predicted the development of T2DM. In *Study IV*, the association of *ADIPOR2* SNPs with cardiovascular risk was analysed with Cox regression with appropriate covariates.

The genetic analyses were performed by comparing all three genotype groups (additive inheritance model; used in all studies), by comparing the minor allele carriers to those homozygous for the major allele (dominant inheritance model; *Studies II* and *IV*) or by comparing the major allele carriers with those homozygous for the minor allele (recessive inheritance model; *Study II*). Analyses involving longitudinal data were performed separately in both DPS study groups. Moreover, subgroup analyses were performed when a significant interaction was found between genotype and gender, genotype and study group, or genotype and WC. A p-value of <0.05 was considered to be statistically significant. Correction for multiple hypotheses testing with the false discovery rate (FDR) method was performed using Q-value software (373) for single SNP analyses in *Studies II* and *IV*. π_0 was estimated with bootstrap method, with a λ range from 0 to 0.9 by 0.05.

5 Results

This thesis comprises a series of candidate gene studies performed in the participants of the DPS (*Studies I-IV*). In addition, allele-specific mRNA expression was analysed in AT and PBMC samples derived from Genobin study participants (*Study IV*). With the varying number of variants analysed per each of the four candidate genes, significant associations were found between several variants and various quantitative traits or disease risks. In this results part, the genetic associations are presented so that all variants associating with given or related phenotypes are considered in the same section.

5.1 GENOTYPE FREQUENCIES

The genotype counts and minor allele frequencies of *ADRAB2*, *ADIPOQ*, *ADIPOR1* and *ADIPOR2* gene polymorphisms are shown in Table 7 with the minor allele frequencies in the HapMap CEU reference population (13). All SNPs except the *ADIPOQ* SNP rs16861210 and the *ADIPOR2* SNP rs11061946 were in HWE ($p>0.05$). Since the q-values were high for both analyses ($q=0.683$ and $q=0.377$, respectively), these SNPs were included in subsequent analyses. The pairwise LD between genotyped *ADIPOQ*, *ADIPOR1* and *ADIPOR2* SNPs in the DPS study population are presented in Figures 2-4.

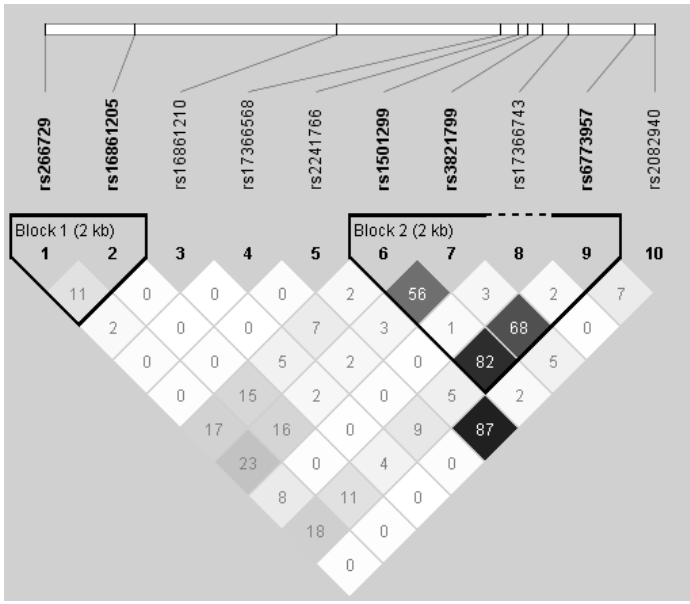

Figure 2. Schematic presentation of the ADIPOQ (Study II) gene indicating the locations of the analysed variants, haploblocks and the pairwise LD (r^2).

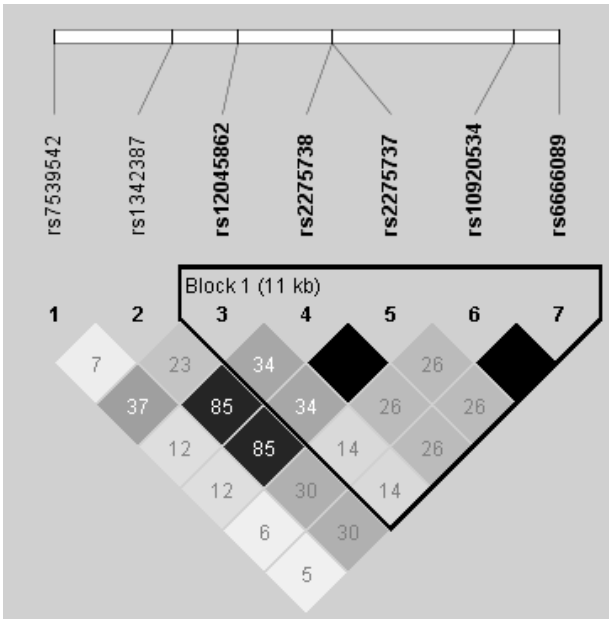

Figure 3. Schematic presentation of the ADIPOR1 (Study III) gene indicating the locations of the analysed variants, haploblocks and the pairwise LD (r^2).

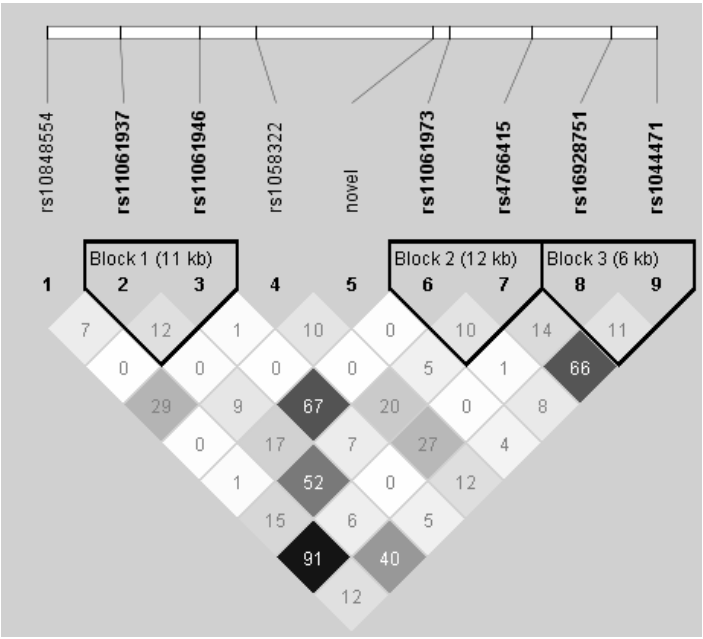

Figure 4. Schematic presentation of the ADIPOR2 (Study IV) gene indicating the locations of the analysed variants, haploblocks and the pairwise LD (r^2).

Table 7. Genotype counts and allele frequencies of the ADRA2B, ADIPOQ, ADIPOR1 and ADIPOR2 variants studied in the DPS population.

	SNP rs-identifier (major/minor allele)	Genotype (count)			Location within gene	MAF in DPS	MAF in HapMap CEU
		0	1	2			
ADRA2B	Glu12/9	145	257	104		Glu9 0.459	-
ADIPOQ	rs266729 (C/G)	219	240	48	5' promoter region	G 0.331	G 0.300
	rs16861205 (G/A)	337	151	19	Intron 1	A 0.186	A 0.084
	rs16861210 (G/A)	471	33	3	Intron 1	A 0.038	A 0.095
	rs17366568 (G/A)	371	122	14	Intron 1	A 0.148	A 0.119
	rs2241766 (T/G)	460	45	2	Exon 2	G 0.048	-
	rs1501299 (G/T)	234	220	53	Intron 2	T 0.321	T 0.279
	rs3821799 (C/T)	149	255	103	Intron 2	T 0.455	T 0.438
	rs17366743 (T/C)	468	39	-	Exon 3	C 0.038	C 0.075
	rs6773957 (G/A)	206	233	68	3' UTR	A 0.364	A 0.376
	rs2082940 (C/T)	466	39	2	3' UTR	T 0.042	T 0.102
ADIPOR1	rs10920534 (C/T)	263	184	32	Intron 1	T 0.259	-
	rs2275738 (G/A)	167	234	106	Intron 1	A 0.440	A 0.438
	rs12045862 (C/T)	246	214	47	Intron 2	T 0.304	T 0.215
	rs1342387 (G/A)	139	224	116	Intron 3	A 0.477	T 0.459
	rs7539542 (C/G)	222	236	49	3' UTR	G 0.329	G 0.289
ADIPOR2	rs10848554 (G/C)	392	107	8	5' promoter region	C 0.121	C 0.138
	rs11061937 (T/C)	225	221	61	Intron 1	C 0.338	C 0.304
	rs11061946 (C/T)	451	51	5	Intron 1	T 0.060	T 0.080
	rs1058322 (C/T)	244	213	50	Intron 1	T 0.309	T 0.285
	rs11061973 (G/A)	424	78	5	Intron 2	A 0.087	A 0.162
	rs4766415 (A/T)	137	263	107	Intron 2	T 0.470	T 0.487
	rs16928751 (G/A)	398	104	5	Exon 7	A 0.112	A 0.115
	rs1044471 (C/T)	143	254	110	3' UTR	T 0.467	T 0.478

