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TITTA SALOPURO

# Studies on Selected Human Obesity Candidate Genes

## Genetic Variation and Adipose Tissue Expression

Doctoral dissertation

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School of Public Health and Clinical Nutrition
Department of Clinical Nutrition and Food and Health Research Centre
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## ABSTRACT

Obesity and insulin resistance are central components of metabolic syndrome (MetS), which is a risk factor for type 2 diabetes (T2DM) and cardiovascular diseases. The genes encoding uncoupling proteins (*UCP*), β-adrenergic receptors (*BAR*) and leptin receptor (*LEPR*) are involved in energy expenditure and lipid and glucose metabolism and long-term weight regulation, and can thus be regarded as candidate genes of obesity. The present study was undertaken to investigate the association of gene variants in the *UCP1, UCP2, UCP3, B2AR, B3AR,* and *LEPR* genes with body weight and the incidence of T2DM in the Finnish Diabetes Prevention Study. In addition, new putative candidate genes were explored by microarray analysis of the transcriptome of adipose tissue in response to weight reduction in individuals presenting features of MetS.

The combination of the *Gln27Gln* genotype in the *B2AR* gene and the *Arg64* allele in the *B3AR* gene was associated with the risk of T2DM. The *G(-3826)G* genotype in the *UCP1* gene associated with higher weight during the follow-up. *Lys109Lys, Gln223Gln* genotypes and *3'UTR Ins* allele in the *LEPR* gene were associated with T2DM risk, while the *3'UTR Del/Del* genotype associated with higher weight during the follow-up. Four variants in *UCP3* associated with total and LDL-cholesterol concentrations, and four variants in the *UCP2-UCP3* gene region associated with measures of abdominal obesity. Furthermore, a risk haplotype for waist-to-hip ratio and for serum total and LDL-cholesterol concentration was found in *UCP2-UCP3* gene region. Long-term moderate weight reduction in subjects with features of MetS resulted in downregulation of gene expression in adipose tissue, where specifically the expression of genes involved in extracellular matrix and cell death was reduced. One of the genes exhibiting the most remarkable change was tenomodulin, which is an interesting novel candidate gene to be investigated in detail.

In conclusion, genetic variation in *B2AR, B3AR*, and *LEPR* may indicate a risk of T2DM, whereas genetic variation in *UCP1* and *LEPR* may modify the risk of obesity. Variation in *UCP2-UCP3* gene region is associated with lipid levels and abdominal obesity, which are features of MetS. Moderate weight reduction involves alterations in the extracellular matrix of adipose tissue, as well as in death of adipocytes.

National Library of Medicine Classification: QS 532.5.A3, QU 120, QU 477, QU 500, QU 75, QU 95, WK 810, WK 820
Medical Subject Headings: Adipose Tissue; Blood Glucose/metabolism; Body Weight/genetics; Cholesterol, LDL; Diabetes Mellitus, Type 2; Finland/epidemiology; Gene Expression Regulation; Genetic Variation; Genotype; Lipids/metabolism; Metabolic Syndrome X; Obesity; Receptors, Adrenergic, beta; Receptors, Leptin; Risk Factors; Subcutaneous Fat, Abdominal

*Dedicated to the memory of*
*our dear daughter Hilla*

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Lahti, July 2009

Titta Salopuro

**ABBREVIATIONS**

| | |
|---|---|
| aa | amino acid |
| AC | adenylate cyclase |
| ADP | adenosine diphosphate |
| AGRP | agouti-related protein |
| AIR | acute insulin response |
| apoA | apolipoprotein A |
| ARC | arcuate nucleus |
| AT | adipose tissue |
| ATP | adenosine triphosphate |
| ATPIII | Adult Treatment Panel III |
| AUC | area under curve |
| B2AR | β2-adrenergic receptor |
| B3AR | β3-adrenergic receptor |
| BAR | β-adrenergic receptor |
| BAT | brown adipose tissue |
| BMI | body mass index |
| BMR | basal metabolic rate |
| bp | basepair |
| BP | blood pressure |
| cAMP | cyclic adenosine monophosphate |
| CART | cocaine and amphetamine regulated transcript |
| CDKAL1 | CDK5 regulatory subunit associated protein 1-like 1 |
| CDKN2 | cyclin-dependent kinase inhibitor 2A |
| cDNA | complementary DNA |
| CEU | Utah residents with ancestry from Northern and Western Europe |
| CHD | coronary heart disease |
| CI | confidence interval |
| CNS | central nervous system |
| CVD | cardiovascular disease |
| cRNA | complementary RNA |
| CRP | C-reactive protein |
| dBP | diastolic blood pressure |
| Del/Ins | deletion/insertion polymorphism |
| DNA | deoxyribonucleic acid |
| DPP | Diabetes Prevention Program |
| DPS | Diabetes Prevention Study |
| ECM | extracellular matrix |
| EE | energy expenditure |
| EGIR | European group for the study of insulin resistance |
| FA | fatty acid |
| FDR | false discovery rate |
| FFA | free fatty acid |
| FPG | fasting plasma glucose |
| FSIGT | frequently sampled intravenous glucose tolerance test |
| FTO | fat mass and obesity associated gene |
| Gi | inhibitory GTP-binding protein |
| GLM | general linear model |
| glu | glucose |
| GO | gene ontology |
| GPCR | G protein-coupled receptor |
| Gs | stimulatory GTP-binding protein |
| GSIS | glucose stimulated insulin secretion |
| GTP | guanosine triphosphate |
| GWAS | genome-wide association study |
| HapMap | haplotype map of the human genome |
| HDL | high density lipoprotein |
| HHEX-IDE | haematopoietically expressed homeobox- insulin-degrading enzyme |
| HOMA-IR | homeostasis model assessment for insulin resistance |
| HOMA-IS | homeostasis model assessment for insulin secretion |
| HR | hazard ratio |
| HRR | hazard risk ratio |
| HSL | hormone sensitive lipase |
| IDF | International Diabetes Federation |
| IDPP | Indian Diabetes Prevention Programme |
| IFG | impaired fasting glucose |
| IGF2BP2 | insulin-like growth factor 2 mRNA-binding protein 2 |
| IGT | impaired glucose tolerance |
| IL-6 | interleukin-6 |
| IR | insulin resistance |
| IRS | insulin receptor substrate |
| kb | kilo base pair |
| KCNJ11 | potassium channel, subfamily J, member 11 |
| KO | knockout |
| LC | leader cistron |
| LD | linkage disequilibrium |

| | |
|---|---|
| LDL | low density lipoprotein |
| LEP | leptin |
| LEPR | leptin receptor |
| MBEI | model based expression index |
| MC4R | melanocortin 4 receptor |
| MetS | metabolic syndrome |
| MP | menopausal |
| mRNA | messenger RNA |
| NA | noradrenaline |
| NADH | nicotinamide adenine dinucleotide |
| NGT | normal glucose tolerance |
| NPY | neuropeptide Y |
| OGTT | oral glucose tolerance test |
| OR | odds ratio |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PKA | protein kinase A |
| POMC | proopiomelanocortin |
| PPAR | peroxisome proliferative activated receptor |
| QPCR | quantitative real-time PCR |
| REE | resting energy expenditure |
| RFLP | restriction fragment length polymorphism |
| RMR | resting metabolic rate |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RQ | respiratory quotient |
| RR | risk ratio |
| SAT | subcutaneous AT |
| sBP | systolic blood pressure |
| Sg | glucose effectiveness |
| Si | insulin sensitivity |
| SLC30A8 | solute carrier family 30 member 8 |
| SMR | sleeping metabolic rate |
| SNP | single nucleotide polymorphism |
| T2DM | type 2 diabetes mellitus |
| TCF7L2 | transcription factor 7 like 2 |
| TG | triglyceride |
| TNF-α | tumour necrosis factor-α |
| TRH | thyrotropin-releasing hormone |
| TSH | thyroid stimulating hormone |
| UCP | uncoupling protein |
| UTR | untranslated region |
| VAT | visceral AT |
| WAT | white AT |
| WC | waist circumference |
| WHO | World Health Organization |
| WHR | waist-to-hip ratio |
| WHtR | waist-to-height ratio |
| VLCD | very low calorie diet |
| VLDL | very low density lipoprotein |
| WR | weight reduction |

## LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, which will be referred to in the text by their Roman numerals (I-IV):

I Salopuro T, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Laakso M, Uusitupa M, for the Finnish Diabetes Prevention Study Group. Common variants in $\beta_2$- and $\beta_3$-adrenergic receptor genes and uncoupling protein 1 as predictors of the risk for type 2 diabetes and body weight changes. The Finnish Diabetes Prevention Study.
Clin Genet, 2004; 66: 365-367.

II Salopuro T, Pulkkinen L, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Laakso M, Uusitupa M, for the Finnish Diabetes Prevention Study Group. Genetic variation in leptin receptor gene is associated with type 2 diabetes and body weight: The Finnish Diabetes Prevention Study.
Int J Obes, 2005; 29: 1245-1251.

III Salopuro T, Pulkkinen L, Lindström J, Kolehmainen M, Tolppanen AM, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Tuomilehto J, Laakso M, Uusitupa M, for the Finnish Diabetes Prevention Study Group. Variations in *UCP2* and *UCP3* genes associate with WHR and serum lipid concentrations: The Finnish Diabetes Prevention Study.
Submitted to BMC Medical Genetics.

IV Kolehmainen M, Salopuro T, Schwab US, Kekäläinen J, Kallio P, Laaksonen DE, Pulkkinen L, Lindi VI, Sivenius K, Mager U, Siitonen N, Niskanen L, Gylling H, Rauramaa R, Uusitupa M. Weight reduction modulates expression of genes involved in extracellular matrix and cell death: the GENOBIN study.
Int J Obes, 2008; 32(2): 292-303.

In addition, some previously unpublished data are presented.

**CONTENTS**

# 1. INTRODUCTION

Obesity is a severe health problem in today's society, where energy intake too easily exceeds the needs of energy expenditure due to the ready availability of energy-dense food supplies and sedentary lifestyles. In particular, the central type of obesity and ectopic fat deposition in insulin-sensitive tissues (muscle, liver and pancreas), has been shown to associate with metabolic aberrations, such as insulin resistance. Obesity and insulin resistance are central components of metabolic syndrome, which is a disorder where several disadvantageous metabolic characteristics cluster, and predispose to type 2 diabetes and cardiovascular diseases. Most risk factors of type 2 diabetes, such as overweight, abdominal obesity, sedentary habits, and unhealthy diet, can be modified by lifestyle changes, whereas the contributions of age, race, genetic factors, and low birth weight as risk factors cannot be altered (1).

The metabolic abnormalities in obesity derive from multiple environmental and genetic factors, several of which are already known. The vast majority of obesity cases are polygenic, where a multitude of genes interact with each other and with environmental factors to induce the disorder. Each of these relatively common genetic variants has only a small or modest effect on the predisposition, though in extremely rare cases of monogenic obesity only a single gene variation is responsible for the disorder. Association studies, linkage studies and genome-wide association studies have been used as tools to find new and to study the existing candidate genes of obesity. Many candidate genes of obesity can be regarded also as candidate genes for diabetes due to the partly overlapping etiology.

This series of studies deals with two lifestyle intervention cohorts, in an attempt to study the association of known candidate genes with type 2 diabetes in Finnish Diabetes Prevention Study, and to search for new candidate genes in a weight reduction study Genobin, where the effect of weight reduction on gene expression profiles of adipose tissue was explored by microarray technology. Candidate genes used in the association studies include the uncoupling proteins 1-3, $\beta_2$- and $\beta_3$-adrenergic receptors and the leptin receptor, which are involved in energy expenditure and lipid and glucose metabolism (uncoupling proteins and adrenergic receptors), or long-term weight regulation (leptin receptor).

# 2. REVIEW OF LITERATURE

## *2.1. Obesity*

### 2.1.1. Definition and prevalence

Obesity can be defined as an excess of body fat. A surrogate marker for body fat content is the body mass index (BMI), which is determined by weight (kg) divided by height squared ($m^2$) (Table 1). In terms of percent of total body fat, which can be measured in several ways e.g. by skin-fold thicknesses, bioelectrical impedance or underwater weighing, obesity can be defined as ≥25% in men and ≥35% in women (2).

Total body adipose tissue (AT) is situated in two major compartments: subcutaneous AT (SAT) and internal AT (2, 3). Internal AT is further divided into visceral (VAT) and nonvisceral components, where VAT refers to the intrathoracic and intra-abdominopelvic compartments (4). The most practical way to estimate central obesity in clinical practice is to measure waist circumference (WC), because WC strongly correlates with the amount of abdominal VAT accumulation (5) and an excess of abdominal fat is most tightly associated with the metabolic risk factors, cardiovascular disease (CVD) and especially to type 2 diabetes (T2DM) (6, 7). Abdominal obesity is defined as WC ≥ 102/88 cm (men/women) in the United States, (2) and as WC ≥ 100/90 cm (men/women) in Finland (8). The guidelines from International Diabetes Federation (year 2007) suggest that Europid individuals with WC ≥ 94/80 cm (men/women) may be at a higher risk for impaired glucose tolerance (IGT) and T2DM, and they need to have their level of risk further investigated (9).

Table 1. The classification of overweight and obesity according to the body mass index (BMI).

| **BMI (kg/m2)** | **Classification** | **Risk of co-morbidities** |
|---|---|---|
| < 18.5 | Underweight | Low |
| 18.5-25 | Normal range | Average |
| > 25 | Overweight, obesity | |
| 25-30 | Pre-obese | Increased |
| 30-35 | Obesity class I | Moderate |
| 35-40 | Obesity class II | Severe |
| > 40 | Obesity class III, morbid obesity | Very severe |

Adopted from World Health Organisation: Technical Reports Series 2000 (10)

International data indicate that the obesity epidemic is a serious global health problem. In 2003-2004, 32% of adults in USA were obese and 66% were overweight or obese (11). During the last decades, the prevalence of obesity doubled in adults in the United States (11) and in Finland (12). In Finland, 20/19% (men/women) were obese and 68/52% (men/women) were overweight or obese in the year 1997 (8), whereas 22/21% (men/women) were obese in 2002 (13). The prevalence has increased especially in individuals with low socio-economic status (13). According to the definition of obesity and depending on the age of the child, up to 10–20% of school age children in Finland are overweight (12, 14). An overweight child carries a high risk to become an overweight adult and to be at risk of associated health problems (14).

### 2.1.2. Multifactorial etiology

Obesity has always existed in human populations, but until recently it has been comparatively rare (15). In only a few decades, the industrialised world has changed from a calorie-poor to a calorie-rich environment. Obviously, the recent unlimited availability of low-cost, calorie-dense food, along with increasing sedentary lifestyles, has played a major role in the adult obesity pandemic (15). Obesity results from the interaction of many factors, including genetic, metabolic, behavioural, and environmental influences (16) (Figure 1). The rapidity by which obesity is increasing suggests that behavioural and environmental influences, rather than genetic changes, have fuelled the epidemic.

Despite popular stereotypes portraying obesity as a problem of gluttonous behaviour and poor willpower, there is a considerable body of evidence that genetic factors play an important role in determining body weight (17). Family clustering of obesity is known to exist. The correlation of BMI is much higher between monozygotic than dizygotic twins (0.74 versus 0.32, respectively), whereas it is 0.24 for siblings and 0.19 for parents and offspring (18). The BMI of adopted children correlates with that of their biological parents (19). These and other data suggest an estimated heritability of 50% to 90% (17). Even though the susceptibility to obesity is determined to a significant extent by genetic factors, a favourable “obesigenic” environment is necessary for its phenotypic expression (20).

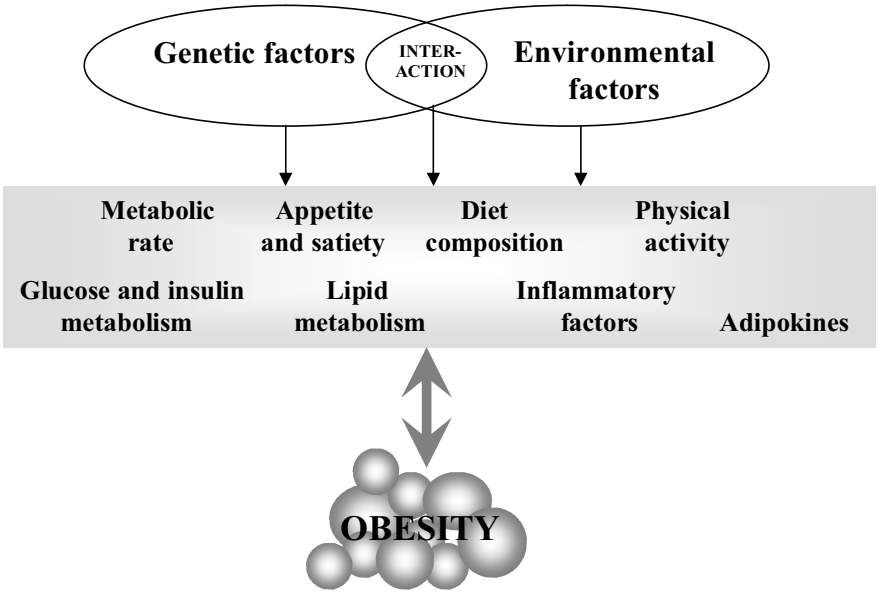



Figure 1. Factors influencing the development of obesity.