MAF, minor allele frequency; UTR, untranslated region

5.2 ASSOCIATIONS WITH BODY SIZE

5.2.1 *ADIPOQ* SNPs (*Study II*)

The *ADIPOQ* variations rs266729, rs16861205, rs1501299, rs3821799 and rs6773957 were associated with baseline body weight and BMI when adjusted for age and sex. The p-values for all SNPs are presented in $-\log_{10}$ scale in Figure 5. The rs266729 G, the rs16861205 G, the rs1501299 G, the rs3821799 C, and the rs6773957 G allele associated dose-dependently with higher baseline body weight (*Study II*: Table 3). For rs266729 a significant sex–genotype interaction was found for weight and significant differences were observed in women.

The same four SNPs were associated with the four-year follow-up measurements of weight in additive inheritance model (rs16861205: p=0.028/q=0.049, rs1501299:p=0.041/q=0.049, rs3821799: p=0.002/q=0.049, and rs6773957: p=0.045/q=0.049) and the associations were even stronger when the dominant inheritance model was used (rs16861205: p=0.008/q=0.049, rs1501299: p=0.014/0.049, rs3821799: p=0.001/q=0.049, and rs6773957: p=0.014/q=0.049). For rs266729, significant differences were only observed in women (p=0.007/q=0.038 for the dominant inheritance model, and p=0.027/q=0.309 for sex–genotype interaction). Similar genotype associations were found for BMI. The longitudinal body weight and BMI data are shown for rs3821799 in Figure 6.

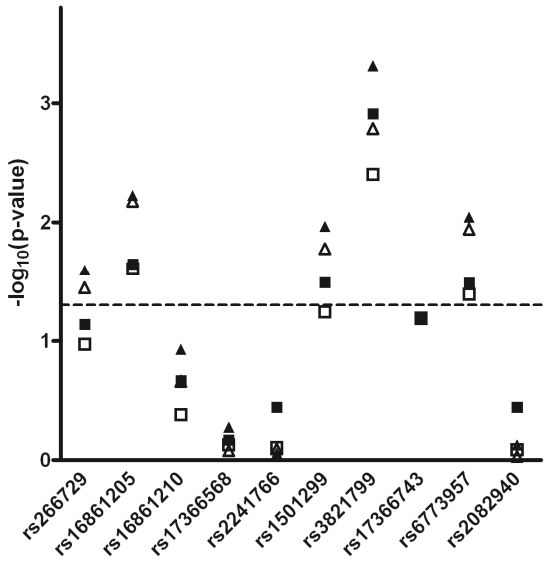

Figure 5. Association of ADIPOQ SNPs with baseline body weight and BMI (Study II). The p-values are for ANOVA adjusted for age and sex, and are presented on $-log_{10}$ scale. Points above the line indicate p-values below the threshold of p<0.05. Black symbols, body weight; open symbols, BMI; Squares, additive inheritance model; triangles, dominant inheritance model.

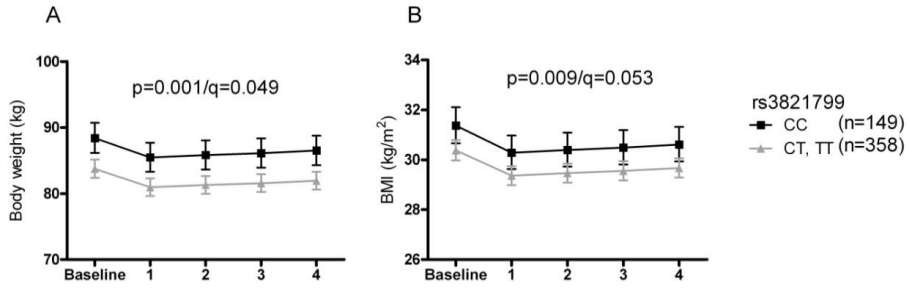

Figure 6. Body weight (A) and BMI (B) from baseline to year 4 according to ADIPOQ SNP rs3821799 (Study II). Data are mean±SEM; p-values for dominant inheritance model are adjusted for age, sex and study group.

In the multiple SNP model including rs266729, rs16861205, rs1501299, rs3821799 and rs6773957, a trend for significant association with baseline body weight was observed for rs3821799 (p=0.063). Following the removal of the three least significant SNPs rs3821799 (p=0.007) remained significantly associated with baseline body weight.

5.2.2 *ADIPOR1* SNPs (*Study III*)

ADIPOR1 SNPs rs10920534, rs22757538 and rs1342387 were significantly associated with multiple anthropometric measurements, such as body weight, height, BMI, WC, hip circumference, WHR, sagittal diameter, and horizontal diameter either in all individuals or in men and women separately. The rs10920534 TT, the rs22757538 GG, and the rs1342387 GG genotypes were associated with larger body size. In addition, carriers of the rs12045862 T allele

were taller compared with those with the CC genotype. Figure 7 presents the p-values (on a –$\log_{10}$ scale) for *ADIPOR1* SNPs for all DPS participants and for men and women separately.

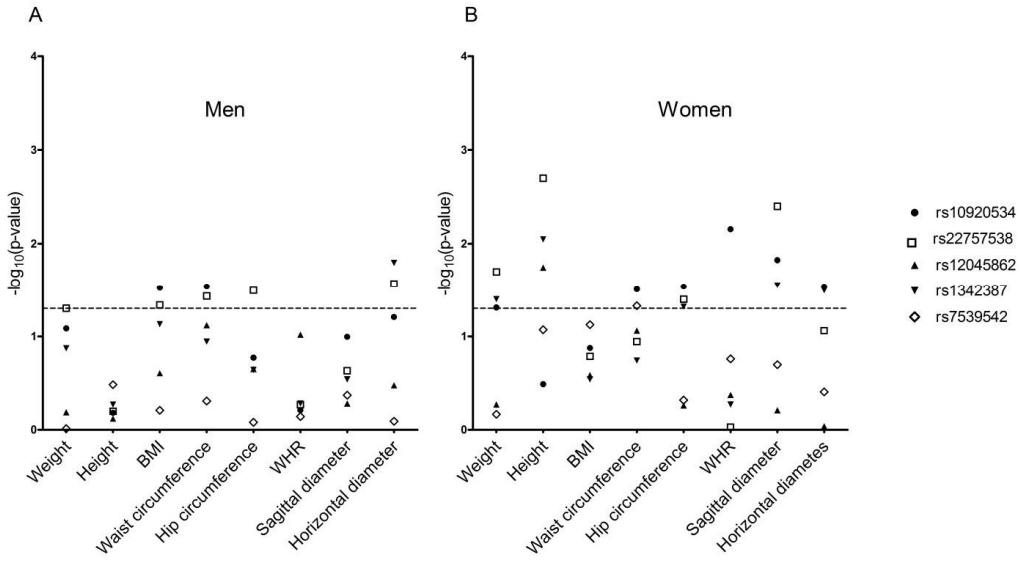

Figure 7. Association of ADIPOR1 SNPs with various baseline body size measures in men (A) and women (B) (Study III). The p-values are from Kruskal-Wallis test or ANOVA adjusted for age and sex (in all), for comparison among all three genotype groups. Points above the line indicate p-values below the threshold of p<0.05.

Genotype differences in body size measures were further reinforced by analysing the longitudinal data from baseline to year three. In the entire study population, significant differences were seen according to rs10920534 (*Study III*: Figure 2), rs2275738 and rs134287 in body weight (p=0.002, p=0.001 and p=0.007, respectively) and WC (p=0.001, p=0.003 and p=0.012, respectively) when adjusted for age and sex. When the follow-up period was extended to year four and analysed using the linear mixed models analysis (adjusted for age, sex and study group), genotype differences remained significant for all three SNPs, as shown for rs10920534 in Figure 8.

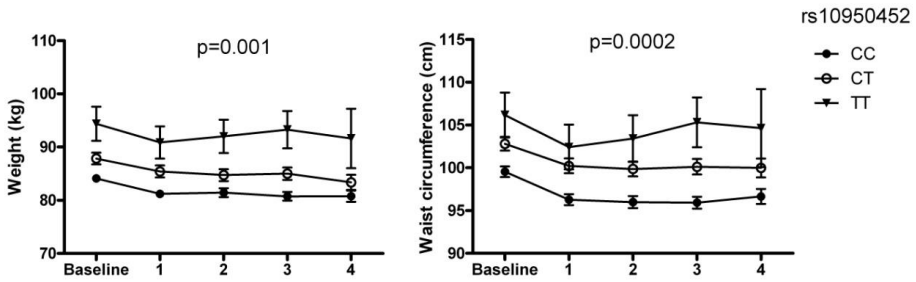

Figure 8. Body weight (A) and waist (B) circumference from baseline to year 4 according to ADIPOR1 rs10920534 (Study III). Data are mean±SEM; p-values for additive inheritance model are adjusted for age, sex and study group.

In addition to single SNP analyses, we examined the covariate adjusted effects of five-marker haplotypes on baseline body size measurements by comparing all other haplotypes with frequency over 0.03 with the most frequent haplotype (*Study* III: Table 3). The results of the haplotype analyses supported the results of single SNP analyses by showing associations between large body size measurements and those haplotypes that harboured alleles associated with larger body size.

5.3 ASSOCIATION OF *ADIPOQ* SNPS WITH SERUM ADIPONECTIN LEVELS (*STUDY II*)

In a subgroup of DPS study participants, three *ADIPOQ* SNPs associated with baseline serum adiponectin levels, adjusted for age, sex and baseline WHR (Figure 9 and Table 5 in *Study II*). Lower baseline serum adiponectin concentrations were associated with the rs16861210 G allele, the rs17366568 A allele and the rs6773957 G allele. Moreover, the rs2241766 TT genotype and the rs2082940 CC genotype showed a trend for association with lower levels. In the multiple SNP model including rs16861210, rs17366568, rs6773957 and either rs2241766 or rs2082940, the first two SNPs remained significantly associated with baseline adiponectin levels (p=0.012 and p=0.003, respectively).

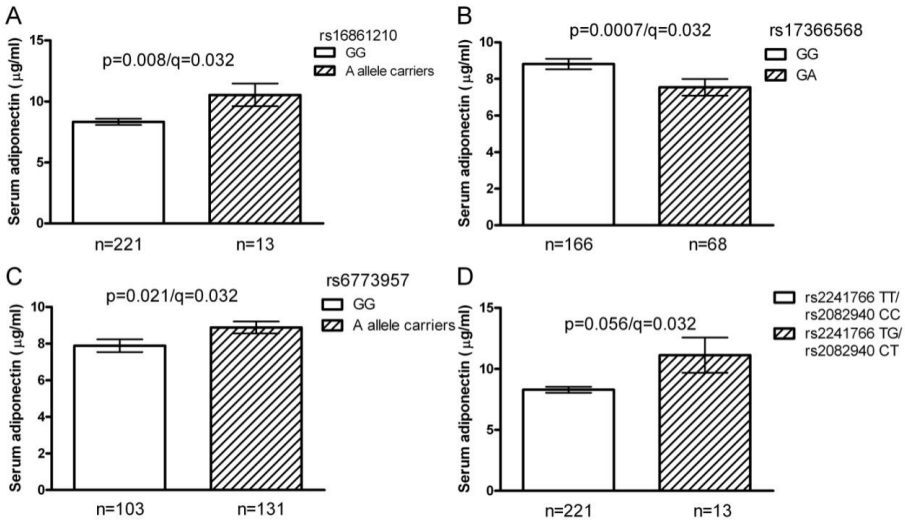

Figure 9. Baseline serum adiponectin levels according to ADIPOQ SNPs (Study II): rs16861210, rs17366568, rs6773957, and rs2241766/rs2082940 (with identical results). P-values for the dominant inheritance model, adjusted for age, sex and baseline WHR.

5.4 ASSOCIATIONS WITH INSULIN AND GLUCOSE METABOLISM, AND THE RISK OF T2DM

Altogether 72 individuals for whom genotype data were available converted from IGT to T2DM (21 in the intervention group and 51 in the control group) during the three-year study period, with 58% relative risk reduction in the lifestyle intervention group (129, 362). During the extended follow-up of 0-11 (median 7) years number of individuals that converted to T2DM

was 184 (74 in the intervention group and 110 in the control group), with 43% relative risk reduction in the lifestyle intervention group (81).

Genetic variants in *ADRA2B*, *ADIPOQ* and *ADIPOR2* associated with the risk of T2DM and the results from extended follow-up period are presented for these variants in Table 8. There were no significant differences in genotype associations between the study groups and the data is presented for the entire DPS population.

5.4.1 *ADRA2B* 12Glu9 polymorphism and the risk of T2DM (*Study I*)

A statistically significant interaction between study group *ADRA2B* 12Glu9 variant was observed for the risk of T2DM (p=0.003, adjusted for age, sex, WC, three-year weight change, study group and fasting plasma glucose). When the study groups were analysed separately, individuals carrying the Glu9 allele had increased risk of developing T2DM compared with those with the Glu12/12 genotype in the control group (OR=2.68, 95% CI 1.02-7.09, p=0.047 for Glu12/9 and OR=5.17, 95% CI 1.76-15.21, p=0.003 for the Glu9/Glu9 genotype). On the contrary, the Glu9/9 genotype was associated with a lower risk of T2DM in the intervention group (OR=0.09, 95% CI 0.01-0.99, p=0.049).

Table 8. Summary of the hazard ratios for ADRA2B, ADIPOQ and ADIPOR2 variants associating with the risk of T2DM in DPS.

Gene	Variant	Genotype	HRR, (95% CI), p/q[a]	HRR, 95% CI, p/q[b]
ADRA2B	12Glu9	Glu12/12 (139)	1	1
		Glu12/9 (244)	1.460, (1.024-2.081), 0.036 [c]	1.446, (1.021-2.049), 0.038[c]
		Glu9/9 (98)	1.243, (0.801-1.929), 0.331 [c]	
ADIPOQ	rs266729	CC (219)	1	1
		CG (240)	0.695, (0.513-0.942), 0.019/0.203	0.682 (0.510-0.912), 0.010/0.203
		GG (48)	0.615, (0.350-1.081), 0.091/0.330	
	rs2241766	TT (460)	1	1
		TG (45)	1.521, (0.944-2.452), 0.085/0.330	1.634 (1.035-2.579), 0.035/0.216
		GG (2)	5.448, (1.329-22.333), 0.019/0.203	
	rs2082940	CC (466)	1	1
		CT (39)	1.383, (0.812-2.357), 0.233/0.668	1.518 (0.918-2.512), 0.104/0.334
		TT (2)	5.368, (1.310-21.997), 0.020/0.203	
ADIPOR2	rs11061946	CC (428)	1	1
		CT (49)	0.767, (0.449-1.309), 0.331/0.802	0.926, 0.572-1.1499, 0.753/0.918
		TT(5)	4.263, (1.528-11.893), 0.006/ 0.514	
	rs11061973	GG (401)	1	
		GA (76)	1.044, (0.700-1.558), 0.831/0.934	1.149, 0.786-1.681, 0.474/0.885
		AA (5)	4.425, (1.585-12.352), 0.005/0.514	

[a]Additive inheritance model, adjusted for age, sex, baseline fasting glucose concentration, baseline WHR and study group; [b]Dominant inheritance model, adjusted for age, sex, baseline fasting glucose concentration, baseline WHR and study group; [c] FDR not applied

A trend for interaction between 12Glu9 and baseline WC was observed for T2DM incidence (p=0.052). Subsequently, men and women were categorised according to the cut-off values of 102 cm for men and 88 cm (102) and the association between 12Glu9 and T2DM incidence was analysed in these groups separately. In the high WC group, the Glu9 allele was dose-dependently associated with a higher incidence of T2DM (p=0.011, for comparison among three genotype groups), whereas in the low WC group the Glu9 allele was associated with a lower incidence of T2DM (p=0.002, for comparison among three genotype groups) (*Study I*: Figure 1). The Glu12/9 and the Glu9/9 genotypes associated with a higher risk of T2DM (OR=2.30, 95% CI, p=0.063, and OR=3.80, 95% CI 1.41-10.26, p=0.008, respectively) when compared with the Glu 12/12 genotype in the high WC group. In the low WC group, the Glu12/9 genotype demonstrated a trend for lower T2DM risk in comparison with the Glu12/12 genotype, but statistical significance was not reached by using the additive inheritance model.