In the 1960s, Neel proposed the 'thrifty gene' hypothesis, whereby genes that predispose to obesity would have had a selective advantage in populations that frequently experienced starvation (21). People who possess these genes in today's obesigenic environment might be those that 'overreact' - not just becoming slightly overweight, but extremely obese, such as the high-risk groups of Pima Indians and Pacific Islanders. This has led to a second hypothesis, which states that only those with fewer risk alleles of obesity susceptibility genes (the former non-survivors) are now able to resist our obesigenic environment and remain at a normal weight without conscious effort (20).

The present work deals with the genetic basis of common obesity and gene-lifestyle interactions, and therefore, other aspects such as socio-economic, cultural and behavioural factors possibly leading to obesity will not be reviewed here.

### 2.1.3. Adipose tissue

AT is a heterogeneous tissue containing different cell types, including mature adipocytes, preadipocytes, endothelial cells, vascular smooth muscle cells, leukocytes, monocytes and macrophages (22). The functions of adipocytes can be grouped generally

into three partially overlapping categories: i) lipid metabolism, ii) glucose metabolism and iii) endocrine functions; such as modulating the immune response, appetite and energy balance, blood pressure (BP) control, haemostasis, angiogenesis, bone mass, and thyroid and reproductive function. (23-25). Adipocyte differentiation (adipogenesis) derives from multipotent mesenchymal cells, which become committed to the adipoblast lineage (26). Expansion of fat stores requires new blood vessels (27), which appears to be regulated in part by factors produced within AT, such as leptin and matrix metalloproteinases (28).

**Adipose tissue as an energy storage**

AT is the largest energy reservoir in the body. The major function of adipocytes is to store triglycerides (TG) in lipid droplets in periods of energy excess (lipogenesis) and to mobilise this energy reserve during times of deprivation (lipolysis). When energy intake chronically exceeds energy expenditure (EE), the resulting imbalance causes an expansion of adipocytes (20), whereas situations of negative energy balance involve loss of adipocyte TG stores and apoptosis (28).

The short-term control of lipogenic and lipolytic processes is modulated by hormonal signals, whereas the long-term changes are accomplished by altering the size of adipocytes (29). The number of adipocytes is set during childhood and adolescence and stays relatively constant throughout adulthood, although there is constant and continual turnover (30). In obese individuals, turnover is higher compared to their leaner counterparts; approximately 10% of adipocytes are renewed annually at all adult ages and levels of BMI (30). The number of adipocytes has been shown to be a major determinant for the fat mass in adults (30).

Lipolytic regulation is complex due to the heterogeneity of fat depots, gender differences, and the diversity of hormones (catecholamines, insulin, thyroid, growth, and sex hormones) and other factors (dieting, exercise, age) governing this process (31). The lipolytic pathway acts through a stimulatory guanosine triphosphate (GTP) -binding protein (Gs) and the antilipolytic pathway through inhibitory GTP-binding protein (Gi) (32, 33). Activation of β-adrenergic receptors (BAR) 1-3 leads to enhanced release of fatty acids (FA), whereas $\alpha_2$-AR activation leads to a decrease in lipolytic activity (34). In human white fat cells, $\beta_2$-AR (B2AR) is the dominating lipolytic receptor (35).

**Adipose tissue as secretory organ**

An active role for the adipocyte in energy metabolism was demonstrated with the discovery of leptin (36), a secretory product that originates almost exclusively from the adipocyte and serves a hormonal function in mediating satiety by activating receptors situated in the hypothalamus. It is now recognised that AT produces multiple bioactive peptides, termed 'adipokines', which act locally and distally via autocrine, paracrine and endocrine mechanisms (37) (Table 2). The adipokine signals are integrated in a network with other tissues and organs such as the liver, skeletal muscle, adrenal cortex, brain, and the sympathetic nervous system (25). Many of these adipokines are classical cytokines such as interleukin-6 (IL-6) or tumour necrosis factor- α (TNF-α), and others including leptin, are structurally related to cytokines (27). Excess adiposity is associated with increased serum IL-6, TNF-α and C-reactive protein (CRP) concentrations and obesity is also characterized as low-grade inflammation state (38).

Table 2. The most common adipokines and their main effects.

| **Adipokine** | **Function/Effect** |
|---|---|
| Leptin | Food intake, reproduction, angiogenesis, immunity |
| Adiponectin | Insulin sensitizer, anti-inflammatory |
| Resistin | Inflammation, IR |
| Angiotensinogen | Electrolyte homeostasis, BP |
| Tumour necrosis factor-α | Inflammation, atherosclerosis, IR |
| Interleukin-6 | Inflammation, atherosclerosis, IR |
| Adipsin | Stimulates TG storage, inhibits lipolysis |
| Acylation stimulating protein | Influences the rate of TG synthesis |
| Fasting-induced adipose factor | Lipid metabolism, angiogenesis |
| Plasminogen activator inhibitor 1 | Vascular homeostasis, blood clotting |
| Tissue factor | Major cellular initiator of the coagulation cascade |
| Monocyte chemoattractant protein1 | Inflammation, cell adhesion |
| Transforming growth factor-β | Cell adhesion and migration, growth and differentiation |
| Visfatin | IR, proinflammatory |
| Vaspin | IR |
| Retinol binding protein 4 | Lipid metabolism |
| C-reactive protein | Inflammation, atherosclerosis, IR |
| Apelin | IR |
| Hepcidin | Inflammation, iron homeostasis |

| | |
|---|---|
| Adipophilin | Lipid accumulation, atherosclerosis |
| Lipocalin 2 (39) | IR |

IR, insulin resistance; BP, blood pressure; TG, triglyceride. Modified from Ahima et al. 2006 (40), Ronti et al. 2006 (25), Frühbeck et al. 2001 (29), and Lago et al. 2007 (41).

**Adipose tissue depots**

AT differs in its adipogenic potential and lipolytic response depending on its regional distribution (26). There is strong evidence that an excess of VAT is more strongly related to metabolic risk factors than its counterparts in other fat compartments (42, 43). SAT nonetheless is a much larger (~80% of total body fat) compartment than VAT (10% of total body fat) (44). The former is usually divided into gluteofemoral and truncal SAT, of which the truncal fat is more strongly related to metabolic risk factors than gluteofemoral fat, and it may have a greater impact on risk factors than VAT because of its greater mass (2).

In addition to depot-specific differences, a further distinction must be made between brown and white adipocytes. Brown adipocytes are found only in mammals, and differ from white adipocytes in that they express uncoupling protein 1 (*UCP1*) gene. Morphologically, brown adipocytes are multilocular, contain less overall lipid and are particularly rich in mitochondria (24). Recent results indicate that brown adipose tissue (BAT) is present and active in human adults (45-48).

### 2.1.4. Comorbidities

Although affected individuals often focus on the social stigma of obesity, this condition is much more than a cosmetic problem; it is strongly associated with a reduction in life expectancy of ~8 y, as well as with an increased risk of several serious diseases (Table 3), including T2DM, coronary heart disease (CHD) and certain cancers such as breast and colon cancers (49). Numerous large-scale studies in humans have shown that overall mortality rises steadily as a function of increasing body weight (17). Even small increases in weight across a population can have a devastating impact on public health. Close to 300 000 deaths each year in the United States are believed to be attributable to obesity, making obesity the second leading cause of preventable death in that country (50). It has been reasonably argued that obesity is beginning to overtake infectious diseases and undernutrition as the most significant contributor to illness in global terms (51).

Table 3. Relative risk for health problems associated with obesity (BMI>30 vs. BMI<25).

| **Greatly increased** | **Moderately increased** | **Slightly increased** |
|---|---|---|
| **Relative risk >> 3** | **2 < relative risk < 3** | **1 < relative risk < 2** |
| T2DM | CHD | Cancer (breast, endometrial, colon) |
| Breathlessness | Osteoarthritis (knees) | Fetal defects associated with maternal obesity |
| Dyslipidaemia | Hypertension | Impaired fertility |
| Gallbladder disease | Hyperuricaemia and gout | Increased anaesthetic risk |
| Insulin resistance | | Low back pain due to obesity |
| Sleep apnea | | Polycystic ovary syndrome |
| | | Reproductive hormone abnormalities |

T2DM, Type 2 diabetes mellitus; CHD, coronary heart disease.
Adopted from World Health Organisation: Technical Reports Series 2000 (10).

Each disease whose risk is increased by being overweight can be classified into one of two pathophysiological categories (52). The first category of disabilities arises from the increased AT mass itself. These include the embarrassment of obesity and the behavioural responses it produces, osteoarthritis, and sleep apnea. The second category is risks that result from the metabolic changes associated with excess AT. These include T2DM, gallbladder disease, hypertension, CVD, and some forms of cancer.

Some individuals are more predisposed than others to obesity-associated diseases, but presently it is difficult to identify the 'at risk' individuals who would benefit the most from individualised monitoring and care (15, 53). Physiologically, metabolically deleterious forms of obesity associate with a preferential accumulation of centrally located fat (43) and with ectopic fat deposition in insulin-sensitive tissues, such as muscle, liver and pancreas. This aberrant fat content strongly correlates with severe generalised insulin resistance (IR) and the development of a chronic inflammatory state, partly due to the infiltration of the AT by macrophages (15, 53). It should also be noted that different subtypes of obesity exist. For example, one can identify two types, 1) metabolically healthy, but obese, and 2) metabolically obese, but normal weight individuals (54). Even though they have normal BMI, in some individuals (prevalence around 15%) the fat mass and plasma TG as well as VAT and liver fat content can be high, predisposing to T2DM, metabolic syndrome (MetS), and CVD (54).

### 2.1.5. Metabolic syndrome

The MetS (syndrome X, the insulin resistance syndrome) is a common metabolic disorder that results from increasing prevalence of obesity (53). The constellation of

metabolic abnormalities includes glucose intolerance, IR, central obesity, dyslipidaemia, and hypertension (Table 4) (53). These conditions co-occur in an individual more often than might be expected by chance. MetS is associated with increased risk of CVD, T2DM, and also susceptibility to polycystic ovary syndrome, fatty liver, cholesterol gallstones, asthma, sleep disturbances, and some forms of cancer (55).

Table 4. Comparison of definitions of the MetS.

| **WHO, 1998** (56) | **EGIR, 1999** (57) | **ATPIII, 2001** (58) | **IDF, 2005** (59) |
|---|---|---|---|
| T2DM or IFG or IGT or IR<br>Plus ≥2 of following | IR-hyperinsulinaemia<br>Plus ≥2 of following | ≥3 of following | Central obesity[a]<br>Plus any 2 |
| BMI >30 or<br>WHR >0.9/0.85 (M/F) | WC ≥94/80 cm (M/F) | WC >102/88 cm (M/F) | |
| TG ≥1.7 mmol/l or HDL-C <0.9/1.0 mmol/l (M/F) | TG >2.0 mmol/l or HDL-C <1.0 mmol/l | TG ≥1.7 mmol/l | TG >1.7 mmol/l |
| | | HDL-C <1.0/1.3 mmol/l (M/F) | HDL-C <1.0/1.3 mmol/l (M/F) |
| BP >140/90 mm Hg | BP ≥140/90 mm Hg and/or medication | BP ≥135/85 mm Hg or medication | BP ≥130/85 mm Hg or medication |
| Albumin excretion >20μg/min | FPG ≥6.1 mmol/l | FPG ≥6.1 mmol/l | FPG ≥5.6 mmol/l or T2DM diagnosis |

[a] Ethnicity specific waist circumference as the measure of central obesity

WHO, World Health Organization; EGIR, European group for the study of insulin resistance; ATPIII, Adult Treatment Panel III; IDF, International Diabetes Federation; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IR, insulin resistance; BMI, body mass index; WHR, waist-to-hip ratio; WC, waist circumference; M, male; F, female; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; BP, blood pressure; FPG, fasting plasma glucose.

There are several definitions for MetS, which agree in their essential components but differ in the detail and criteria (Table 4) (53, 55, 57-60). Furthermore, there has been a debate about the very existence of MetS as a single entity, since metabolic and cardiovascular abnormalities which are extremely common in elderly people do not appear to cluster together under a single common factor (61). Regardless of the diagnostic criteria used, there is full agreement that beneficial lifestyle changes, with the emphasis on weight reduction, constitute the first-line therapy for MetS (62). In patients in whom lifestyle changes fail to reverse metabolic risk factors, consideration should be given for treating the specific abnormalities in these risk factors with drugs in combination with lifestyle modification (55).

The role of AT is crucial in the pathophysiology of MetS (63). VAT is more active in terms of accepting and releasing free fatty acids (FFA) in a metabolically restricted circulation. The liver processes the blood from this area and thus the rest of the body is

partly protected from an excessive exposure to FFA. As long as massive energy storage takes place in SAT, the buffering capacity of VAT is preserved, but when the storage capacity of the SAT becomes exhausted, the visceral area is forced to take over and its buffering capacity is then blunted (63). When AT capacity is overwhelmed by buffering demands, an increase in TG rich particles occurs, and new surrogate storage depots are used for the excess of fat, e.g. the liver (64) and to a lesser extent skeletal muscle. Fatty liver disease further leads to steatohepatitis as the generic response of an overloaded system. The functional capacity of the AT varies among subjects explaining the incomplete overlapping between the MetS and obesity (63).

## *2.2. Type 2 diabetes (T2DM)*

### 2.2.1. Definition and prevalence

T2DM is a complex disease characterised by elevated levels of serum glucose, caused mainly by impairment in both insulin secretion and insulin functional pathways. T2DM accounts for ~90-95% of all those with diabetes (65). The epidemic of T2DM observed in recent years is a clear indication of the importance of environmental factors in diabetes onset; in particular, obesity and physical inactivity are well-established risk factors. Currently, T2DM can be divided into several subcategories, with grouping by mode of inheritance and by major pathway affected. This includes a collection of monogenic disorders that can be subdivided into maturity-onset diabetes of the young, syndromes of IR, and mitochondrial diabetes (66).

American Diabetes Association has defined criteria for the diagnosis of diabetes (65) as follows:

fasting plasma glucose (FPG) < 5.6 mmol/l = normal fasting glucose
FPG 5.6-6.9 mmol/l = impaired fasting glucose (IFG)
FPG ≥ 7.0 mmol/l = provisional diagnosis of diabetes
(must be confirmed by second test).

With oral glucose tolerance test (OGTT) the following criteria are used:

2-h postload glucose < 7.8 mmol/l = normal glucose tolerance (NGT)
2-h postload glucose 7.8-11.1 mmol/l = IGT
2-h postload glucose ≥ 11.1 mmol/l = provisional diagnosis of diabetes
(must be confirmed by second test).

Patients with IFG and/or IGT are now referred to as having ‘pre-diabetes’ indicating the relatively high risk for the development of diabetes (65, 67).

The prevalence of diabetes in all age-groups worldwide was estimated to be 2.8% in 2000 and is projected to be 4.4% in 2030 (most of which will be T2DM) (68). The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030, with India, China and USA being the top three countries estimated to have the highest numbers of people with diabetes (68). Extreme examples of high prevalence are encountered in the Nauru Islanders of the tropical Pacific (41%), Pima Indians in Arizona (50%) and urban Wanigela people in Papua New Guinea (37%) (69). It has been evaluated that diabetes is likely to be the fifth leading cause of death worldwide, similar in magnitude (5.2% of all deaths) to the numbers reported for victims of HIV/AIDS (70).