When follow-up was extended to mean 6.4 years and analysis method was changed to Cox regression, to match *Studies II-IV*, a significant (at p<0.05 level) difference in risk of T2DM was still seen according to 12Glu9 genotype in the whole DPS population (Table 8) with the dominant inheritance model. The genotype-study group interaction was no longer significant, and when the study groups were analysed separately the association between *ADRA2B* genotype and T2DM risk was not significant in either group. Interestingly, with the extended follow-up, a significant interaction between genotype and baseline WC was still observed (p=0.033, for the dominant inheritance model). In the low WC group, the Glu9 allele carriers had lower (HR=0.38, 95% CI 0.16-0.92, p=0.032) and in the high WC group higher (HR=1.83, 95% CI 1.22-2.76, p=0.003) risk of T2DM compared with individuals with the Glu12/12 genotype.

5.4.2 Association of *ADRA2B* 12Glu9 with insulin secretion (*Study I*)

In a subpopulation of study participants (n=83), a frequently sampled intravenous glucose tolerance test (FSIGT) was performed at baseline. In this subgroup the, Glu9 allele was associated dose-dependently with low AIR and DI (p=0.004 and p=0.003, respectively, adjusted for age, sex, and BMI) (Figure 10). Moreover the exponential relationship between AIR and SI was only seen individuals with the Glu12/12 genotype (r^2=0.223, p=0.006), but not in individuals carrying the Glu9 allele (*Study I*: Figure 2).

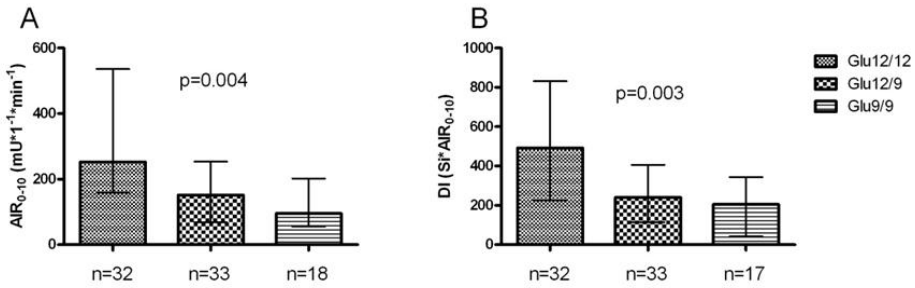

Figure 10. Baseline AIR (A) and DI (B) according to ADRA2B 12Glu9 genotype (Study I). Values are medians (interquartile ranges). The p-values are for ANOVA comparison among all three genotype groups, adjusted for age, sex and BMI.

5.4.3 *ADIPOQ* SNPs and the risk of T2DM (*Study II*)

The *ADIPOQ* SNP rs266729 G allele associated with a decreased risk for conversion from IGT to T2DM (Table 8). In addition, the homozygous minor allele carriers (n=2) of rs2241766 and rs2082940 (GG and TT, respectively) had increased risk of developing T2DM (Table 8). In a multiple-SNP model including all three SNPs, only rs266729 associated with T2DM risk (HR=0.693, 95% CI 0.518-0.928, p=0.014 fot the G allele carriers compared with individuals with the CC genotype).

5.4.4 *ADIPOR1* SNPs and baseline insulin concentrations (*Study III*)

Three *ADIPOR1* SNPs associated with insulin levels and genotype differences were more often seen in men (Figure 11). The T allele of rs10920534 and the C allele of rs12045862 associated with higher two-hour insulin levels in men (p=0.027 and p=0.001, respectively, for comparison among three genotype groups, adjusted for age and BMI), but women carrying the rs12045862 T allele had higher two-hour insulin levels (p=0.029). Moreover, rs12045862 C allele and rs7539542 C allele associated with higher fasting serum insulin in men (p<0.001 and p=0.001, respectively). None of the *ADIPOR1* SNPs analysed predicted conversion from IGT to T2DM.

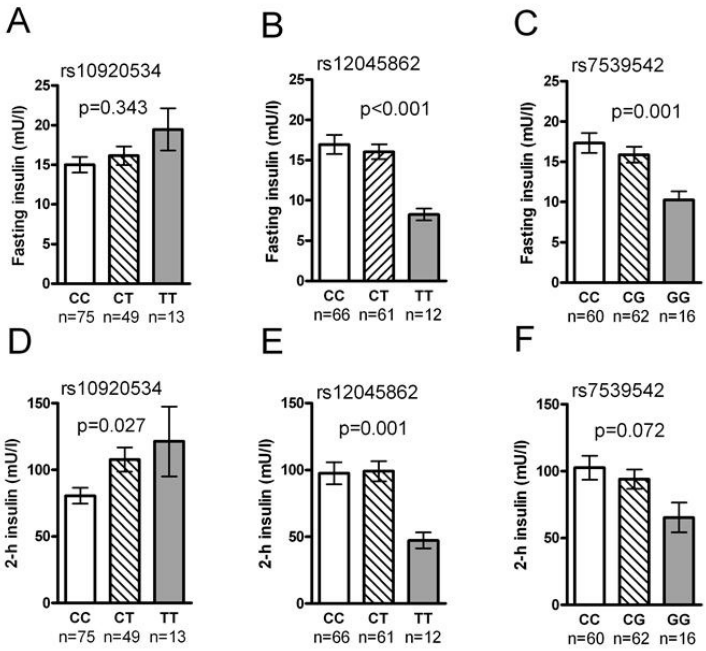

Figure 11. Baseline fasting and two-hour insulin according to ADIPOR1 rs10920534 (A and D), rs12045862 (B and E), and rs7539542 (C and F) in men participating DPS (Study III). Data are mean±SEM. ANOVA across all three genotypes were adjusted for age and BMI.

5.4.5 *ADIPOR2* SNPs and the risk of T2DM (*Study IV*)

The rare minor alleles of two *ADIPOR2* SNPs associated with the risk of T2DM. Subjects homozygous for the rs11061946 T allele and the rs11061973 A allele (n=5) had significantly higher risk of T2DM compared with subjects homozygous for the major alleles (Table 8). When study groups were analyzed separately, results were similar in both study groups.

5.5 ASSOCIATION OF *ADIPOR2* VARIANTS WITH CVD (*STUDY IV*)

After a median follow-up period of 10.2 years, there were 100 CVD events among the DPS participants who were available for genetic analyses (50/241 in the intervention and 50/232 in the control group) (363). Four *ADIPOR2* SNPs were associated significantly with the risk of CVD (Figure 12). The rs10848554 C allele associated with an increased risk of CVD when compared with the GG genotype (HR=1.61, 95% CI: 1.04-2.50, p=0.032/q=0.085). Additionally, carriers of the rs11061937 C allele had significantly lower risk of a CVD event than those with the TT genotype (HR=0.59, 95% CI: 0.39-0.88, p=0.019/q=0.085). Finally, the rs1058322 T allele and the rs16928751 A allele were dose-dependently associated with higher risk of CVD (HR=1.75, 95% CI: 1.14-2.68, p=0.010/q=0.085 and HR=1.80, 95% CI: 1.12-2.82, p=0.010/q=0.085 for dominant inheritance models, respectively). The analyses were performed separately in the intervention and in the control group, and similar results were found in both. Moreover, no significant interaction between *ADIPOR2* variants and sex or baseline BMI on CVD risk was found. In the multiple SNP model, including all four SNPs, two variants (rs11061937 and rs1058322) remained significant predictors of CVD risk (p=0.014 and p=0.020, respectively).