### 2.2.2. Pathophysiology of T2DM

The pathogenesis of T2DM is complex and in most cases clearly requires defects in both pancreatic β-cell function and tissue insulin sensitivity (71). A degree of hyperglycaemia sufficient to cause pathological and functional changes in various target tissues, without clear clinical symptoms, may be present for a long period of time before diabetes is actually diagnosed (65, 67). During this asymptomatic period, it is possible to demonstrate an abnormality in glucose metabolism by measurement of plasma glucose in the fasting state or after a challenge with an oral glucose load (72). The chronic hyperglycaemia in diabetes leads to long-term damage, dysfunction, and failure of several organs, especially the eyes, kidneys, nerves, heart, and blood vessels (65). The major cause (75-80%) of death among individuals with diabetes is CVD (9).

T2DM is said to be a "2-hit" disease in which IR is accompanied by β-cell defects preventing the compensatory upregulation of insulin secretion (73). The genetic and molecular bases for the reduction in insulin sensitivity and β-cell function are not fully understood, but it seems that body fat distribution and especially visceral fat are major determinants of IR while reductions in β-cell mass contribute to β-cell dysfunction (74). It has become apparent that insulin sensitivity is influenced by a number of different factors such as genetic factors (75), age (76), acute exercise (77), physical fitness (78), diet (79), medications (80), obesity (81), and body fat distribution (82-85). Once hyperglycaemia exists, β-cell dysfunction is clearly present in subjects with T2DM (71). One way to quantify this change is to measure the decrease in the insulin response to intravenous glucose (74).

Normally, β-cell size stays relatively constant and, as such, β-cell mass is maintained at an optimal level through most of adulthood (86). However, β-cells adapt to obesity and IR by increasing their mass. The onset of T2DM is accompanied by a progressive

decrease in β-cell mass (87), which is due to the marked increase in β-cell apoptosis (88-90). As a result, the body can no longer adapt to any increases in metabolic load. Several mechanisms can trigger the increase in β-cell apoptosis, such as the development of endoplasmic reticulum stress (91), chronic hyperglycaemia, chronic hyperlipidaemia, oxidative stress (92), and cytokines (86). Pancreatic β -cell dysfunction is genetically determined and appears early in the course of the disease (93).

### 2.2.3. Pathways from obesity to T2DM

Obesity increases the risk of diabetes to a much greater extent than it increases the risk of other diseases, independently of age, race and physical activity (94). The relationship between obesity and diabetes displays such an interdependence that the term 'diabesity' has been coined (95). It is estimated that about 60-90% of all patients with T2DM are or have been obese, though only 10% of obese individuals are diabetic (96).

In the obesity-diabetes relationship, two factors are recognised to be the major components: insulin deficiency and IR. Chronic hyperglycaemia, by means of the glucotoxicity phenomenon, impairs insulin sensitivity, while sustained hyperinsulinaemia inhibits both insulin secretion and insulin action. (96). Based on Randle's hypothesis from 1960s (97), the dominant factor in obesity is the permanent elevation of plasma FFA and consequently the predominant utilisation of lipids by the muscle leading to a diminution of glucose uptake and, therefore, IR (96, 97) (Figure 2). The partial reversibility of the evolution of obesity towards diabetes is well demonstrated by lifestyle changes and multidisciplinary weight loss programmes (98-100).

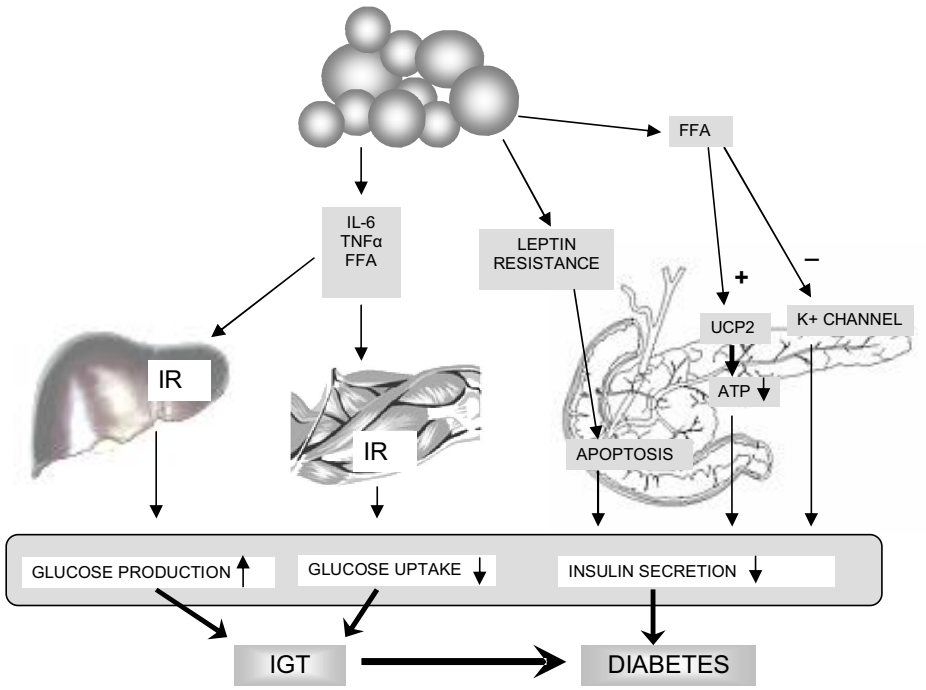



Figure 2. Potential mechanisms of obesity-induced insulin resistance (IR). Elevated rate of fat breakdown and excessive food intake lead to increased release of circulating free fatty acids (FFAs). These have a detrimental action on the uptake of glucose by skeletal muscle, production of glucose by the liver, and systemic dyslipidaemia. Initially, the β-cells of the pancreas compensate for these processes by producing more insulin. However, with time, there is a failure of the β-cells to continue to secrete huge amounts of insulin leading to the development of hyperglycaemia, impaired glucose tolerance (IGT), and hence T2DM. K+ channel is an essential part of insulin signalling, and it can be disturbed by FFA. FFAs also enhance expression of uncoupling protein 2 (UCP2), which diminishes ATP production necessary for insulin secretion. FFAs can also induce β-cell apoptosis via endoplasmic stress response. Leptin has been shown to have anti-apoptotic effects in β-cells, which may also be diminished in the leptin-resistant state. Modified from Hajer et al. 2008 (101).

As well as FFA, AT in obese individuals also releases increased amounts of glycerol, hormones, proinflammatory cytokines and other factors that are involved in the development of insulin secretion defects and IR (102). In addition to adipocyte-derived factors, increased release of TNF-α, IL-6, monocyte chemoattractant protein and additional products of macrophages and other cells that populate AT might also have a role in the development of insulin secretion defects and IR (102).

The distribution of body fat in itself is a critical determinant of insulin sensitivity. Whereas simple obesity is typically associated with IR, insulin sensitivity also varies

markedly in lean individuals because of differences in body fat distribution. Lean individuals with a more peripheral (subcutaneous) distribution of fat are more insulin sensitive than lean subjects with centrally (abdominal and chest areas) distributed fat (102).

### 2.2.4. Lifestyle changes in the prevention of T2DM

Modest weight loss has been shown to have a beneficial effect on cardiovascular risk factors such as hypertension, hyperlipidaemia, and T2DM, and to reduce clustering of these risk factors (103). Recent lifestyle intervention studies in individuals with IGT suggest that even modest beneficial changes in lifestyle can prevent or delay the appearance of T2DM (Table 5).

Table 5. Lifestyle intervention studies in the prevention of diabetes.

| Study | Intervention | n | Relative risk reduction (%) | Duration (years) |
|---|---|---|---|---|
| Malmö 1991 (104) | Lifestyle | 415 | 63 | 6 |
| Da Qing 1997 (100) | Lifestyle [a] | 577 | 31 (1), 46 (2), 42 (3) [a] | 6 |
| DPS 2001 (98) | Lifestyle [b] | 522 | 58 | 3 |
| DPP 2002 (99) | Lifestyle [c] | 1079 | 58 | 3 |
| | Metformin | 1073 | 31 | 3 |
| Japanese 2005 (105) | Lifestyle [d] | 458 | 67 | 4 |
| IDPP 2006 (106) | Lifestyle [e] | 133 | 29 | 3 |
| | Metformin | 133 | 26 | 3 |
| | Lifestyle + Metformin | 129 | 28 | 3 |

[a] Treatment groups: 1)diet only, 2)exercise only, and 3)diet plus exercise
[b] See the text
[c] Goals: ≥7% weight loss, ≥150 min of physical activity per week
[d] Goal: BMI<24 kg/m$^2$ for the standard intervention group, <22 kg/m$^2$ for the intensive intervention group
[e] Healthy diet (reduction in total energy, fat and sugar intake, and increase in fibre intake), and ≥30 min physical activity per day
DPS, Finnish Diabetes Prevention Study; DPP, Diabetes Prevention Program; IDPP, Indian Diabetes Prevention Programme. Modified from Laakso and Uusitupa 2007 (1), and Alberti et al. 2007 (9).

**Malmö Study**

The Malmö study included 181 Swedish men with IGT and 41 with T2DM (104), who aimed to change their dietary habits and level of physical activity. Over six years, their body weight decreased by 2.0-3.3 kg, whereas body weight increased by 0.2-2.0 kg in control subjects (n=193). The incidence of T2DM was 11% in the IGT group and 29% in the control subjects, showing a reduction of 63% during the mean follow-up of

six years. The findings of a 12-year follow-up (107) suggest that a long-term intervention programme, with an emphasis on lifestyle changes, including dietary counselling and physical exercise, will reduce mortality in subjects with IGT.

**Da Qing IGT and Diabetes Study**

In the Chinese Da Qing IGT and Diabetes Study, 577 individuals with IGT were randomised either to one of three active treatment groups or to control group (100). During six years the body weight decreased by 1.9 kg in the combined intervention group, whereas body weight decreased by 0.9 kg in control subjects. During the follow-up, the cumulative incidence of diabetes was 44% in the diet group, 41% in the exercise group, and 46% in the diet-plus-exercise group, compared with 68% in the control group, indicating 43% lower incidence in the combined intervention group, compared with the control group. The benefits of the intervention were still apparent 14 years after the 6 year active period (108).

**Finnish Diabetes Prevention Study**

In the Finnish Diabetes Prevention Study (DPS), 522 middle-aged, overweight subjects with IGT were randomly assigned to either the intervention group or the control group (109). The subjects in the intervention group were given detailed advice about how to achieve the goals of the intervention, whereas the subjects in the control group were given general oral and written information about diet and exercise, but no specific individualised programmes were offered. An oral glucose-tolerance test was performed annually, and the diagnosis of diabetes was confirmed by a second test.

Weight loss during the first year was 4.2 kg in the intervention group and 0.8 kg in the control group, while the loss by the end of year 2 was 3.5 kg and 0.8 kg for the intervention and control groups, respectively. Weight loss was also associated with a significant reduction in the WC and with an improvement in plasma TGs as well as decreases in systolic and diastolic BP (sBP, dBP), emphasising the multitude of effects of a modest weight loss. In a subgroup of DPS (n=52), changes in insulin sensitivity and insulin secretion were studied by means of a frequently sampled intravenous glucose tolerance test (FSIGT) (110). A strong correlation between the 4-year changes in insulin sensitivity ($S_i$) and weight was observed for both intervention and control groups. In the entire study group, $S_i$ improved by 64% in the highest tertile of weight loss but deteriorated by 24% in those who gained weight (lowest tertile) (110).

During the mean follow-up of 3.2 years, the cumulative incidence of diabetes was 11% in the intervention group and 23% in the control group (98). Thus, the risk of

diabetes was reduced by 58% in the intervention group. Recently, an extended follow-up of 7 years on DPS was reported (111) showing sustained lifestyle changes and a reduction in diabetes incidence even after the individual lifestyle counselling had been terminated three years previously. During the total follow-up of 7 years, the cumulative incidence of T2DM was 23% in the intervention group and 38% in the control group, indicating 43% reduction in relative risk.

**US Diabetes Prevention Program**

In the US Diabetes Prevention Program (DPP) (99) 3234 overweight adults of diverse ethnic backgrounds were randomly assigned to metformin (a biguanide-type antihyperglycaemic agent), lifestyle intervention or placebo group. The average weight loss was 2.1, 5.6, and 0.1 kg in the metformin, lifestyle intervention, and placebo groups, respectively. During the average follow-up of 2.8 years, the cumulative incidence of diabetes was 22%, 14%, and 29% in the metformin, lifestyle intervention, and placebo groups, respectively, showing 58% and 31% reduction in the incidence of T2DM for the lifestyle intervention and metformin groups, respectively, when compared to the placebo group.

**Japanese Study**

The Japanese trial consisted of an intensive intervention group (n=102) and a standard intervention group (n=356), including men with IGT (105). Body weight decreased by 2.2 kg in the intensive intervention group and by 0.4 kg in the standard intervention group. The cumulative 4-year incidence of diabetes was 3% in the intensive intervention group and 9% in the standard intervention group and, indicating 67% reduction in the risk of T2DM.

**Indian Diabetes Prevention Programme**

In the Indian Diabetes Prevention Programme (IDPP) (106) 531 subjects with IGT were randomised into four groups: lifestyle modification (LSM), metformin (MET), LMS+MET, and control group. No significant changes in body weight were seen during the intervention. The 3-year cumulative incidences of T2DM were 39%, 41%, 40%, and 55% in the LSM, MET, LMS+MET, and control groups, respectively. The relative risk reduction was 29% with LSM, 26% with MET, and 28% with LSM+MET, as compared with the control group.

## 2.3. Genetics of obesity

### 2.3.1. Methods of genetic studies

Two main approaches have been used to identify the genes underlying polygenic diseases such as obesity: candidate-gene and whole-genome approaches (112, 113). Candidate genes include the functional and positional candidates. Functional candidates are genes that are selected since they have some plausible role in obesity and/or IR based on their known or presumed biological role in energy homeostasis, whereas positional candidate genes are identified because they lie within genomic regions that have been shown to be genetically important in linkage or association studies. Polymorphic markers, single nucleotide polymorphisms (SNPs), or deletion/insertion polymorphisms (Del/Ins) within the gene of interest are genotyped among the studied population, and possible associations with traits of interest, or differences in genotype frequencies between affected vs. non-affected individuals are then studied.

Whole-genome approaches cover genome-wide linkage studies and genome-wide association studies (GWAS). Linkage studies involve typing of families using polymorphic markers that are positioned across the whole genome at a density of ~10-20 cM (~10-20 Mb), followed by calculating the degree of linkage of the marker to a disease trait (113, 114). Finally, positional candidate genes can then be identified by fine-mapping the regions around the peaks of linkage. This approach does not rely on any pre-existing knowledge of the genes that underlie the trait being studied, and it has been successful in identifying single-gene forms of obesity (115).

The novel technique GWAS (114) has been made possible by the International HapMap (haplotype map of the human genome) resource (116), the availability of dense genotyping chips (Affymetrix and Illumina), and large and well-characterized clinical samples (117). Large-scale SNP-discovery projects are currently providing the necessary SNP information to online resources such as the dbSNP database. In GWAS, approximately 300 000-500 000 SNPs are typically analysed in ~1000-2000 cases and ~1000-5000 control subjects (118).

### 2.3.2. Common obesity is a polygenic disease

The 1990s brought the positional identification of a series of murine obesity genes including leptin and its receptor, carboxypeptidase E and agouti, and also the critical regulatory role of melanocortin 4 receptor (MC4R) was established (119). These discoveries were followed by the finding of mutations in homologous genes causing morbid human obesity presenting in childhood. Human Obesity Gene Map 2005 (120)

(http://obesitygene.pbrc.edu) lists 11 genes as single-gene mutations with an obesity phenotype and of these MC4R deficiency represents the most common monogenic obesity disorder that has been identified so far. It is present in 1-6% of obese individuals of different ethnic groups, with a higher prevalence in cases with increased severity and earlier age of onset (113).

In summary, the known monogenic forms of obesity are very rare in the general population, accounting for <1% of cases (115). The role of genetic factors in most human obesities ('common obesity') is complex, being determined by the interaction of several genes (polygenic), each of which may have relatively small effects, i.e., they are 'susceptibility' genes and function in interaction with each other as well as with environmental factors such as nutrients, physical activity and smoking (112).

The Human Obesity Gene Map 2005 (120) incorporates published results up to the end of October 2005. According to this update, 176 human obesity cases due to single-gene mutations in 11 different genes have been reported and 50 loci related to Mendelian syndromes relevant to human obesity have been mapped to a genomic region. The number of human obesity quantitative trait loci derived from genome scans continues to grow, and is now 253 for obesity-related phenotypes from 61 genome-wide scans (120). However, a recent meta-analysis of linkage studies failed to find strong positive loci for BMI (115). Association studies have found 426 positive associations with 127 candidate genes, of which 22 genes are each supported by at least five positive studies, and 12 genes supported by at least ten studies (Table 6). The obesity gene map shows putative loci on all chromosomes except Y (120).