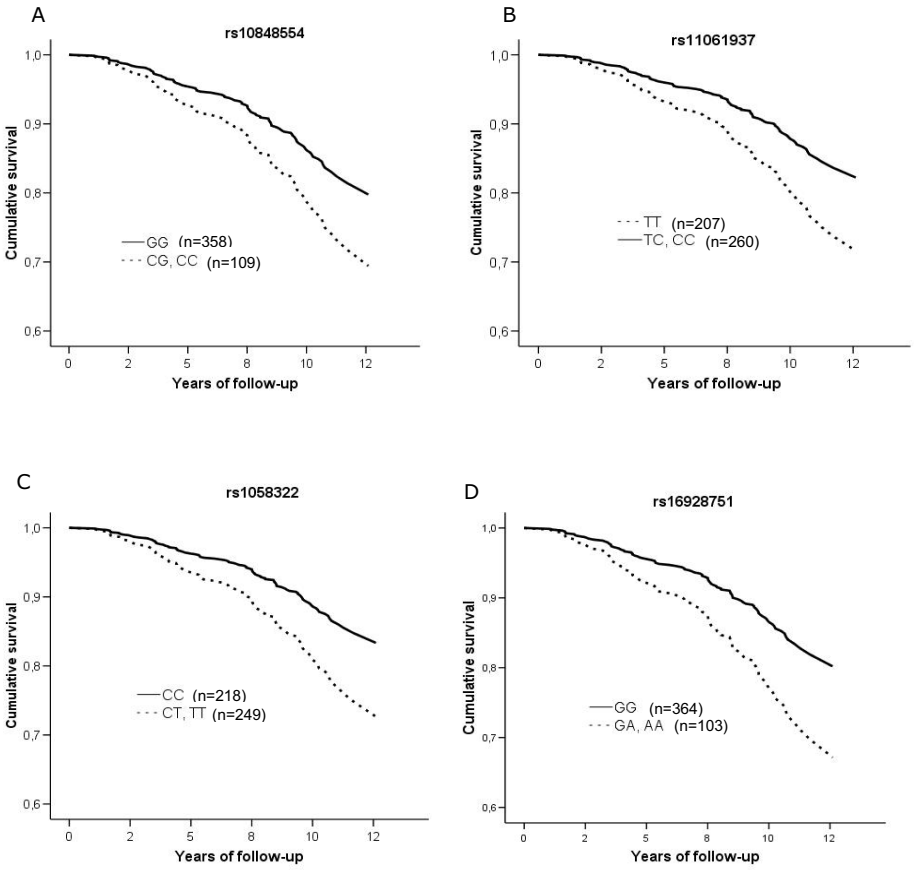

Figure 12. Cox survival curves for CVD incidence according to ADIPOR2 rs10848554 (A), rs11061937 (B), rs1058322 (C), and rs16928751 (D) in DPS participants (Study IV). Dominant inheritance model, adjusted for age, sex, baseline BMI, study group, CVD at baseline, systolic blood pressure, smoking status, and baseline total-to-HDL cholesterol ratio.

5.6 ALLELE DIFFERENCES IN THE MRNA EXPRESSION OF *ADIPOR2* (*STUDY IV*)

In the PBMCs, the expression of the *ADIPOR2* at the mRNA level differed according to the rs1058322 genotype (p=0.029/q=0.328 adjusted for age, sex and baseline BMI) with individuals carrying the T allele demonstrating lower expression levels than those with the CC genotype (*Study IV*: Figure 2 B). A trend for dose-dependent effect was seen when using the additive inheritance analysis model (p=0.091/q=0.328). None of the other SNPs included in the present study associated with the mRNA expression levels in PBMCs, and no differences in expression levels according to *ADIPOR2* SNPs were seen in AT samples.

6 Discussion

6.1 METHODOLOGICAL ISSUES

6.1.1 Study population

The major strength of this study series is the use of clinically well-defined and carefully selected study populations. The DPS population is a homogeneous group of overweight and middle-aged Finnish individuals with IGT who are at high risk of developing T2DM and CVD (129). Likewise, the participants of the Genobin study (*Study IV)* were overweight and middle-aged Finnish individuals with either IGT or IFG and additional features of metabolic syndrome. In the DPS, the prospective study design and longitudinal follow-up data are superior to case-control or cross-sectional samples, which are typically used in candidate gene studies (374). Another advantage is that, compared with more admixed populations of central European background, the Finnish population is characterised by relatively low genetic diversity and a high degree of LD. This genetic homogeneity may offer benefits in performing genetic association studies, even though some caution for population substructures may be necessary (375, 376). The genetic homogeneity of the Finnish population is due to the demographic history of Finland, which is characterised by a small founder population, long-standing isolation, several population bottlenecks and recent expansion (377).

The main limitation of the study populations used was the moderate sample size of the DPS and the small sample size of the Genobin population. This may weaken the statistical power to find true associations and increase the chance of false positive findings, particularly in the case of gene variants with low MAF. Moreover, in two studies only a subset of the DPS participants was used for genetic association analyses: in *Study I*, the FSIGT was performed at baseline for 83 individuals, and in *Study II* the baseline serum adiponectin levels were measured from 243 individuals, further decreasing the statistical power. The Genobin study was originally designed as a clinical intervention study and the number of participants may be considered too small for genetic studies. However, allele-specific differences could be detected in the mRNA expression levels that were biologically plausible and may be considered statistically significant.

6.1.2 Candidate gene approach

Generally, the results of genetic association studies of complex diseases have proven difficult to replicate. Several explanations may underlie the inconsistent results. First, the allele frequencies and LD patterns vary between populations with different ethnic backgrounds. Another reason is that multiple environmental and genetic factors, and their interactions, affect quantitative traits and the risk of complex diseases, such as obesity and T2DM. Furthermore, the effect sizes of individual variants are usually small, and different combinations of predisposing and protective variants may lead to various outcomes. In addition, direct comparison of individual studies is challenging, since different combinations of SNPs are usually investigated in each study.

During the past decade, the nature of genetic association studies has changed dramatically due to technological development, and decreasing costs of genotyping and sequencing. Nowadays GWASs dominate the field and significantly larger study populations are required for sufficient statistical power. However, as GWASs have certain limitations, candidate gene studies performed with carefully selected candidate genes in high-quality study populations still hold their value, especially when the results are replicated in independent study populations. Moreover, the candidate gene approach is likely to be used in the future for more

accurate localisation of true functional variant(s) after an association between genomic region and phenotype is revealed by GWAS (61).

6.1.3 Candidate gene and SNP selection

Candidate genes in these studies were selected based on previous literature and the biological information available on gene products and their functions in metabolic processes.

In early candidate gene studies typically only one or few markers, often known or suspected to be functional, per gene were genotyped. However, the international HapMap project (13) has yielded information on LD patterns in different human populations and it is now possible to select minimal set of tagSNPS to capture most of the genetic variation in a given region.

This general trend of the field is reflected in this series of studies. *Study I* may be regarded as a candidate polymorphism study, since a single functional variant was genotyped, whereas in *Studies II-III*, a set of tagSNPs was selected to cover the maximal amount of common variation in the candidate gene loci.

6.1.4 Genotyping accuracy

The genotyping methods used in *Studies I-IV* vary according to the technological development in the field. In each study, an appropriate number of samples were replicated in order to confirm genotyping accuracy. Moreover, as a genotyping quality check, each variant was tested for HWE. A significant deviation from HWE may indicate genotyping error, but may also result from population stratification, non-random mating, selection, inbreeding or presence of common deletion polymorphisms (378). The genotype frequencies of two SNPs, the *ADIPOQ* rs16861210 and the *ADIPOR2* rs11061946, deviated from HWE at a significance level of $p<0.050$. However, the FDR for both was high ($q>0.150$), and therefore both SNPs were included in subsequent analyses. It is likely that the violation of HWE is due to change, as is often the case for variants with low genotype counts (378). In addition, it is noteworthy that the HWE should always be tested in a control population, since deviation may actually be an indication of disease association (379).

6.1.5 Statistical issues

Performing multiple statistical tests in genetic association studies is a common problem and it increases the probability of false positive results. A general practice for applying correction for multiple comparisons is lacking, and the available correction methods are generally not well suited for hypothesis-driven candidate gene approach. In the context of correlated tests, such as testing multiple SNPs that are in LD, or related phenotypes, the conservative Bonferroni method, in which p-values are multiplied by total number of tests, may lead to rejection of true associations in studies with limited power (57, 378). Therefore, replication of the associations in independent study populations is considered to be the optimal way to separate genuine associations from false positives (380).

In these studies, the unadjusted p-values were considered cautiously with the significance level at $p<0.05$. Additionally, in *Studies II* and *IV* the FDR method, which estimates the proportion of false positive tests among all positives, was used for correction of multiple comparisons. The FDR derived q-values were considered together with the p-value in these studies. A result with p-value below the traditional $\alpha=0.05$ level, but with high q-value might be expected to be a false positive finding. Since a common threshold for q-value has not been established, the exact q-values are reported and those below 0.150 are considered suggestive of a true association.

In *Study I*, the association of the *ADRA2B* variant with the T2DM incidence was analysed by logistic regression after three years of follow-up. However, in subsequent studies the follow-up was extended to seven years and the conversion from IGT to T2DM was analysed by Cox regression analysis, which takes into account the survival time until the development of T2DM and is capable of handling information regarding individuals who dropped out of the study.

Similarly, in *Study III*, the longitudinal analyses were performed with the three-year follow-up data using the GLM repeated measures procedure, whereas in *Study II*, the longitudinal data during four years of follow-up were analysed using linear mixed models analysis, which can take into account subjects with missing data. In this thesis, the data from *Studies I* and *III* were re-analysed using same follow-up duration and statistical methods that were used in later studies and the results remained essentially similar to the original studies.

6.2 CONSIDERATION OF MAJOR RESULTS

6.2.1 Association studies on *ADRA2B* gene

The results of *Study I* suggest that the *ADRA2B* 12Glu9 variant is associated with AIR and DI, the markers of beta cell function, in individuals with IGT. The Glu9 allele associated with lower AIR and DI, but also with a higher risk of T2DM in individuals who had high WC or were not included in the lifestyle intervention group of the DPS. In contrast, the risk of T2DM risk may be slightly decreased, in individuals carrying the Glu9, who were involved in the lifestyle intervention or who had low WC.

As a product of insulin sensitivity and first phase insulin secretion measures, DI describes the ability of beta cells to compensate for decreased insulin sensitivity by increasing insulin secretion (132, 136). It can be assumed that the capacity of beta cells to compensate for insulin resistance is already disturbed to some extent in participants of the DPS who all had IGT. Nevertheless, the Glu9 allele was dose-dependently associated with lower DI and similar genotype difference was seen in insulin secretory capacity (AIR), even when not adjusting for insulin sensitivity. Finally, indicating impaired beta cell function, the normal exponential relationship between AIR and SI was absent in carriers of the Glu9 allele.

Measures of insulin secretion have been shown to have high heritability (381), and most of the genetic variants associated with T2DM identified so far are related to insulin secretion rather than to insulin sensitivity of target tissues (160, 189, 382). This study was the first to report the association between *ADRA2B* 12Glu9 with beta cell function, AIR and risk of T2DM. The observation is strengthened by the observation that the association with the risk of conversion from IGT to T2DM remained significant in the whole study population and in individuals with high WC after the extended follow-up time.

The 12Glu9 variant involves three glutamic acid residues in the third intracellular loop of the α2B-AR (343). The deletion allele has been shown to have functional consequences by impairing the agonist-promoted receptor desensitisation *in vitro* (343). Since complex neural mechanisms are involved in the regulation of insulin secretion (341) and blockage of α2-ARs has been shown to improve glucose-potentiated insulin secretion in T2DM (383), it may be hypothesised that the Glu9 allele might cause prolonged inhibition of insulin secretion through impaired receptor desensitisation.

The Glu9 allele was not a significant predictor of T2DM in individuals who were involved in lifestyle intervention or who were less abdominally obese. Compared with individuals in the control group, those in the intervention group achieved significantly larger weight loss leading to improvement in insulin sensitivity (362, 368). These results suggest that the improvement in insulin sensitivity, due to beneficial lifestyle changes, benefits more the carriers of the Glu9 allele, whose beta cell capacity may be genetically impaired. Similarly, central obesity is connected to insulin resistance (132), and the Glu9 allele increased the risk of T2DM only in individuals with high WC. This interactive effect of 12Glu9 with baseline WC was still detectable after the extended follow-up time. Interestingly, others have also reported similar interactions between 12Glu9 and lifestyle (358-360).

Up until the time of writing, the association with beta cell function has not been replicated. Furthermore, *ADRA2B* SNPs has not been identified by GWAS for T2DM or traits relating to beta cell capacity (70). However, Papazoglou *et al.* (384) reported that among individuals with

T2DM, the Glu9 allele associated with earlier onset of T2DM and a variant in the gene encoding the α2A-AR subtype was recently implicated in insulin secretion and the risk of T2DM (385, 386).

6.2.2 Association studies on *ADIPOQ* gene

Several SNPs in the *ADIPOQ* locus were associated with body weight, the risk of T2DM, and serum adiponectin concentrations in the participants of the DPS study. These results lend support to the role of *ADIPOQ* variations in conditions linked to T2DM and obesity, by replicating earlier findings, but also presenting novel associations. Another aim of *Study II* was to examine whether *ADIPOQ* SNPs modify the effect of lifestyle intervention on various metabolic traits. Gene-lifestyle interactions were not found, however, and the results were basically similar when the study groups were analysed separately.

The studies investigating association of *ADIPOQ* variants with metabolic traits have given highly inconsistent results in various study populations. The strongest and most consistent evidence exists for association of *ADIPOQ* variants with circulating adiponectin levels (203, 237, 240, 241, 298, 299, 305, 308, 309, 387) supporting the strong genetic control of adiponectin. On the contrary, the role of *ADIPOQ* variants in obesity and T2DM is more controversial (218). At the time of the writing of this thesis, five GWAS on circulating adiponectin have been reported in the NHGRI Catalogue of Published Genome-Wide Association Studies and four of these have identified the *ADIPOQ* locus with genome-wide significance (70). By contrast, none of the GWAS for T2DM or obesity have identified *ADIPOQ* variants.

In the DPS population, the C allele of the promoter variant rs266729 was associated with an increased risk of T2DM, whereas the G allele associated with body weight particularly in women. Others have also reported an association between rs266729 and obesity-related phenotypes (305, 309), the risk of T2DM (388), or insulin resistance (305, 318), but mostly with opposing risk alleles. In accordance with the results of the present study, the C allele associated with an increased risk of T2DM in a German study population (310) and lower insulin sensitivity in a cohort of Caucasian adolescents and their parents (312). Although associations between circulating adiponectin levels and rs266729 and other promoter SNPs have been reported widely (240, 241, 299, 308, 309), an association between rs266729 and adiponectin levels was not found in the DPS, possibly due to lack of statistical power. It is currently unclear whether rs266729 has any functional relevance or is in LD with a yet unidentified functional variant(s). A recent functional study suggested that rs266729 may alter the binding site of transcriptional stimulatory protein (389). Another study reported that rs266729 and two other promoter SNPs have an important role in regulating *ADIPOQ* promoter activity (313). On the other hand, results of three other functional studies suggest that rs266729 does not directly influence the transcription efficiency (240, 305). Two studies have found that the effect of this variant may also be sensitive to dietary factors (318, 390). Santos *et al.* (390) found an interactive effect of rs266729 and the percentage of energy derived from fat on obesity, whereas in another study interactive effect with plasma levels of saturated fatty acids was found on homeostasis model assessment of HOMA-IR (318).

Rs16861205 was associated with body weight at baseline and during the four-year follow-up in the DPS. The association between body weight and rs16861205 has not been reported previously, and this intronic SNP does not have any obvious functional role. These observations are therefore likely explained by LD with another variant of functional significance.