Table 6. Genes showing associations with obesity-related phenotypes in ≥10 replicative association studies.

| Gene | Gene name | Location | Phenotypes studied |
|---|---|---|---|
| *ADIPOQ* | Adiponectin | 3q27 | BMI, obesity, weight, WHR, VAT, WC |
| *B2AR* | β2-adrenergic receptor | 5q31-q32 | BMI, obesity, weight, fat mass, fat %, WHR, leptin, VAT and SAT, adipocyte size, lipolysis |
| *B3AR* | β3-adrenergic receptor | 8p12-p11.2 | BMI, obesity, weight, fat mass, fat %, WHR, VAT and SAT |
| *GNB3* | G-protein, polypeptide 3 | 12p13.31 | BMI, obesity, weight, fat mass, fat %, lipolysis, waist and hip circumference |
| *LEP* | Leptin | 7q31.3 | BMI, obesity, weight, leptin |
| *LEPR* | Leptin receptor | 1p31 | BMI, obesity, weight, fat mass, fat %, leptin, abdominal fat, lean mass, adipocyte size, 24h EE, WC |

| | | | |
|---|---|---|---|
| *NR3C1* | Nuclear receptor subfamily 3, group C, member 1 | 5q31 | BMI, obesity, weight, WHR, leptin, VAT, lean mass |
| *PPARG* | Peroxisome proliferative activated receptor γ | 3p25 | BMI, obesity, weight, fat mass, fat %, WHR, leptin, VAT and SAT, lean mass |
| *HTR2C* | Serotonin receptor 2c | Xq24 | BMI, obesity, weight |
| *UCP1* | Uncoupling protein 1 | 4q28-q31 | BMI, obesity, weight, fat %, WHR, RMR, meal-induced thermogenesis |
| *UCP2* | Uncoupling protein 2 | 11q13.3 | BMI, obesity, weight, fat mass, fat %, glucose/lipid oxidation, 24h EE, RQ, REE, 24h SMR, skinfold thickness |
| *UCP3* | Uncoupling protein 3 | 11q13 | BMI, weight, fat mass, fat %, WHR, leptin, lean mass, caloric intake, RQ, REE, RMR, skinfold thickness |

BMI, body mass index; WHR, waist-to-hip ratio; VAT, visceral adipose tissue; WC, waist circumference; SAT, subcutaneous adipose tissue; EE, energy expenditure; RMR, resting metabolic rate; RQ, respiratory quotient; REE, resting EE; SMR, sleeping metabolic rate. Modified from Rankinen et al. 2006 (120), and Bell et al. 2005 (113).

Recent studies using the GWAS approach have identified SNPs in the fat mass and obesity associated gene (*FTO)* (121-124) associated with obesity in several populations. *FTO* was originally found as a T2DM susceptibility gene but was then shown to predispose to T2DM through an effect on BMI (122). Very recently, reports from an Indian population have shown that the T2DM predisposing effect of FTO might also be independent of the effect of BMI (125). As a gene of unknown function in an unknown pathway, *FTO* provides an example of how GWAS can point to previously unsuspected candidate genes (121). In addition to BMI, *FTO* is also associated with hip circumference and weight (121), in children, adults, and elderly, with no difference between males and females (122). The at-risk haplotype yielded a proportion of attributable risk of 20-22% for common obesity (122, 123).

## 2.4. Genetics of T2DM

Multiple lines of evidence support the view that a genetic component plays an important role in the pathogenesis of T2DM (66). This can be seen in i) the spectrum of T2DM prevalence in different ethnic groups, ii) familial aggregation studies and iii) twin studies, and iv) the heritability of intermediate phenotypes (126). The complex genetic pattern of T2DM, including the variable age of onset, reduced penetrance, locus and allelic heterogeneity, and the likelihood of phenocopies (multiple different genetic and

nongenetic causes), as well as the likelihood that many genes interacting with the environment contribute to T2DM have confounded the search for T2DM susceptibility loci (126).

Over the last decade 27 genome-wide linkage scans and a huge number of candidate gene studies have been published in multiple ethnic groups in an attempt to discover genes having an impact on the T2DM risk (66). Despite the differences, eight susceptibility regions show at least some evidence for replication and appear likely to harbor one or more susceptibility genes, namely chromosomes 1q, 2q, 3q, 8p, 11q, 12, 18p and 20 (126). Some of the genes possibly corresponding for the high lod scores in these susceptibility loci have been identified, such as calpain 10 (chr. 2q) (127), protein tyrosine phosphatase 1B and heteronuclear factor 4A (chr. 20) (128-130), liver pyruvate kinase and calsequestrin 1 (chr. 1q) (131, 132), and adiponectin (chr. 3q) (133-135). Despite the initial identification through a linkage signal, which would be expected to select relatively strong effects, each of these variants above has only a modest individual effect. Thus, it is believed that i) the effects of individual variants are likely to be small; ii) multiple variants probably contribute to replicated linkage signals; and iii) most variants are noncoding and regulatory rather than altering the protein structure (126).

Very large number of studies has been conducted on candidate genes identified through physiological pathways, and for some genes, reasonable replication has been achieved. In each case, similar to the situation of those genes identified through linkage, the effect of these variants is relatively small, with odds ratio (OR) <1.4 (126). In these functional candidate gene studies, the variants are more often in a coding region of the gene. The most consistent findings as a T2DM risk variant have been the *P12* allele in the peroxisome proliferative activated receptor γ2 (*PPARγ2)* gene (136-138) and the *K23* allele in the Kir6.2, a subunit of the β-cell ATP-sensitive K-channel (*KCNJ11*) (139-142). Promising findings have also been made with *G971R* variant in the insulin receptor substrate (*IRS-1*) gene, *G482S* variant in the *PPARγ* coactivator-1α gene, and *K121Q* variant in the plasma cell glycoprotein 1 gene (126). Most recently, a novel T2DM susceptibility gene, transcription factor 7 like 2 gene (*TCF7L2*, chr. 10q), which has displayed impressive associations (the strongest known OR of 1.7) in several populations, was found (143, 144).

In 2007, five GWAS provided new insights into T2DM etiology, with six new gene regions identified (117, 144-148). These five studies had several features in common: i) relatively large sample size (in combination >18000 individuals), ii) all participants were of Northern European ancestry, and iii) extensive follow-up case-control studies

(118). These novel, confirmed genetic regions altering the risk of T2DM include: haematopoietically expressed homeobox- insulin-degrading enzyme (*HHEX-IDE*), solute carrier family 30 member 8 (*SLC30A8*), CDK5 regulatory subunit associated protein 1-like 1 (*CDKAL1*), cyclin-dependent kinase inhibitor 2A (*CDKN2*), insulin-like growth factor 2 mRNA-binding protein 2 (*IGF2BP2*) and *FTO* genes, of which *FTO* was shown to mediate its effects through a primary effect on adiposity (118).

## 2.5. Candidate genes of the present study

The present series of studies is part of a series of genetic association studies conducted on obesity and diabetes candidate genes in different populations. Traditionally, genes that play a role in EE, lipid turnover, insulin signalling, adipogenesis and molecules secreted by AT have been the focus of the research group's efforts. As soon as follow-up data on DPS was available, it was possible to investigate genetic association in this study longitudinally. So far over 40 genes with one or more variants have been examined in DPS (149), with positive associations between obesity and/or diabetes related traits and gene variants in *PPARγ2* (150), *α2B-adrenergic receptor* (151), *SUR1, Kir6.2* (142), *TNFα, IL-6* (152), *IGF-1R, IRS-1, IRS-2* (153), *hepatic lipase* (154), *SLC2A2* (155), *TCF7L2* (156), *adiponectin receptor 1* (157), *ghrelin* (158, 159), *ghrelin receptor* (160), *tenomodulin* (161, 162), and *FTO* (163) genes.

The selection of genes for the present studies was based partly (*B3AR, UCP1*) on previous findings of the research group (164-166). The supplementation with *B2AR, UCP2* and *UCP3* to these was a natural choice, since these genes all deal with energy metabolism, and belong to the same superfamilies of G protein-coupled receptors (BARs) and anion carrier proteins (UCPs). The leptin receptor (*LEPR)* gene was added to this series of studies due to increasing interest and evidence of its importance as a genetic modifier of obesity and T2DM risk.

### 2.5.1. β2-adrenergic receptor (*B2AR*)

Catecholamines are important regulators of fat cell lipolysis in human white AT (WAT) (32, 33). Noradrenaline (NA) and adrenaline regulate lipolysis through lipolytic β-adrenergic receptors 1-3 and antilipolytic $\alpha_2$-adrenergic receptors (32, 33).

BARs are members of G protein-coupled receptor (GPCR) superfamily, which includes nearly 2000 members classified into over 100 subfamilies (167). All GPCRs have an extracellular N-terminal segment, seven transmembrane (TM) domains, which form the TM core, three exoloops, three cytoloops, and a C-terminal segment (167).

B2ARs are widely distributed and, as part of the sympathetic nervous system, they play important roles in regulating cardiac, vascular, pulmonary, metabolic, central nervous system (CNS), and reproductive functions (168).

Despite the variety of functions involving B2AR, knockout (KO) mice are normal and fertile, but they become hypertensive in response to adrenaline infusion or to the cardiovascular stress induced by exercise (168). Studies with *B1/B2AR* double KO mice suggest that in the mouse, BAR stimulation of cardiac inotropy and chronotropy is mediated almost exclusively by the B1AR, whereas vascular relaxation and metabolic rate are controlled by all three BARs (169).

The *B2AR* gene was cloned in 1986 (170), and it was localised to chromosome 5q31-q32 (171). The gene is intronless (172), and it encodes a protein of 413 amino acids (aa) (171). Receptor transcripts have a 5'-leader region harboring an open reading frame that encodes a 19-aa peptide (172), which has been shown to inhibit translation of B2AR mRNA thus regulating cellular expression of the receptor (173). This peptide, termed as 5'-leader cistron (5'LC), harbors a variant *Arg19Cys*, which increases expression of *B2AR* (174). Recently, crystal structure of the human B2AR has been resolved and this represented a major achievement in view of its low natural abundance, inherent structural flexibility, and instability in detergent solutions (175, 176). Previously, knowledge on the GPCR structure was based on data obtained with rhodopsin (177).

Several variants within the coding block of *B2AR* have been identified: *Arg16Gly, Gln27Glu, Val34Met,* and *Thr164Ile*. In studies utilizing site-directed mutagenesis and recombinant expression, amino acid changes at positions 16, 27, and 164 have been found to alter receptor function (178, 179). The *Ile164* displays altered coupling to adenylyl cyclase (178), the *Gly16* displays enhanced agonist-promoted downregulation, and the *Glu27* form is resistant to downregulation (179). Thus, *Gly16* and *Glu27* may have opposite effects on agonist-mediated desensitization of the B2AR (180). *Gln27Glu* variant has been found to be associated with increased body weight (181-185), hypertriglyceridaemia (186, 187) and susceptibility to T2DM (185, 188). *Gln27Glu* variant may have different impact on obesity according to gender (184) and physical activity (189). Positive associations of different *B2AR* variants with obesity/diabetes related traits are listed in the Table 7, whereas the negative studies (187, 190-196) are not included.

Table 7. Positive associations of *B2AR* gene variants with obesity and diabetes related traits.

| Ref | Subjects (number/ethnicity) | SNP | Risk allele | Association |
|---|---|---|---|---|
| (181) | 140 women<br>Swedish Caucasian | *Arg16Gly*<br>*Gln27Glu* | *Arg*<br>*Glu* | Lower receptor agonist sensitivity<br>Higher risk of obesity, higher fat mass, and larger fat cells |
| (192) | 1152<br>French Caucasian | *Gln27Glu* | *Gln* | Higher weight, BMI, and WHR among physically inactive men<br>Interaction with physical activity |
| (183) | 278 men<br>Japanese | *Gln27Glu* | *Glu* | Higher frequency among obese |
| (184) | 138 men, 109 women<br>Swedish Caucasian | *Gln27Glu* | *Gln*<br>*Glu* | Higher frequency among obese men<br>Higher frequency among obese women |
| (185) | 104 T2DM, 26 obese NGT, 478 lean NGT<br>Japanese | *Gln27Glu*<br>*Arg16Gly* | *Glu*<br>*Arg* | Higher risk of obesity and T2DM<br>Higher risk of obesity in women |
| (197) | 124 obese, 450 lean<br>Japanese | *5'LC-Cys19Arg* | *Arg* | Higher BMI and TG<br>Higher frequency among T2DM (n=113) |
| (182) | 180 men<br>Swedish Caucasoid | *Gln27Glu* | *Glu* | Higher BMI, cholesterol, TG, and VLDL levels |
| (198) | 119 obese, 717 lean<br>French Caucasoid | *Arg16Gly*<br>*Gln27Glu* | *Arg*<br>*Gln* | Additive effect with *Gln27* on adiposity<br>Higher risk of obesity (men) |
| (199) | 743<br>French-Canadian | *Arg16Gly*<br><br>*Gln27Glu* | *Arg*<br><br>*Glu* | Higher frequency among obese, higher total and LDL-cholesterol<br>Higher total and LDL-cholesterol<br>Combination with ***Arg****16Gly* results in higher abdominal SAT |
| (188) | 461 T2DM, 593 NGT<br>Swedish Caucasoid | *Gln27Glu* | *Gln* | Higher frequency among T2DM<br>Higher insulin and FFA |
| (200) | 12 male twin pairs | *Gln27Glu* | *Gln* | Higher weight and SAT gain, increase in insulin-AUC, and plasma leptin during overfeeding |
| (186) | 251 men<br>Japanese | *Arg16Gly*<br>*Gln27Glu* | *Gly*<br>*Glu* | Lower HDL-cholesterol<br>Higher TG, higher prevalence of fatty liver |
| (201) | 604<br>Caucasoid | *Arg16Gly* | *Arg* | Higher BMI<br>Interaction with physical activity on FFA levels |
| (202) | 112 obese, 127 lean<br>Spanish Caucasoid | *Gln27Glu* | *Glu* | Higher risk of obesity (men, n=40), specially among those with low HDL-cholesterol |
| (203) | 15 obese women<br>Spanish Caucasoid | *Gln27Glu* | *Glu* | Lower lipolysis and fat oxidation promoted by exercise |
| (204) | 130 T2DM, 130 NGT<br>Asian | *Arg16Gly* | *Arg* | Higher risk of diabetes<br>Earlier onset of diabetes |
| (205) | 205 black, 415 white | *Arg16Gly* | | Risk allele is modified by interaction with *B3AR Trp64Arg* on fat reduction after endurance training (black) |
| (206) | 159 obese, 154 lean<br>Caucasoid | *Gln27Glu* | *Glu* | Interaction with carbohydrate intake on the risk of obesity |
| (207) | 666<br>Caucasoid | *Gln27Glu* | *Glu* | Higher BMI among obese men (n=79)<br>Higher 2h glucose |

| | | | | |
|---|---|---|---|---|
| (208) | 260 black, 482 white | *Arg16Gly* | *Arg*<br>*Gly* | Higher fat accumulation (n=39)<br>Lower reduction in BMI and fat mass during endurance training |
| | | *Gln27Glu* | *Gln* | Higher fat accumulation (n=58) |
| (209) | 1576<br>several ethnicities | *Arg16Gly* | *Gly*<br>*Arg* | Higher sBP, risk of hypertension<br>Higher BMI, higher risk of obesity |
| | | *Gln27Glu* | *Gln* | Higher risk of obesity |
| (210) | 47 T2DM<br>Japanese | *Arg16Gly* | *Gly* | Higher fasting insulin and HOMA-IR |
| (211) | 834 white<br>345 African American | *Arg16Gly* | *Gly* | Interaction with *B1AR Arg389**Gly*** on increase in BMI over years (in men) |
| (212) | 160 men<br>Japanese | *Arg16Gly* | *Gly* | Higher frequency of weight gain and BP elevation, higher plasma NA |
| | | *Gln27Glu* | *Glu* | Higher frequency of BP elevation in combination with *B3AR **Trp**64Arg*<br>Higher plasma NA |
| (213) | 329 adolescents<br>Asian | *G(1053)C* | *C* | Higher BMI, interaction with *B3AR Trp64**Arg*** on BMI |
| (214) | 155 men<br>Japanese | *Arg16Gly* | *Gly* | Higher HOMA-IR, fasting insulin, NA, body fat mass, and BP |
| (215) | 154 obese men<br>Japanese | *Arg16Gly* | *Gly* | Higher frequency among subjects with rebound weight gain (n=36)<br>Higher NA, leptin, fat mass, and BMI |
| (216) | 642<br>Caucasoid | *5'LC-Cys19Arg* | *Cys* | Higher TG and LDL-cholesterol |
| | | *Arg16Gly* | *Arg* | Higher TG and LDL-cholesterol |
| | | *Gln27Glu* | *Gln* | Higher TG and LDL-cholesterol |
| (217) | 123 obese, 206 lean men<br>Japanese | *Arg16Gly* | *Gly* | Higher frequency among obese<br>Higher fat mass, WHR, and leptin |
| | | *Gln27Glu* | *Glu* | Higher frequency among obese<br>Higher fat mass, WHR, and leptin |
| (218) | 55 obese men<br>Japanese | *Arg16Gly* | *Gly* | Higher weight gain and BP elevation<br>Higher fat mass, WHR, and leptin |
| (219) | 342 T2DM, 305 NGT<br>Italian Caucasoid | *Arg16Gly* | *Arg* | Combination with *UCP3 C(-55)**T*** results in higher prevalence of T2DM |
| | | *Gln27Glu* | *Gln* | Higher risk of diabetes |
| (220) | 149 obese hypertensive, 139 lean hypertensive, 149 control<br>Chinese | *Gln27Glu* | *Glu* | Higher frequency among obese hypertensive |

Ref, reference; SNP, single nucleotide polymorphism; BMI, body mass index; WHR, waist-to-hip ratio; T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; TG, triglyceride; VLDL, very low density lipoprotein; LDL, low density lipoprotein; SAT, subcutaneous adipose tissue; FFA, free fatty acid; AUC, area under curve; HDL, high density lipoprotein; BP, blood pressure; sBP, systolic BP; HOMA-IR, homeostasis model assessment for insulin resistance; NA, noradrenaline. The risk allele in 'association'-column is written in bold.