Rs16861210 and rs17366568 associated strongly with baseline serum adiponectin levels in a subgroup of the DPS participants. Although both SNPs are located in intron 1, they are not in LD with each other and probably represent independent genetic signals. Neither rs16861210 nor rs17366568 have any known functional role, but three SNPs located immediately on either side of rs17366568 were recently predicted to affect transcription factor binding sites (243). Previous studies have not examined the association between rs16861210 and adiponectin levels. However, consistent with the results of the present study, the A allele of rs17366568 associated

with lower adiponectin levels in white, but not black women (316). Moreover, a recent GWAS found that rs17366568 explained 3.8% of variation in adiponectin levels, while altogether 6.7% of the variation was explained by at least nine independent SNP groups in the *ADIPOQ* locus (243).

Rs2241766 is one of the most extensively studied *ADIPOQ* SNPs. It a synonymous SNP located in the exon 2, and is in strong LD with rs2082940, which is located in the 3'UTR region. Both rs2241766 and rs2082940 have low MAF and the only two individuals in the DPS who were homozygous for the rs2241766 minor allele were also homozygous for the rs2082940 minor allele and *vice versa*. The results were basically identical for these two SNPs: the minor alleles (G and T, respectively) associated with a higher risk of T2DM, but paradoxically with higher adiponectin levels, particularly in men. Although the association between the rs2241766 G allele and T2DM related traits (66, 291, 292, 300) and between the T allele and low adiponectin levels (298, 299, 387) have also been reported by others, these contradictory results should be interpreted with caution due to the low MAFs of these SNPs.

Rs1501299 is another commonly studied *ADIPOQ* SNP with an unknown functional role. Consistent with the observed association between rs1501299 and body weight in the DPS population, a number of earlier studies have reported association with obesity-related traits (298, 305). The results are, however, conflicting as the risk allele varies among populations, and some studies have failed to replicate these findings (307).

The strongest association for body weight in the DPS was found for rs3821799. The genotype differences in weight remained significant during the four-year follow-up regardless of the study group. Moreover, rs3821799 remained significant predictor of baseline body weight in a multi SNP model with all the SNPs associating with body weight. This SNP is intronic, its possible functional role is not known, and it has not been examined in previous association studies have.

The rs6773957 G allele associated with both high body weight and low serum adiponectin levels in the DPS participants. Association of this SNP with body weight has not been reported in earlier studies, but in a recent genome-wide linkage and association scan rs6773957, and another SNP in LD with it, were found to be strongly associated with adiponectin levels (237). Furthermore, two recent studies have reported an association between the rs6773957 G allele and low circulating adiponectin levels (203, 241). The functional role of this variant is currently unknown, but given the consistent results seen in various study populations and the location of this SNP in the 3'UTR, where it may have an effect on mRNA stability or translational efficiency, future studies are warranted.

In conclusion, the results of *Study II* supported the well established role of *ADIPOQ* SNPs in explaining the variation in circulating adiponectin levels, but also a more contradictory role in other traits related to T2DM and obesity.

6.2.3 Association studies on *ADIPOR1* gene

In *Study III*, consistent and significant association between *ADIPOR1* SNPs and various body size measures were found. Moreover, three *ADIPOR1* SNPs associated with fasting and post-challenge insulin levels with sex-specific differences, but none of the SNPs examined were associated with T2DM risk.

Several earlier association studies have focused on *ADIPOR1* variants reporting associations with various traits including insulin resistance (302, 312, 318, 320, 326, 332), high liver fat (332), and obesity or fat distribution (306, 306, 318, 330). Few studies have found association between *ADIPOR1* variations and T2DM (324, 333), but similar to the results of *Study III* most have reported negative results (321, 325, 331, 334, 335). On the other hand, whereas in this and a few other studies (318, 330) significant associations between *ADIPOR1* variants and several body size measurements were found, others have failed to detect such associations (316, 321, 325, 333). Overall, the results are inconsistent, and some studies have suggested that factors such as ethnicity (312), obesity status (326, 391) or genetic background (306) may influence the effect of

these variants and, thus explain the discrepancies. The results of *Study III* suggest that the influence of *ADIPOR1* variants on body size and insulin levels may depend on sex, but Collins *et al.* (325) were unable to be find evidence for such interactive effect in a large population-based study. Other possible explanations for contradictory results include differences in study design or in allele frequencies and LD patterns between populations.

More specifically, three SNPs (rs10920534, rs2275738 and rs1342387) were associated with various body size measurements at baseline and with WC and body weight during the three-year follow-up. None of these variants have any known functional role and so far these results have not been replicated in other populations. Rs6666089 is located in the *ADIPOR1* promoter region and is in complete LD with an intronic SNP rs10920534 in DPS. Rs6666089 has been included in several association studies, but did not associate with BMI or other body size measurements (316, 321, 330, 333). Likewise rs2275738 and rs2275737, which are in complete LD, and the intronic variant rs1342387 have been associated with body size measures in other populations (316, 325, 330, 333). These three SNPs are in moderate to high LD with each other (*Study II*, Table 1: r2=0.264-0.849 and D'=0.996-1.0) and may be merely markers of a true functional variant(s) in *ADIPOR1* or nearby loci.

In the DPS, rs10920534, rs12045862 and rs7539542 demonstrated sex-specific associations with baseline fasting and 2-h insulin levels. Rs10920534 was associated with the baseline two-hour glucose levels in men. Collins *et al.* (325) did not find association between rs6666089 (which is in complete LD with rs10920534) and insulin levels, but this SNP associated with insulin resistance with adiposity dependent manner in another study population (391). Intriguingly, different alleles of rs12045862 associated with high insulin levels in men and women. The functional role of this intronic SNP is not known and the results of the present study are not supported by other association studies (325). Therefore, the sex-specific effect of this variant remains inconclusive.

Rs7539542 is located in the 3'UTR and has been associated with body size measurements (330) and insulin resistance (312) in other populations, but was not associated with fasting or two-hour insulin levels in large population based study (325). In future, the association of *ADIPOR1* variants with insulin levels, and the observed differences between men and women, needs to be investigated in larger study populations.

Interestingly, a strong association between *ADIPOR1* variants and height was observed in the female participants of the DPS. Height is a complex trait with heritability estimates ranging from 0.68 to 0.84 (392). Currently, genetic variants in at least 180 loci are known to influence adult height (393). The mechanistic link between adiponectin pathway and adult height is unknown, but birth length and circulating adiponectin levels do correlate positively (217).

In summary, *ADIPOR1* variants were associated with body size, fat distribution and insulin levels in individuals with IGT. The observed associations differed between men and women. The mechanism of these sex-specific differences is unknown, but may be related to the well documented sexual dimorphism in circulating adiponectin levels (201, 216).

6.2.4 Association studies on *ADIPOR2* gene

In *Study IV*, a significant association between four *ADIPOR2* SNPs (rs10848554, rs11061937, rs1058322 and rs16928751) and the risk of CVD was found. When all four SNPs were included in the same model only rs11061937 and rs1058322 remained significant predictors of CVD risk, indicating independent effects of these two variants. This view is supported by the observation that rs11061937 was in low LD (r^2 ranging from 0.0090 to 0.071) with the other three SNPs, whereas rs1058322 was in moderate LD with rs10848554 and rs16928751, and rs10848554 and rs16928751 were in high LD with each other (*Study IV*: Table 3). In addition, two *ADIPOR2* SNPs (rs11061946 and rs11061973) associated with the risk of T2DM. Individuals (n=5) homozygous for the minor alleles of these two SNPs had a higher risk of converting from IGT to T2DM.

Several studies have investigated the association of *ADIPOR2* variants with traits relates to T2DM or CVD risk, but the results are somewhat inconsistent, and only a few studies have included the same SNPs that were genotyped in DPS. Earlier studies have reported associations between *ADIPOR2* SNPs and plasma TG concentrations (319, 327, 328, 330), and T2DM incidence or related traits (312, 322, 327, 331, 333). On the other hand, a number of studies have failed to replicate association with T2DM (320, 321, 324, 325, 334) and insulin resistance (318, 332).