### 2.5.2. β3-adrenergic receptor (*B3AR*)

In mature brown adipocytes, NA interacts with all three types of adrenergic receptors: β, $\alpha_1$, and $\alpha_2$; these receptor types are associated with activation of different signalling pathways, the most significant of which is the pathway for β–adrenergic stimulation of

thermogenesis (221). B3AR increases thermogenesis through the action of UCP1 in brown fat cells and stimulates the mobilisation of lipids from the white fat cells (35). Whereas B1AR and B2AR subtypes are most active in WAT lipolysis, B3AR is less active, particularly in the SAT where it can activate lipolysis only to a minor extent (222). However, in VAT, the B3AR has a strong lipolytic action, and it has been suggested that this is of importance for some of the metabolic aberrations seen in obese subjects (222).

Mice lacking B3AR display a modest increase in body fat, indicating that B3AR plays a role in energy balance (223). B1AR but not B2AR mRNA levels are upregulated in WAT and BAT of B3AR-deficient mice, implying that B3AR mediates physiologically relevant signalling and that cross-talk exists between *B3AR* and *B1AR* gene expression (223). Mice that lack all three known *BARs* had a reduced metabolic rate and became slightly obese on a Chow diet (224). On a high-fat diet, they developed massive obesity that was entirely due to a failure to express diet-induced thermogenesis (224). Their BAT was unresponsive to both physiological (cold exposure) and pharmacological (β-agonist) stimulation (225). In AT of obese (*fa/fa*) Zucker rats, the amount of B3AR was reduced by 71% as compared with lean control animals (226).

B3AR was cloned in 1989, and shown to be expressed mostly in BAT and WAT, but also in ileum, liver, soleus muscle (227), and pancreatic β-cells (228). The *B3AR* gene is located in chromosome 8p12-p11.2 (229), consisting of two exons and a single intron which encode a protein of 408 aa (230).

In 1995, a *Trp64Arg* variant, situated in the first intracellular loop of the receptor, was identified and reported to be associated with increased body weight, clinical features of IR, and early development of T2DM (231-233). This variant has been shown to have functional significance; the *Arg64* variant causes a10-fold decreased receptor responsiveness to activation by a B3AR agonist compared with the *Trp* allele (234). Reduced receptor function may lead to decreased lipid mobilisation from the fat depots and/or decreased thermogenesis in BAT, thus predisposing to obesity. Individuals homozygous for the *Arg64* allele secrete significantly less insulin in response to a glucose infusion, have higher fasting glucose levels, and have lower glucose effectiveness (Sg), compared with those homozygous for the *Trp* allele (235). Transfection studies with rat β-cells have shown that cells transfected with the *Arg64* variant secrete less insulin, both spontaneously and after exposure to human B3AR agonist, and their glucose responsiveness is significantly reduced (228). These findings may help explain the earlier onset of T2DM observed in several populations of individuals with the *Arg* allele.

The coexistence of common variants in the *B3AR (Trp64Arg)* and *B2AR (Gln27Glu)* genes has been reported to be associated with T2DM (188), and the interaction with *B2AR Arg16Gly* variant has contributed to the changes in fat mass and abdominal obesity (205). However, the results of meta-analyses have been contradictory in defining the association between *Trp64Arg* variant and BMI, i.e. two studies finding the variant carriers to exhibit higher BMI than normal homozygous subjects (236, 237), one study finding the association only in East Asians but not in Europeans (238), and one study finding no association (239). One meta-analysis suggested that *Trp64Arg* variant has moderate effects on IR in Asian populations and in obese and diabetic subgroups (240). Positive associations of *B3AR Trp64Arg* variant with obesity/diabetes related traits are listed in the Table 8, whereas the negative studies (190, 196, 214, 241-248) are not included.

Table 8. Positive associations of *Trp64Arg* variant in *B3AR* gene with obesity and diabetes related traits.

| **Ref** | **Subjects (number/ethnicity)** | **Risk allele** | **Association** |
|---|---|---|---|
| (233) | 390 T2DM, 252 NGT<br>Pima Indian | *Arg* | Lower age at onset of T2DM |
| (231) | 185 obese, 94 lean<br>French Caucasoid | *Arg* | Higher weight gain during adult life |
| (232) | 128 T2DM, 207 NGT<br>Finnish Caucasoid | *Arg* | Lower age at onset of T2DM<br>Higher WHR, insulin response in OGTT, dBP, and lower rate of glucose disposal (in NGT) |
| (249) | 159 T2DM, 191 NGT<br>Japanese | *Arg* | Higher BMI, fasting and 2h insulin in OGTT (in NGT) |
| (250) | 254 obese, 151 lean<br>Finnish Caucasoid | *Arg* | Earlier onset of obesity |
| (251) | 238 obese, 91 lean<br>French Caucasoid | *Arg* | Higher weight gain,<br>additive effect with *UCP1 A(-3826)**G*** |
| (252) | 131 obese, 256 lean, women<br>Japanese | *Arg* | Greater VAT area, higher body fat, sBP, glucose, insulin, cholesterol, and TG levels<br>Lower HDL-cholesterol |
| (253) | 25 T2DM, 36 NGT<br>421<br>Mexican American | *Arg* | Lower age at onset of T2DM<br>Higher 2h insulin in OGTT |
| (164) | 170 obese<br>Finnish Caucasoid | *Arg* | Combination with *UCP1 A(-3826)**G*** results in lowered BMR |
| (254) | 45 sib-pairs<br>Mexican American | *Arg* | Higher BMI, fat mass, and WC |
| (255) | 27 obese women<br>Caucasoid | *Arg* | Lower exogenous glucose infusion in clamp study<br>Lower total glucose disposal in clamp study |
| (256) | 77 obese women<br>Finnish Caucasoid | *Arg* | Combination with *UCP1 A(-3826)**G*** results in lower weight reduction during VLCD, and increased weight during maintenance period |

| | | | |
|---|---|---|---|
| (234) | 208<br>Swedish Caucasoid | *Arg* | Higher BMI, lower sensitivity for receptor agonist in VAT adipocytes |
| (166) | 70 T2DM, 123 NGT<br>Finnish Caucasoid | *Arg* | Combination with *UCP1 A(-3826)**G***<br>results in higher weight gain |
| (235) | 62<br>several ethnicities | *Arg* | Lower insulin secretion and glucose effectiveness<br>Higher fasting glucose |
| (199) | 743<br>French Canadian | *Arg* | Higher total and SAT abdominal fat area in men<br>Interaction with *α2-AR* **6.7**/6.3kb on<br>total and SAT abdominal fat area in men |
| (257) | 24 obese women<br>Caucasoid | *Arg* | Lower reduction of VAT during weight reduction |
| (258) | 909 women<br>Caucasoid | *Arg* | Interaction with *α2b-AR Glu12/**Glu9*** on<br>fat mass and percent fat |
| (188) | 461 T2DM, 593 NGT<br>Swedish Caucasoid | *Arg* | Higher 2h glucose and FFA |
| (259) | 453<br>Mexican American | *Arg* | Interaction with *PPARγ2 Pro12**Ala*** on<br>higher BMI, insulin, and leptin levels |
| (260) | 476<br>Japanese-American | *Arg* | Higher WHR, insulin, and HOMA-IR<br>(among obese men) |
| (205) | 205 black, 415 white | *Trp,*<br>*Arg* | Risk allele is modified by interaction with *B2AR Arg16Gly* on fat reduction after endurance training (in black) |
| (261) | 62<br>several ethnicities | *Arg* | Lower RMR and higher thermic effect of feeding |
| (262) | 105 obese children<br>Japanese | *Arg* | Higher BMI and lower HDL-cholesterol,<br>apoA-I, and apoA-II levels (in boys) |
| (263) | 76 women<br>Japanese | *Arg* | Lower weight reduction |
| (264) | 224 obese<br>Asian | *Arg* | Combination with *UCP3 C(-55)**T*** resulted in lower reduction in VAT, and glucose and FFA levels |
| (265) | 296 neonates<br>Chinese | *Arg* | Higher insulin and insulin-to-glucose ratios among small for gestational age babies (n=76) |
| (266) | 332<br>Caucasoid | *Arg* | Higher leptin level among women (n=172) |
| (211) | 340 African American,<br>813 white | *Arg* | Interaction with *B1AR Arg389**Gly*** on higher increase in BMI among women (n=658) |
| (267) | 295 obese, 147 lean children<br>Hungarian | *Arg* | Higher weight, body fat, sBP, and insulin levels |
| (212) | 160 men<br>Japanese | *Trp* | Higher frequency of BP elevation |
| (213) | 329 adolescents<br>Asian | *Arg* | Higher BMI, body fat, and plasma leptin<br>Interaction with *B2AR G(1053)**C*** on BMI |
| (268) | 295 men<br>Japanese | *Arg* | Interaction with high energy intake<br>on higher risk of obesity |
| (218) | 55 obese men<br>Japanese | *Arg* | Higher weight gain, BP elevation, fat mass,<br>WHR, and plasma NA level |
| (220) | 149 obese hypertensive,<br>139 lean hypertensive,<br>149 control, Chinese | *Arg* | Higher frequency among obese hypertensive<br>vs. other groups (in men) |

Ref, reference; T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; WHR, waist-to-hip ratio; OGTT, oral glucose tolerance test; dBP, diastolic blood pressure; BMI, body mass index; VAT, visceral adipose tissue; sBP, systolic blood pressure; TG, triglyceride; HDL, high density lipoprotein;

BMR, basal metabolic rate; WC, waist circumference; VLCD, very low calorie diet; SAT, subcutaneous adipose tissue; FFA, free fatty acid; HOMA-IR, homeostasis model assessment for insulin resistance; RMR, resting metabolic rate; apoA, apolipoprotein A; NA, noradrenaline. The risk allele in 'association'-column is written in bold.

### 2.5.3. Uncoupling protein 1 (*UCP1*)

The uncoupling proteins 1, 2, and 3 (UCP1-3) are members of the superfamily of anion carrier proteins situated in the inner membrane of mitochondria (269). UCP1 is a dimeric protein, the three-dimensional structure of which is not known. It is believed to consist of six αα-helices connected by loops with the third matrix side loop containing the purine nucleotide-binding domain (270). Both the N- and C-terminal amino acids face the intramembrane space (270).

Brown adipocytes possess abundant mitochondria with densely packed cristae, and they are unique in expressing UCP1, 'thermogenin', which functions to uncouple mitochondrial respiration in the inner mitochondrial membrane (271). Mitochondrial adenosine triphosphate (ATP) synthesis from adenosine diphosphate (ADP), driven by electron flow from reduced substrate (mainly NADH) to oxygen, defines oxidative phosphorylation (272). Oxidation is coupled by the electron transport chain to pumping of protons from the mitochondrial matrix, generating a proton motive force (or electrochemical potential difference) for protons across the inner membrane of the mitochondria. This force drives the protons back into the matrix through an ATP synthase, which couples proton transport across the membrane to phosphorylation of ADP (272). UCP1 is able to dissipate the proton gradient, thereby uncoupling fuel oxidation from the availability of ADP. Thus, the physiological consequence of UCP1 activity is unrestrained oxidation of fuels with the sole byproduct being the generation of heat (271). Mild uncoupling of respiration can also prevent the accumulation of oxygen radicals generated by mitochondria, and control the $NAD^+$/NADH ratio and thereby regulate ketogenesis, lipogenesis and amino acid synthesis, which are dependent on the levels of these cofactors (272).

UCP1 expression is increased by cold acclimation and overfeeding, and reduced in fasting and genetic obesity (273). UCP1 is activated by FFA and inhibited by purine nucleotides (274). In response to a cold environment, NA released by sympathetic nerves stimulates both lipolysis – thus increasing the intracellular FFA availability – and the transcription of the *UCP1* gene (Figure 3) (274). The ability of NA to stimulate lipolysis and thermogenesis in adipocytes is controlled largely by B3AR (275).

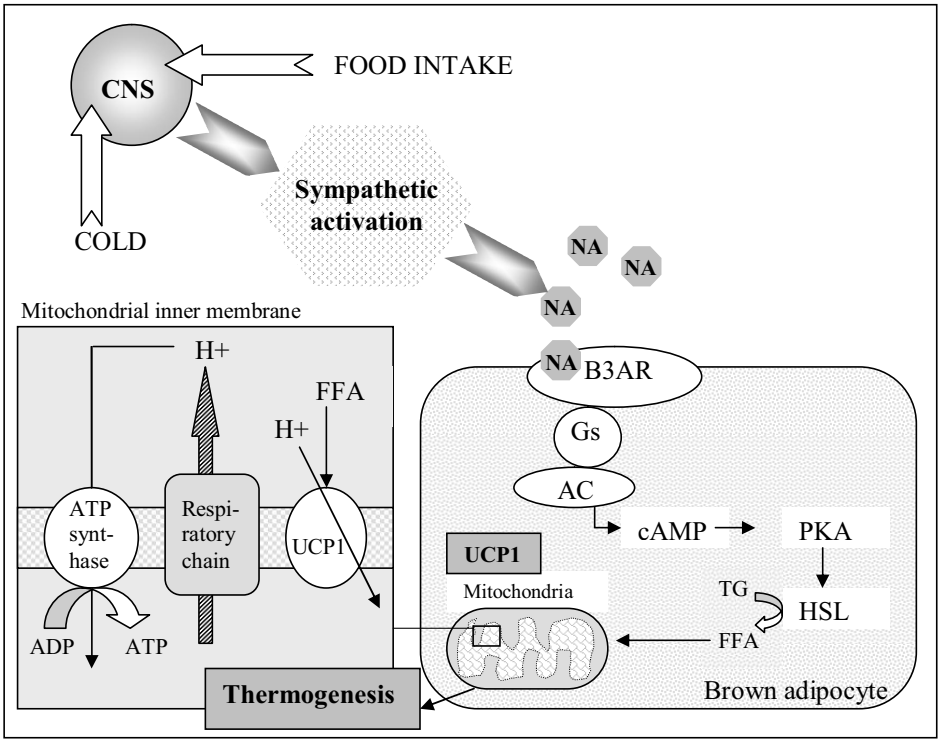



Figure 3. The role of UCP1 in thermogenesis of brown adipose tissue. In response to several stimuli, the catecholamines released by the sympathetic system increase the intracellular concentration of cAMP and, as a result, the lipolytic activity is enhanced, resulting in an increase in the intracellular FFA concentration, which activates both *UCP1* gene transcription and UCP1 activity. Activation of UCP1 reduces the proton gradient generated by the respiratory chain and decreases the membrane potential, which in turn accelerates respiration. Increased respiration and shunting of ATP synthesis induce thermogenesis. AC, adenylate cyclase; B3AR, $\beta_3$-adrenergic receptor; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; FFA, free fatty acid; Gs, stimulatory guanosine triphosphate binding protein; HSL, hormone sensitive lipase; NA, noradrenaline; PKA, protein kinase A; TG, triglyceride; UCP1, uncoupling protein 1. Modified from Yasuda et al. 2006 (276), Argiles et al. 2002 (274), and Pecqueur et al. 2001 (277).

Transgenic mice with genetic ablation of BAT develop obesity, and as obesity progresses, also hyperphagia develops (278). When fed with a Western diet, they develop also morbid metabolic complications, such as IR, hyperglycaemia, and hyperlipidaemia (279). BAT phenotype of the adipose organ in rodents is important for the prevention of obesity and diabetes (280).

Human *UCP1* was cloned in 1988 (281). The *UCP1* gene is located on chromosome 4q28-q31, having six exons all of which are translated, encoding a protein of 305 aa (282).

There are many common exonic variants (*Arg40Trp, Ala64Thr, Val137Met, Met229Leu, Lys257Asn*), which have not been associated with obesity (270). However, the *A(-3826)G* variant in the 5'UTR close to an enhancer region, has been found to be associated with obesity and metabolic disorders (166, 256, 283). The simultaneous existence of the variants in the *B3AR (Trp64Arg)* and *UCP1 (A(-3826)G)* genes are known to have an additive effect on low basal metabolic rate (BMR) (164) and weight gain (166, 251, 256). Positive associations of *UCP1* variants with obesity/diabetes related traits are listed in the Table 9, whereas the negative studies (245, 284, 285) are not included.

Table 9. Positive associations of *UCP1* gene variants with obesity and diabetes related traits.

| **Ref** | **Subjects (number/ethnicity)** | **SNP** | **Risk allele** | **Association** |
|---|---|---|---|---|
| (251) | 238 obese, 91 lean French Caucasoid | *A(-3826)G* | *G* | High weight gain during adult life, additive effect with *B3AR Trp64**Arg*** |
| (286) | 153 obese Caucasoid | *A(-3826)G* | *G* | Lower UCP1 mRNA in VAT |
| (164) | 170 obese Finnish Caucasoid | *A(-3826)G* | *G* | Combination with *B3AR Trp64**Arg*** resulted in lower BMR |
| (256) | 77 obese women Finnish Caucasoid | *A(-3826)G* | *G* | Combination with *B3AR Trp64**Arg*** resulted in lower weight reduction during VLCD and faster weight gain after it |
| (166) | 70 T2DM, 123 NGT Finnish Caucasoid | *A(-3826)G* | *G* | Combination with *B3AR Trp64**Arg*** resulted in higher weight gain |
| (283) | 526 obese women Australian Caucasoid | *A(-3826)G* | *G* | Higher BMI |
| (287) | 12 male twin pairs | *A(-3826)G* | *G* | Worse recovery from overfeeding |
| (285) | 25 T2DM, 25 NGT Japanese | *A(-112)C* *Met229Leu* | *C* *Leu* | Higher frequency among diabetic subjects Higher frequency among diabetic subjects |
| (288) | 118 obese Polish Caucasoid | *A(-3826)G* | *G* | Higher TG and FFA levels, Lower HDL-cholesterol level |
| (266) | 332 Spanish Caucasoid | *A(-3826)G* | *G* | Higher weight among women |
| (289) | 93 T2DM Japanese | *A(-112)C* | *C* | Higher HOMA-IR and hepatic lipid content |
| (290) | 295 T2DM, 113 offspring, 120 NGT Czech Caucasoid | *A(-3826)G* | *G* | Higher BMI among offspring of T2DM |

Ref, reference; SNP, single nucleotide polymorphism; VAT, visceral adipose tissue; BMR, basal metabolic rate; VLCD, very low calorie diet; T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; BMI, body mass index; TG, triglyceride; FFA, free fatty acid; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance. The risk allele in 'association'-column is written in bold.

### 2.5.4. Uncoupling protein 2 (*UCP2*)

UCP2 is widely distributed with the greatest amounts found in spleen, thymus, pancreatic β-cells, heart, lung, WAT, BAT, stomach, testis, and macrophages, and lesser amounts found in brain, kidney, liver, and muscle (269, 291). UCP2 is a key component of β-cell glucose sensing, since it seems to regulate glucose stimulated insulin secretion (GSIS) (270, 292). It has been suggested that increased UCP2 expression might lead to a reduced risk of obesity, but increased risk of diabetes; thus, UCP2 has been called the 'adiposity angel and diabetes devil' (Figure 4) (293). Furthermore, UCP2 regulates FFA metabolism in multiple tissues, and negatively modulates the production of reactive oxygen species (ROS) (270, 292).

Mice lacking *UCP2* following targeted gene disruption are viable and appear normal (294). No differences in body weight were seen compared to wild-type littermates. UCP3 mRNA levels were unchanged, whereas UCP1 mRNA in WAT was increased (294). Immunological studies showed that ROS production in macrophages was higher than in the wild-type mice, and that UCP2 is active in the limitation of ROS and in antioxidant defence (294).

Overexpression of UCP2 in rat pancreatic islets resulted in severe blunting of GSIS (295), whereas glucose-stimulated mitochondrial membrane hyperpolarization and the ATP content of islets were reduced, suggesting that the low ATP level led to reduced glucose-induced depolarization, thereby causing reduced insulin secretion (296). Studies on mice revealed that UCP2 deficiency resulted in higher islet ATP levels and increased GSIS (297). UCP2 was markedly upregulated in islets of *ob/ob* mice, a model of obesity-induced diabetes, whereas *ob/ob* mice lacking UCP2 had restored first-phase insulin secretion, increased serum insulin levels, and greatly decreased levels of glycaemia (297). It was concluded that UCP2 is a critical link between obesity, β-cell dysfunction and T2DM (297). Furthermore, short-term inhibition of UCP2 expression ameliorated the hyperglycaemic syndrome in two distinct animal models of obesity and diabetes by modifying both insulin secretion and insulin signal transduction in AT (298).

Endogenously produced mitochondrial superoxide activates the UCP2-mediated proton leak, thus lowering ATP levels and impairing GSIS (299). Furthermore, hyperglycaemia- and obesity-induced loss of glucose responsiveness is prevented by a reduction in mitochondrial superoxide production or gene KO of *UCP2* (299). Therefore, one of the key missing elements in hyperglycaemia-induced β-cell dysfunction could be the activation of UCP2 by superoxide (299). It is believed that the

physiological role of UCP2 is to prevent excessive superoxide generation through a feedback loop (300).

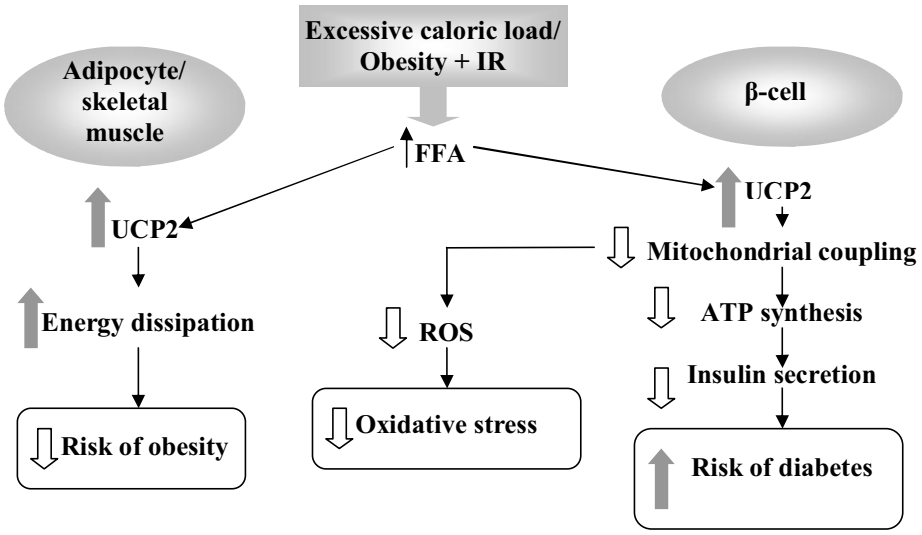



Figure 4. UCP2 expression increases with high fat feeding and/or in response to obesity and insulin resistance (IR). Effects of the increased expression of UCP2 are different in adipocytes and skeletal muscle vs. pancreatic β-cells. FFA, free fatty acid; ROS, reactive oxygen species. Modified from O'Rahilly 2001 (293) and Langin 2001 (301).

*UCP2* and *UCP3* were identified in 1997 by reverse cloning, i.e. by 'mining'databases of expressed sequence tags or searching sequences for similarity to *UCP1* in complementary DNA (cDNA) libraries (302, 303). *UCP2* and *UCP3* have 59% and 57% identity, respectively, with *UCP1*, and 73% identity with each other (269). The transcription initiation of *UCP2* is placed only 7 kb downstream of the *UCP3* stop codon in the chromosome 11q13 (304). There are 8 exons in *UCP2* gene, of which 1 and 2 are untranslated (304).

Three common variants exist in the *UCP2* gene, one situated in the promoter region (*G(-866)A*), one is a missense variant in exon 4 (*Ala55Val*), and one is located in the untranslated exon 8 (*3'UTR 45 bp Del/Ins*). It has been shown that the functional *G(-866)A* variant, situated in a multifunctional *cis* regulatory site, acts as a binding site for a pancreatic transcription factor *PAX6* (305). This variant has been associated with increased AT mRNA expression and obesity (306), β-cell function and diabetes (305), insulin secretion (307), energy metabolism (308), and CHD (309). In a recent meta-analysis of genome-wide linkage studies, suggestive evidence for chromosome 11q13.3-

22.3 was observed for BMI-defined obesity (115). Positive associations of different *UCP2* variants with obesity/diabetes related traits are listed in the Table 10, whereas the negative studies (310-313) are not included.

Table 10. Positive associations of *UCP2* gene variants with obesity and diabetes related traits.

| Ref | Subjects (number/ethnicity) | Variant | Risk allele | Association |
|---|---|---|---|---|
| (314) | 82<br>790<br>Pima Indian | *Ala55Val*<br>*Del/Ins* | *Ala+Val*<br>*Del+Ins* | Lower SMR than in heterozygotes<br>Lower SMR than in heterozygotes<br>Lower 24h EE than in heterozygotes<br>Higher BMI than in heterozygotes (older subgroup) |
| (315) | 596 South Indian<br>83 British Caucasoid | *Del/Ins* | *Ins* | Higher BMI in women |
| (287) | 12 male twin pairs | *Ala55Val*<br><br>*Del/Ins* | *Val*<br><br>*Del* | Poorer recovery from overfeeding, high RQ<br>Greater increase in TSH response to TRH<br>Poorer recovery from overfeeding<br>Higher weight increase after overfeeding |
| (306) | 340 obese, 256 lean, 791 control<br>Caucasoid | *G(-866)A* | *G* | Higher BMI, lower *UCP2* mRNA expression in AT |
| (305) | 201 obese T2DM, 391 obese<br>Caucasoid | *G(-866)A* | *A* | Lower disposition index<br>Higher risk of diabetes<br>Higher activation of the promoter in β-cell |
| (307) | 301<br>Italian Caucasoid | *G(-866)A* | *A* | Lower glucose stimulated insulin secretion (Studied also in human islets, n=10) |
| (309) | 465 T2DM men<br><br>2695 men<br>British Caucasoid | *G(-866)A* | *A* | Lower antioxidant status<br>Higher oxidative stress (patients with CHD)<br>Higher CHD risk |
| (308) | 296 obese, 568 lean, children<br>Caucasoid | *G(-866)A* | *A* | Lower lipid oxidation, higher carbohydrate oxidation (obese subgroup, n=147) |
| (316) | 681 T2DM<br>Caucasoid | *G(-866)A* | *A* | Higher levels of TG, cholesterol, LDL-cholesterol |
| (317) | 483 T2DM, 181 offsprings, 565 NGT<br>100 morbidly obese<br>Italian Caucasoid | *G(-866)A* | *A* | Lower insulin sensitivity, studied by clamp<br>Higher frequency among T2DM women<br>*UCP2* mRNA correlates with IR in SAT |
| (318) | 283 T2DM, 388 NGT<br>125 NGT<br>36<br>several ethnicities | *G(-866)A*<br>*Ala55Val*<br>*Del/Ins*<br>*Haplotype* | *A*<br><br><br>*GVI/AVD* | Lower UCP2 mRNA in SAT (n=36)<br><br><br>Higher BMI, TG, and insulin levels |
| (319) | 157 obese, 150 lean<br>Spanish Caucasoid | *Del/Ins* | *Ins* | Higher risk of obesity |
| (320) | 504 T2DM, 133 NGT<br>Asian | *Ala55Val* | *Ala+Val* | Higher risk of T2DM in combination with *PPARγ* ***C****(161)T* than in *Ala55Val* heteroz. |
| (321) | 413 T2DM, 172 NGT<br>Japanese | *G(-866)A* | *A* | Higher promoter activity in β-cell line<br>Earlier need for insulin therapy<br>Higher frequency of insulin treatment |
| (322) | 746 T2DM, 327 NGT<br>Italian Caucasoid | *G(-866)A* | *G* | Higher risk of T2DM |

| | | | | |
|---|---|---|---|---|
| (323) | 565 T2DM, 299 NGT<br>Pima Indian | *G(-866)A*<br>*Del/Ins* | *G*<br>*Del* | Lower 24h metabolic rate (n=185)<br>Lower 24h metabolic rate (n=185) |
| (324) | 2936 men<br>Caucasoid | *G(-866)A* | *A* | Higher risk of T2DM, especially in obese<br>Additive effect with *UCP3 C(-55)**T*** |
| (325) | 658 women<br>Asian | *G(-866)A*<br>*Ala55Val* | *A*<br>*Val* | Lower HDL-cholesterol<br>Lower HDL-cholesterol |
| (326) | 458 obese women<br>Asian | *G(-866)A*<br>*Ala55Val* | *A*<br>*Val* | Lower VLCD-induced BMI reduction<br>Lower VLCD-induced BMI reduction |
| (327) | 150<br>Caucasoid | *Ala55Val* | *Val* | Lower circulating leptin level |
| (328) | 3457 T2DM,<br>French Caucasoid | *G(-866)A* | *G* | Higher risk of CHD |

Ref, reference; SMR, sleeping metabolic rate; EE, energy expenditure; BMI, body mass index; RQ, respiratory quotient; TSH, thyroid stimulating hormone; TRH; thyrotropin-releasing hormone; AT, adipose tissue; T2DM, Type 2 diabetes mellitus; CHD, coronary heart disease; TG, triglyceride; LDL, low density lipoprotein; NGT, normal glucose tolerance; IR, insulin resistance; SAT, subcutaneous adipose tissue; HDL, high density lipoprotein; VLCD, very low calorie diet. The risk allele in 'association'-column is written in bold.

### 2.5.5. Uncoupling protein 3 (*UCP3*)

Both UCP3 mRNA and protein are found only in skeletal muscle, and to a lesser extent in heart (269). UCP3 mRNA is also found in BAT, but there is controversial data about whether or not the protein has been identified in BAT (269). The functions proposed for UCP3 include regulation of redox state, regulation of FA and glucose metabolism and control of ROS formation (274, 329, 330). The abundant and relatively selective expression of UCP3 in skeletal muscle suggests that it may be a mediator of adaptive thermogenesis in humans (331).

UCP3 expression has been shown to increase in human skeletal muscle during fasting, a state characterised by a marked increase in circulating plasma FA concentrations and FA oxidation rates, suggesting a role for this gene in the regulation of FA homeostasis (332). Acute exercise induces upregulation of UCP3 mRNA and protein levels, whereas endurance training results in the downregulation of UCP3 (333, 334). When oxidative capacity is high, such as in physically well trained subjects, FAs can easily be oxidised and therefore are not likely to accumulate inside the mitochondria, and only a little amount of UCP3 is needed to export FA anions from the mitochondrial matrix (335).

The UCP3 protein content has been shown to be 50% lower in T2DM patients compared with healthy control subjects, evidence of a role for UCP3 in T2DM, consistently with mouse studies (336). Also ageing, which is characterised by mitochondrial dysfunction, is accompanied by a reduction in UCP3 levels (335). UCP3 expression varies two- to three-fold and may be a determinant of the variability in rates

of EE and the degree of obesity. Indeed, BMI has been shown to be negatively correlated with the expression levels in Pima Indians (337).