More specifically, the rs16928751 variant has been previously associated with fasting TG levels in a small study population of individuals with metabolic syndrome (328), and T2DM in a case-control study (322). Rs1044471, located in the 3' UTR, was associated with T2DM in an Amish population (333), but not in the DPS or in a Korean case-control population (320).

Probably the most extensively studied *ADIPOR2* SNP is an intronic variant rs767870, which was associated with CAD, intima media thickness and endothelial function in a cross-sectional study population (317). Moreover it was associated with fasting plasma TG concentrations in Mexican-American subjects (330), and in a population based sample of 3050 Finnish subjects (319). Rs767870 has also been associated with measures of liver fat content and its surrogate markers (319), and T2DM in a case-control study of 1498 Caucasian subjects (331). Unfortunately, rs767870 was not genotyped in the present study. However, three of the SNPs that associated with CVD risk in the DPS are in moderate or high LD with rs767870 in the HapMap CEU population (394) potentially capturing the same genetic information (rs10848554: r^2=0.774 and D'=0.916, rs1058322: r^2=0.337 and D'=0.895, and rs16928751: r^2=0.838 and D'=1.0). Importantly, rs767870 is not in high LD with rs11061937 (r^2=0.073 and D'=1.0) or the two T2DM associated SNPs rs11061946 (r^2=0.014 and D'=1.0) and rs11061973 (r^2=0.036 and D'=1.0), which therefore likely represent independent genetic signals.

ADIPOR1 and *ADIPOR2* expression may be regulated in a tissue specific manner and may be influenced by genetic factors. At the moment, however, the regulatory mechanisms at mRNA and protein levels are poorly understood, and the results of different studies are contradictory. Halvatsiotis *et al.* (317) observed that the A allele of the rs767870 was associated with higher levels of *ADIPOR2* protein expression in peripheral monocytes. Interestingly, in the present study differences in *ADIPOR2* mRNA expression levels were seen according to the rs1058322 genotype in PBMCs derived from individuals with metabolic syndrome. Individuals carrying the T allele, who had an increased risk of CVD in the DPS population, demonstrated decreased mRNA expression. The PBMCs are important factors in inflammation, which is closely connected to both CVD and T2DM (395). Moreover, monocytes and macrophages are the target cells of the antiatherogenic and anti-inflammatory effects of adiponectin (283) and SNPs affecting *ADIPOR2* expression tissue-specifically may disturb these interactions directly. The mechanisms by which *ADIPOR2* variants might influence the regulation gene expression or the risk of CVD and especially T2DM are currently hypothetical. The potential role of rs1058322, located in intron 1, in the tissue-specific regulation of gene expression is currently unknown. It may instead be a marker in LD with yet uncharacterised functional variant(s).

In conclusion, the results of *Study IV* are in agreement with previous findings suggesting a role for *ADIPOR2* gene in susceptibility to CVD and T2DM, possibly through independent genetic effects. Different variants in the *ADIPOR2* locus may act independently or in concert to induce subtle, and possibly tissue specific, alterations in gene expression. The observation of allele-specific differences according to rs1058322 variant in mRNA expression levels further support this view.

6.3 FUTURE PERSPECTIVES

Over the past years, the understanding of the genetics of several complex diseases and traits has increased dramatically. The research has shifted from candidate gene studies to GWAS, and towards using ever larger study populations.

The rationale for investigating the genetic component of complex diseases and traits, such as T2DM, CVD and obesity, is obvious. The prevalence of obesity has increased rapidly over the past decades and it is associated with severe co-morbidities, such as T2DM, CVD, which are the leading causes of morbidity and mortality worldwide, and account for large part of the financial burden on the health care system (396).

Despite advances, a significant portion of the heritability of complex obesity-related traits remains to be identified. In the near future, the search for the heritable component will be extended to rare variants, CNVs, the epigenome modifications and interactions between genetic and non-genetic factors. The next-generation sequencing methods will soon enable sequencing of whole genomes, which in turn will facilitate identifying inter-individual differences genetic predisposition to complex diseases. It is likely that significant genetic heterogeneity underlies complex conditions and therefore classification to new disease subcategories may become necessary.

The prediction of disease risk based on genetic factors has proven limited, and perhaps not applicable to clinical practice, due to strong non-genetic influences and the fact that a large number of variants with moderate effect sizes are involved with most individuals carrying both risk and protective alleles (397). However, knowing one's genetic risk may be an important factor motivating towards healthier lifestyle choices. In fact some studies have suggested that a genetic risk score may improve prediction in younger individuals for whom medical and lifestyle interventions may be most beneficial (398).

The most important aspect of recognizing genetic variants associating with complex diseases is, however, the recognition of novel etiological pathways and biological processes involved in disease pathogenesis, which will lead to more accurate precision of the disease subtype and serve as targets for future preventive and therapeutic strategies (6, 56).

It is also worth noting that the information on environmental exposures relating to complex traits and diseases is often incomplete and non-systematic. Another less obvious advantage of identifying new genetic variants associating with complex traits is that when new molecular pathways to various diseases are recognised, they will also give clues of crucial environmental exposures relating to each disease (399).

6.4 SUMMARY AND CONCLUDING REMARKS

In *Study I* an association between the functional *ADRA2B* gene 12Glu9 variant and AIR and DI was found in a DPS subpopulation. The risk allele of this variant also associated with an increased risk of converting from IGT to T2DM in the whole DPS population, particularly in individuals with central obesity. These results appear biologically plausible, given that α2-ARs regulate many physiological functions in various tissues, including insulin secretion in beta cells, and this variant leads to impaired receptor function. However, this finding has not been replicated in later association studies. Therefore, it has to be concluded that the role of the *ADRA2B* 12Glu9 variant in beta cell function and the risk for T2DM remains unclear. In this regard, studies on rare genetic variants may yield additional information.

Studies II-IV concentrated on the role of genes in the adiponectin pathway in various metabolic outcomes. Convincing evidence indicates that decreased levels of circulation adiponectin are associated with several adverse metabolic conditions. Moreover, it has been clearly demonstrated that adiponectin has direct insulin-sensitising, anti-atherogenic and anti-inflammatory effects. The genetic component of circulation adiponectin levels is strong and at

this point it is clear that *ADIPOQ* gene variants contribute significantly to adiponectin levels, even though the mechanisms remain unsolved. In *Study II* five *ADIPOQ* variants associated with serum adiponectin levels. It is unclear whether any of these variants have functional effects, but the present study, as well as several earlier studies, suggests that more than one variant affecting circulating adiponectin levels exist in the *ADIPOQ* locus. The association of *ADIPOQ* variants with other metabolic traits remains unclear and may be population specific.

The role of *ADIPOR1* and *ADIPOR2* gene variants in body size and various metabolic traits and disease risk is even less clear as the results vary across study populations. In *Study III* associations between *ADIPOR1* SNPs and various body size measures were found. In addition, *ADIPOR1* SNPs associated with insulin levels in men. Some later studies have reported similar results, whereas in others the results have been negative. *ADIPOR1* variants with effects on gene expression or on receptor function remain to be identified.

In *Study IV*, an association between *ADIPOR2* variants and the risk of CVD was found, and the results were supported by tissue and allele-specific differences in the mRNA expression levels. These results are in accordance with a few other association studies investigating *ADIPOR2*. Nevertheless, the possible effect of rs1058322 on gene expression needs to be investigated in larger study populations and at the protein level.

Lastly, an important aim of this series of studies was to investigate the possible gene-lifestyle interactions. In *Study I*, the role of lifestyle x *ADRA2B* gene variant interaction was significant, but for the adiponectin pathway genes, the response to lifestyle was essentially similar regardless of genotype.

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Niina Siitonen

Candidate Gene Studies on Body Size, Type 2 Diabetes and Related Metabolic Traits

Genetics of ADRA2B, ADIPOQ, ADIPOR1 and ADIPOR2 in the DPS Study Population

The present study utilised a candidate gene approach to identify gene variants associated with type 2 diabetes, obesity and related traits in individuals with impaired glucose tolerance. The candidate genes included in the present study, *ADRA2B*, *ADIPOQ*, *ADIPOR1* and *ADIPOR2*, were selected based on biological plausibility and the previous literature. The results of this thesis suggest that genetic differences in susceptibility to obesity and its comorbidities may exist. Moreover, individuals with different genotypes may respond differently to beneficial lifestyle changes.

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