*UCP3* KO mice appear to be healthy animals (338). However, the respiration activities in skeletal muscle mitochondria are abnormal. Mitochondria are more coupled, meaning that UCP3 has a proton transport activity and consequently functions as a genuine UCP. At the cellular level, skeletal muscle *UCP3* KO increased the rate of ATP synthesis 4-fold (339). Moreover, mitochondria lacking UCP3 also produced more ROS, which might enhance oxidative damage to lipids, proteins, and DNA, thus accelerating the ageing process (338). Studies on transgenic mice strongly suggest that enhancement of UCP3 expression is a promising approach for the treatment of obesity, since overexpression of human UCP3 in mouse skeletal muscle results in hyperphagia and leanness (340). These mice also exhibit lower FPG and insulin levels and an increased glucose clearance rate, and a striking reduction in AT mass (340).

The human *UCP3* gene maps to the distal segment of 11q13, adjacent to the region housing *UCP2* (331). Given the close proximity and the high degree of similarity between *UCP2* and *UCP3* (~70% at the nucleotide level), it is likely that one *UCP* gene arose from the other via a duplication event (331). The *UCP3* gene contains 7 exons, multiple transcription initiation sites, and it gives rise to two main alternative transcripts, the shorter one having a polyadenylation site in exon 6, which terminates approximately 50% of the transcripts (270). Therefore, human UCP3 exists as long (UCP3L, 312 aa) and short (UCP3S, 275 aa) forms (331).

Although a number of amino acid substitutions have been reported, most are rare, like *Val9Met, Arg70Trp, Val102Ile, Arg143X, Arg282Cys, Arg308Trp*, and a mutation at the junction of exon 6 and intron 6, allowing only the UCP3 short form to be translated from the allele (*exon 6 ivs + 1G/A, GAIVS6*) (270). In addition, many silent or intronic variants exist. A promoter region variant *-55 C/T* is potentially interesting because it is situated only 6 bp apart from the TATA box and 4 bp from a DR1 site, which is a part of a retinoic acid response element (341). The *-55 T* allele associates with higher UCP3 expression (341), which has been positively correlated with SMR and 24 h EE (337). In French subjects, the *-55 T/T* genotype was associated with higher BMI (342), but lower risk of T2DM (343). Positive associations of different *UCP3* variants with obesity/diabetes related traits are listed in the Table 11, whereas the negative studies (344) are not included.

Table 11. Positive associations of *UCP3* gene variants with obesity and diabetes related traits.

| Ref | Subjects (number/ethnicity) | SNP | Risk allele | Association |
|---|---|---|---|---|
| (341) | 67<br>Pima Indian | *C(-55)T* | *C* | Lower *UCP3* mRNA in skeletal muscle (n=18) |
| (343) | 232 obese, 49 T2DM, 894 control | *C(-55)T* | *C*<br>*T* | Higher frequency among T2DM<br>Higher cholesterol, LDL-cholesterol, and apolipoprotein (control group) |
| | 171 T2DM, 124 NGT<br>French Caucasoid | | *C* | Higher frequency among T2DM |
| (342) | 401 obese, 231 lean<br>French Caucasoid | *C(-55)T* | *T* | Higher BMI |
| (345) | 710 South Indian<br>450 Caucasoid | *C(-55)T* | *T* | Higher WHR in females |
| (346) | 419<br>Caucasoid | *C(-55)T* | *C* | Higher BMI |
| (347) | 734<br>Caucasoid | *GAIVS6* | *240 bp allele* | Higher BMI, fat mass, % body fat, and leptin |
| (264) | 224 obese<br>Asian | *C(-55)T* | *T* | Lower reduction in VAT after weight reduction, in combination with *B3AR Trp64**Arg***<br>Lower reduction of glucose and FFA |
| (348) | 162<br>Caucasoid | *C(-55)T* | *T* | Higher WHR |
| (349) | 345 men, 377 women<br>Hispanic and non-Hispanic white | *Tyr210Tyr (C/T)* | *T* | Lower fat and lean mass despite of higher dietary intake (women) |
| | | *Tyr99Tyr (T/C)* | *C* | Higher dietary intake and lean mass (women) |
| | | *C(-55)T* | *T* | Higher dietary intake and lean mass (women) |
| (350) | 1873<br>Caucasoid | *C(-55)T*<br>*Tyr99Tyr*<br>*Tyr210Tyr* | *C* | Higher BMI |
| (219) | 342 T2DM, 305 NGT<br>Italian Caucasoid | *C(-55)T* | *T* | Combination with *B2AR **Arg**16Gly* results in higher prevalence of T2DM in older age |
| (351) | 214 obese, 246 lean, women<br>Asian | *+521G/C*<br>*+1063G/A*<br>*Tyr210Tyr*<br>*Haplotype* | *C*<br>*A*<br>*C*<br>*CGTACC* | Lower weight reduction after VLCD<br>Lower weight reduction after VLCD<br>Lower weight reduction after VLCD<br>Higher weight, WHR, and BMI at baseline<br>Higher weight reduction after VLCD |
| (324) | 2936 men<br>Caucasoid | *C(-55)T* | *T* | Higher risk of diabetes, especially in the obese<br>Additive effect with *UCP2 G(-866)**A*** |
| (352) | 225 obese<br>Spanish Caucasoid | *C(-55)T* | *T* | Higher CRP |
| (325) | 658 women<br>Asian | *Tyr210Tyr* | *T* | Lower HDL-cholesterol |
| (326) | 458 obese women<br>Asian | *+521G/C*<br>*+1063G/A*<br>*Tyr210Tyr* | *C*<br>*G*<br>*C* | Lower BMI and fat mass reduction<br>Lower BMI and fat mass reduction<br>Lower BMI and fat mass reduction |

| | | | | |
|---|---|---|---|---|
| | | *Haplotype* | | Haplotype with *UCP2* associates with lower BMI and fat mass reduction after VLCD |
| (353) | 282 Japanese | *C(-55)T* | *C* | Lower HDL-cholesterol Higher BMI |

Ref, reference; SNP, single nucleotide polymorphism; T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; LDL, low density lipoprotein; BMI, body mass index; WHR, waist-to-hip ratio; VAT, visceral adipose tissue; FFA, free fatty acid; VLCD, very low calorie diet; CRP, C-reactive protein; HDL, high density lipoprotein. The risk allele in 'association'-column is written in bold.

### 2.5.6. Leptin receptor (*LEPR*)

**Finding of leptin**

The "*obese*" locus was first described 6 decades ago (354) and was later shown by positional cloning to be the *LEP* gene that encodes a secreted protein, leptin (36). Mice and humans homozygous for a leptin gene mutation (*Lep* $^{ob/ob}$) develop hyperphagia, early-onset obesity, severe IR, steatosis, hypothalamic hypogonadism, deficits of the thyroid and growth hormone axes, and immunosuppression (40, 355). Circulating concentrations of leptin reflect fat cell stores – increasing with overfeeding and decreasing with starvation. Leptin administration to obese leptin-deficient animals has reversed their hyperphagia, hypothermia, decreased locomotor activity, and all neuroendocrine and immunological abnormalities (356). Leptin therapy also dramatically reduced body weight (mostly fat mass) in four children (357-359) and three adults (360) with leptin deficiency. Leptin is expressed mainly in WAT adipocytes, though low levels are produced in the stomach, mammary gland, placenta, and skeletal muscle (40).

**Leptin acts through leptin receptor**

The diverse nature of leptin is supported by the universal distribution of LEPR. *LEPR* was cloned in 1995 (361). It was identified to be the same gene which was mutated in diabetic *db/db* mouse (362), which had earlier been shown to have a similar phenotype to *ob/ob* mouse, but to be resistant to leptin treatment (363). Also the leptin resistant Zucker *fatty* rat (*fa/fa*) carries a mutation in the *LEPR* gene (364). *LEPR* localises to chromosome 1p31 (365), and the long form has 18 exons (366). LEPR is a single-transmembrane-domain receptor, which displays a structural similarity to the class I cytokine receptor family (367).

The LEPR is produced in several alternatively spliced forms, designated LEPRa – LEPRf, that share the common extracellular domain (>800 aa) and the transmembrane domain (34 aa), and have a variable intracellular domain, characteristic of each of the

isoforms (367). “Short forms” of LEPR may have roles in binding (LEPRe), transport (LEPRa), and clearance (LEPRc and LEPRd) of leptin (368), whereas only LEPRb, the “long form” (1162 aa), encodes all protein motifs capable of activating the janus kinase – signal transduction and transcription (JAK-STAT) pathway, which in turn stimulates transcription of target genes (369).

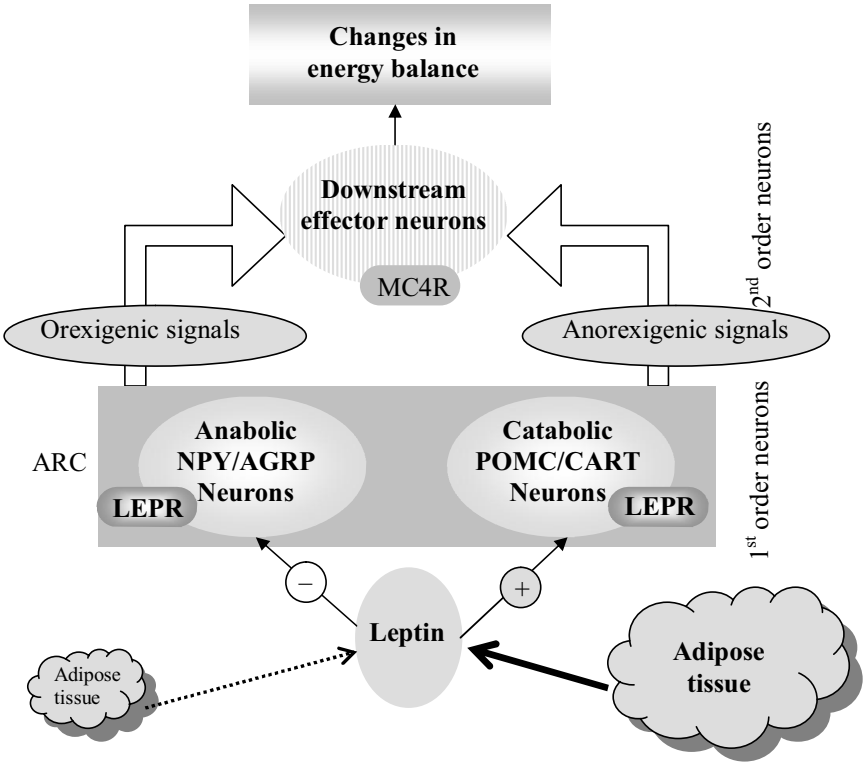



Figure 5. Leptin participates in long-term weight regulation. It is secreted from adipose tissue, circulating at levels that are proportional to body adipose stores, and exerts its effects through the leptin receptor, inhibiting (-) the NPY/AGRP neurons and stimulating (+) the POMC/CART neurons in hypothalamus arcuate nuclei. Activation of the NPY/AGRP has an orexigenic effect, promoting food intake, whereas the POMC/CART neurons have the opposite anorexigenic effect. ARC, arcuate nucleus; AGRP, Agouti-related protein; CART, cocaine and amphetamine regulated transcript; LEPR, leptin receptor; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; POMC, proopiomelanocortin. Modified from Bell et al. 2005 (113) and Crowley 2008 (370).

Within the CNS, the hypothalamus is the main site of leptin action with respect to controlling food intake and EE (371). High levels of the LEPRb are expressed in several

hypothalamic nuclei including the arcuate nucleus, where two neuronal subtypes are critical targets: i) an orexigenic pathway comprising neuropeptide-Y and agouti-related protein -containing neurones and ii) an anorexigenic pathway consisting of proopiomelanocortin and cocaine- and amphetamine-regulated transcript -containing neurones (Figure 5). These neurones subsequently innervate various second order neurone centres where further integration of satiety/adiposity signalling occurs (371).

Leptin resistance is common in obesity, and the mechanisms responsible for this phenomenon may include defects of the leptin receptor, leptin transport, genes involved in leptin signal transduction, or defective transport of leptin across the blood-brain barrier (369). At the level of pancreatic β-cell, leptin resistance may contribute to dysregulation of the adipo-insular axis and promote the development of hyperinsulinaemia and manifest as T2DM in overweight patients (372).

**Genetic variation in leptin receptor**

Mutations in *LEPR* are rare; in a recent study, 300 subjects with hyperphagia and severe early-onset obesity were sequenced for *LEPR* mutations, and only 8 nonsense or missense mutations were found, each resulting in impaired receptor signalling (373). However, the *LEPR* gene is highly polymorphic, and associates with obesity traits in several studies.

A common CTTTA-pentanucleotide *Del/Ins* variant of the 3'-UTR part of the *LEPR* gene is believed to generate an A+U-rich sequence, that should be able to form a stem-loop structure, which may affect mRNA stability in the cell (374). The *Del/Ins* variant has been found to be associated with serum insulin levels (374, 375), serum high density lipoprotein (HDL)-cholesterol and apolipoprotein A (apoA) -I levels (376), and susceptibility to T2DM (375).

A *Lys109Arg* variant in exon 4 in the extracellular domain of the *LEPR* has been associated with glucose homeostasis (377) and insulin response (378), whereas the *Gln223Arg* variant, situated in exon 6 in the extracellular domain of the *LEPR*, has been found to be associated with glucose and insulin homeostasis (378-380), EE (381), body weight, and fat mass (382-386). However, few studies (387-391), one meta-analysis (392) and a pooling analysis of raw data (393) did not find any association of *Lys109Arg* or *Gln223Arg* with BMI or WC. Positive associations of different *LEPR* variants with obesity/ diabetes related traits are listed in the Table 12.

Table 12. Positive associations of *LEPR* gene variants with obesity and diabetes related traits.

| Ref | Subjects (number/ethnicity) | Variant | Risk allele | Association |
|---|---|---|---|---|
| (388) | 10 obese, 10 lean Pima Indian | *A(-36)T* (intron 16)<br>*A(+37)C* (intron 19)<br>*Pro1019Pro (A/G)* | *T*<br>*C*<br>*G* | Higher % body fat<br>Higher % body fat<br>Higher % body fat |
| (374) | 249 obese, 138 lean Finnish Caucasoid | *3'UTR del/ins* | *Del* | Higher serum insulin (n=120) |
| (394) | 12 male twin pairs | *Gln223Arg* | *Gln* | Metabolic abnormalities in response to long-term overfeeding |
| (382) | 319 black, 522 Caucasoid | *Gln223Arg* | *Arg* | Higher adiposity values in Caucasian males |
| (375) | 41 T2DM, 81 NGT, men Caucasoid | *3'UTR del/ins* | *Del* | Higher serum insulin<br>Higher risk of diabetes |
| (385) | 29 obese, 89 lean Greek | *Gln223Arg* | *Arg* | Higher BMI and % body fat |
| (384) | 280 obese women Caucasoid | *Lys109Arg*<br>*Gln223Arg*<br>*Lys656Asn* | *Lys*<br>*Gln*<br>*Asn* | Higher leptin levels<br>Higher abdominal fat<br>Higher hip circumference and SAT |
| (378) | 89 IGT, 269 NGT obese women Caucasoid | *Lys109Arg*<br><br>*Gln223Arg*<br>*Lys656Asn* | *Arg*<br>*Lys*<br>*Gln*<br>*Lys* | Higher glu-AUC (pre-MP, IGT)<br>Higher insulin (post-MP, IGT)<br>Higher insulin (post-MP, IGT)<br>Higher glu, glu-AUC (pre-MP,IGT) |
| (386) | 220 women Caucasoid | *Gln223Arg* | *Gln* | Higher BMI |
| (395) | 192 women | *Lys656Asn* | *Lys* | Lower fat oxidation rate<br>Higher glucose oxidation rate |
| (383) | 336 Brazilian Caucasoid | *Gln223Arg* | *Arg* | Higher BMI among non-smokers |
| (381) | 452 Pima Indian | *Gln223Arg* | *Arg* | Lower 24h EE and physical activity<br>Higher adipocyte cell size |
| (396) | 259 weight gainers, 277 stable weight Dutch Caucasoid | *Lys109Arg*<br>*Gln223Arg* | *Arg*<br>*Arg* | Higher leptin among weight gainers<br>Higher leptin among weight gainers |
| (377) | 143 black, 397 white | *Lys109Arg* | *Lys* | Regular exercise improves glucose homeostasis in *Arg* allele carriers (in whites) |
| (379) | 36 T2DM, 99 IFG, 377 NGT, men Japanese | *Arg223Gln*<br>*Pro1019Pro (A/G)* | *Gln*<br>*A* | Higher insulin and HOMA-IR for subjects with *Arg/Gln* or *Gln/Gln* + *A/A* combination (in NGT) |
| (397) | 1873 Caucasoid | *Lys109Arg*<br>*Lys656Asn*<br>*Pro1019Pro*<br>*Haplotype* | *Lys*<br><br>*GCA* | Higher lean and fat mass<br><br>Association with lean and fat mass |
| (380) | 67 Caucasoid | *Gln223Arg* | *Arg* | Reduction in insulin sensitivity and glucose clearance |
| (376) | 221 men Japanese | *3'UTR del/ins* | *Ins* | Lower HDL-cholesterol and apoA-I levels |
| (398) | 770 IGT Multicentre study | *3'UTR del/ins* | *Del* | Lower weight and WC reduction |

| | | | | |
|---|---|---|---|---|
| (399) | 775 T2DM, 688 NGT Asian | *Lys109Arg* | *Lys* | Higher BMI |

Ref, reference; T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; BMI, body mass index; SAT, subcutaneous adipose tissue; IGT, impaired glucose tolerance; glu, glucose; AUC, area under curve; MP, menopausal; EE, energy expenditure; IFG, impaired fasting glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HDL, high density lipoprotein; apoA, apolipoprotein A; WC, waist circumference.

## 2.6. Transcriptomics of adipose tissue

DNA microarrays provide information on mRNA expression from thousands of genes in tissues of interest simultaneously (113). The collection of genes that are expressed or transcribed from genomic DNA, the transcriptome, is a major determinant of cellular phenotype and function of each tissue (400). Differences in gene expression are responsible for morphological and phenotypic differences, and cellular responses to environmentary stimuli (400). The enormous quantity of data generated from each experiment can be analysed to search pathways or gene clusters that respond to experimentary conditions (401).

The effect of *agouti* ($A^y$) and *obese* ($Lep^{ob}$) mutation on the gene expression profile of AT has been studied using microarrays (402). Over one thousand transcripts were significantly correlated with body mass, with a large proportion of these genes encoding proteins which are characteristic of macrophages. The results suggested, and histological data from both mice and humans confirmed, that the macrophage content of AT correlated with BMI and adipocyte size (402). More recently, in abdominal SAT from Pima Indians, most inflammation-related genes were upregulated in adipocytes of obese subjects, emphasizing the active role of mature adipocytes in obesity-related inflammation (403).

In a comparison of omental AT gene expression of obese and lean subjects, 89 genes were upregulated and 64 genes were downregulated in obese patients (404). The downregulated genes were mostly lipolysis inducer genes and growth factor genes, whereas the upregulated genes encoded mitogen-activated protein kinases, perhaps pointing to an attempt to restrain adipocyte proliferation and differentiation. Downregulation of genes normally associated with adipocyte differentiation was also shown in mice (405, 406), suggesting that the adipocytes are engaged in a dedifferentiation process that could be a consequence of their enlargement induced by obesity.

Effects of short-term weight reduction on AT gene expression profile have been studied in humans. In one study, the effects of different hypocaloric diets on SAT gene

expression were studied in obese women (407), revealing that weight loss of ≈7.5% increased the expression of 52 genes whereas expression of 44 genes was decreased. Most changes after a 10 week diet were modest (<25% of baseline), but all genes regulating the formation of polyunsaturated FAs from acetyl-CoA and malonyl-CoA were markedly down-regulated (30-60% decrease) (407). Weight reduction has been shown to regulate the expression of inflammation-related genes in SAT of obese subjects. Consumption of a VLCD for 28 days was shown to improve the inflammatory profile by decreasing the levels of proinflammatory factors and increasing those of anti-inflammatory molecules in SAT (408). The genes were mostly expressed in the stromavascular fraction, which is known to contain macrophages. Long-term caloric restriction in mice resulted in 345 differentially expressed genes (409, 410). Down-regulated genes were classified as being involved in the cytoskeleton, extracellular matrix (ECM), inflammation, or angiogenesis. Dietary restriction has been shown to extend the life span and retard many age-related changes in laboratory rodents. A comparison of altered genes showed that certain functions, such as metabolism, energy metabolism, stress and immune response, cell growth, and transcription regulation were shared across species (411).

Genomic examination of AT has provided a wealth of information about changes in gene expression in obesity and diabetes. It has been hypothesised that lack of lipogenic adipocytes, whether due to extreme leanness (lipodystrophy) (412) or extreme obesity (*ob/ob* mice), promotes diabetes due to an increase in the lipogenic burden experienced by tissues other than adipose tissue. If the liver is capable of handling the burden, normoglycaemic obesity is achieved; otherwise diabetes will be the consequence (401).

# 3. AIMS OF THE STUDY

The present study was undertaken to investigate the association of selected candidate genes with body weight and body weight changes and the incidence of diabetes in the Finnish Diabetes Prevention Study (DPS). In addition, new putative candidate genes were sought by examining the transcriptome of WAT in response to a weight reduction intervention (Genobin) study. The specific aims of this study were:

1. To study the association of genetic variation in the *B2AR, B3AR,* and *UCP1* genes with the incidence of T2DM and body weight (Study I)
2. To evaluate the association of genetic variation in the *LEPR* gene with the incidence of T2DM and body weight (Study II)
3. To examine the association of genetic variation in the *UCP2* and *UCP3* genes with the incidence of T2DM, body weight and serum cholesterol levels (Study III)
4. To determine the effect of weight loss on the gene expression profile of AT in subjects with features of MetS (Study IV)

# 4. SUBJECTS AND METHODS

## *4.1. Subjects and study designs*

### 4.1.1. The Finnish Diabetes Prevention Study (Studies I-III)

DPS is a randomised, controlled, multicenter study carried out in Finland in 1993-2000 (98, 109, 413). The study subjects were recruited through various methods with a special emphasis on the high-risk groups such as obese subjects and first-degree relatives of T2DM patients (109). The main inclusion criteria were as follows: BMI >25 kg/m$^2$, age 40 to 64 years, IGT based on the mean values of two OGTT measurements. Altogether 522 individuals with IGT were randomised into either a control group or an intensive, individualised diet and exercise intervention group in five outpatient clinics (Helsinki, Kuopio, Oulu, Tampere, and Turku). Randomisation to the intervention and control groups was stratified according to the clinic, sex, and the mean plasma glucose concentration two hours after an oral glucose load (7.8-9.4 or 9.5-11.0 mmol/l). Baseline characteristics of the study subjects are presented in Table 13. The subjects were excluded if they had a previous diagnosis of diabetes other than gestational diabetes. Other exclusion criteria were vigourous exercise, active glucose-lowering treatment other than routine dietary and health advice, and chronic diseases (413).

The individuals in the intervention group received detailed and individually tailored counselling on lifestyle, diet and exercise. The goals were (98):

-reduction in weight of ≥5%
-reduction in total intake of fat to <30% of daily energy intake
-reduction in the intake of saturated fat to <10% of daily energy intake
-increment in fibre intake at least to 15 grams per 1000 kcal
-moderate exercise for at least 30 minutes per day

Each individual in the intervention group had seven individual sessions with a clinical nutritionist during the first year of the study and then one session every three months. The intervention group also received individual guidance on increasing the level of physical activity. The control group received general advice about healthy food and the importance of weight reduction and increased physical activity (98). The primary outcome measure was the development of diabetes, and all the study subjects had an OGTT at each annual visit. If the first OGTT showed diabetic values, the diagnosis was confirmed by another OGTT done at least 1 week after the first one (413). The subject continued in the study if his/her second OGTT showed non-diabetic values. A medical

history was taken and a physical examination done at baseline and at each annual follow-up visit.

### 4.1.2. The Genobin Study (Study IV)

Originally, 75 overweight or obese (BMI 28-40 kg/m$^2$) subjects aged 40 to 70 years were recruited to the study from the Kuopio area in 2004. Volunteers were recruited by newspaper announcements and by inquiries made to subjects who had previously participated in other studies arranged by the Department of Clinical Nutrition and the Research Institute of Exercise Medicine. The subjects had IFG (FPG 5.6-7.0 mmol/l) or IGT (2-h plasma glucose 7.8-11.0 mmol/l) and at least two other features of MetS according to the ATPIII criteria (58) as modified by American Heart Association (414): WC >102/88 cm (males/females), fasting serum TG concentration ≥1.7 mmol/l, fasting serum HDL-cholesterol <1.0/1.3 mmol/l (males/females), BP ≥130/80 mm Hg.

Subjects were randomised into one of the following groups: a weight reduction (WR) group (n=28), aerobic exercise training group (n=15), resistance exercise training group (n=14) or a control group (n=18). Subjects were matched for age, gender and the status of glucose metabolism. In addition, 11 normal-weight subjects (mean age 48±9 years, mean BMI 23.7±1.9 kg/m$^2$) were recruited. For Study IV, only the WR group and the control group were included. Baseline characteristics of the study subjects are presented in Table 13.

At screening, the health status and the medical history of the subjects were examined by interview, and the liver, kidney and thyroid function were examined. OGTT, FSIGT, AT biopsy, BP measurement, anthropometric measurements, and biochemical measurements for serum total and lipoprotein lipids, serum FFA, and leptin were done at baseline and after the intervention period. The biochemical, anthropometric and BP measurements were performed also at the 12 weeks' time point.

The duration of the study varied between 32 and 38 weeks, the mean duration being 33.3 ± 1.1 weeks. The WR group had a 12-week intensive WR period. The aim for the weeks 12-33 was to maintain the achieved weight loss. A clinical nutritionist provided individual instructions to decrease the energy intake level based on an interview and 4-day food records kept by the subjects at baseline. The subjects kept 4-day food records twice during the intensive period and two times during weeks 20 to 33. Subjects were asked to maintain their habitual level of physical exercise. Physical activity was monitored by a questionnaire at the beginning and for weeks 12 and 33 of the study.

The control group subjects were advised to continue their normal lifestyle during the study. They were given general advice for recommended diet and regular physical activity according to current recommendations.

Table 13. Baseline characteristics of the subjects in Studies I-III and in Study IV.

| | DPS | | Genobin | |
|---|---|---|---|---|
| | **Intervention group** | **Control group** | **Weight reduction group** | **Control group** |
| N | 265 | 257 | 28 | 18 |
| Gender (M/F) | 91/174 | 81/176 | 12/16 | 8/10 |
| Age (years) | 55±7 | 55±7 | 59±7 | 61±7 |
| Weight (kg) | 86.7±14.0 | 85.5±14.4 | 92.8±15.1 | 87.9±8.3 |
| BMI (kg/m$^2$) | 31.3±4.6 | 31.0±4.5 | 32.9±3.2 | 32.4±2.5 |
| Waist circumference (cm) | 102±11 | 101±11 | 108±9 | 105±7 |
| Fasting plasma glucose (mmol/l) | 6.1±0.8 | 6.2±0.7 | 6.4±0.5 | 6.5±0.4 |
| 2-h plasma glucose (mmol/l) | 8.9±1.5 | 8.9±1.5 | 6.9±2.0 | 8.0±2.4 |
| Serum cholesterol (mmol/l) | 5.6±1.0 | 5.6±0.9 | 5.2±1.0 | 5.5±1.1 |
| Serum LDL-cholesterol (mmol/l) | 3.6±0.8 | 3.6±0.8 | 3.4±0.9 | 3.6±0.9 |
| Serum HDL-cholesterol (mmol/l) | 1.2±0.3 | 1.2±0.3 | 1.2±0.2 | 1.3±0.2 |
| Serum triglycerides (mmol/l) | 1.7±0.8 | 1.7±0.8 | 1.5±0.6 | 1.8±0.6 |
| Blood pressure (mm Hg) | | | | |
| Systolic | 140±18 | 136±17 * | 137±17 | 137±12 |
| Diastolic | 86±9 | 86±10 | 90±10 | 87±10 |

Data are mean ± SD. * $P$=0.03 for the comparison with the intervention group by two-tailed t-test. DPS, Finnish Diabetes Prevention Study; M, male; F, female; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein. Modified from Tuomilehto et al. 2001 (98), Lindström et al. 2003 (415), and Kolehmainen et al. 2007 (416).

## *4.2. Methods*

### 4.2.1. Anthropometric measurements

Weight and height were measured in light clothing, and BMI was calculated as kg/m$^2$. Waist and hip circumference were measured as described (109), and WHR (not included in Study IV) was calculated. In Studies I-III also the sagittal and transverse diameters were measured as described (109). The long-term weight change was calculated as: [(weight$_{\text{end of follow-up}}$ − weight$_{\text{baseline}}$)/ weight$_{\text{baseline}}$] x 100%. In Study IV, the body composition was analysed by bioelectrical impedance (STA/BIA Body Composition Analyser, Akern Bioresearch Srl, Firenze, Italy).

### 4.2.2. Glucose tolerance tests

A 2-h OGTT was performed after consumption of 75 g of glucose. Samples for glucose and insulin were taken at fasting and 120 min. The FSIGT in study IV was performed by administrating 300 mg/kg body weight glucose as 50% solution through a catheter inserted into an antecubital vein. During the first 10 minutes, blood samples were drawn every two minutes and immediately before (19 min) the 20 min insulin bolus (0.03 U/kg body weight). Plasma glucose and serum insulin concentrations were followed for 180 min, with altogether 25 blood samples being collected. The results for insulin Si, Sg and AIR were derived using the MINMOD Millennium software (417).

### 4.2.3. Biochemical analyses

Plasma glucose was measured at each centre by standard methods in Studies I-III and by hexokinase method (Thermo Clinical Labsystems, Vantaa, Finland) in Study IV. The serum insulin concentration was measured in a central laboratory by a radioimmunoassay method (Pharmacia, Uppsala, Sweden) in Studies I-III and by the chemiluminescence sandwich method (ACS, Bayer A/S, Tarrytown, NY, USA) in Study IV. Very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL were separated by ultracentrifugation (Beckman Optima L-90K, Beckman Coulter, Inc., Fullerton, CA, USA), and the concentrations of total and lipoprotein cholesterol and total TG were analysed by enzymatic methods (Roche Diagnostics, Mannheim, Germany) with Kone Pro Clinical Chemistry Analyser (Thermo Clinical Labsystems, Konelab, Espoo, Finland) (Study IV). Serum total cholesterol, HDL-cholesterol and TG were determined using an enzymatic CHOD-PAP method (Monotest, Boehringer Mannheim GmbH, Mannheim, Germany) in Studies I-III. The Friedewald formula (418) was used to calculate the concentration of LDL-cholesterol. A commercial radioimmunoassay kit was used for the analysis of serum leptin concentration (Linco Research Inc., St Louis, MO, USA) in Study IV.

HOMA-IR was calculated in Studies I-III using the following formula: fasting plasma glucose (mmol/l) x fasting serum insulin (mU/l)/22.5 (419). Homeostasis model assessment for insulin secretion (HOMA-IS) was calculated in Studies I-III as 20 x fasting serum insulin (mU/l)/(fasting plasma glucose [mmol/l] – 3.5) (419).

### 4.2.4. Adipose tissue biopsy (Study IV)

AT samples were drawn by syringe from abdominal SAT under local anesthesia (lidocaine 10 mg/ml, without adrenaline), after an overnight fast, to collect 0,5-5 g of AT. AT samples for the mRNA expression studies were washed twice with phosphate

buffered saline (PBS, Invitrogen, Carlsbad, California, USA) and stored in RNAlater (Ambion, Austin, TX, USA) for 24 h at 4°C. RNAlater was removed and tissues were stored at -80°C until used for RNA extraction.

Part of the AT sample was taken for the cell size measurement. These samples were immediately placed in PBS and the isolation procedure was started within 30 min. Adipocytes were isolated by the modification of Ohisalo et al. (420) of the method of Rodbell (421) in the presence of collagenase (0.5 mg/ml) with constant shaking in water bath at 37°C, and median adipose cell diameter was estimated by direct microscopy (Olympus CH-2, Olympus, Tokyo, Japan) of isolated cells.

## 4.2.5. Genetics and transcriptomics

### 4.2.5.1. DNA extraction and genotyping (Studies I-III)

In study I, DNA was available from 490 individuals (161 men and 329 women). Their mean BMI was 31.3 ± 4.6 kg/m$^2$ and age 55.3 ± 7.1 years. In studies II and III, DNA was available from 507 individuals (166 men and 341 women). Their mean BMI was 31.2 ± 4.5 kg/m$^2$ and age 55.3 ± 7.1 years.

Genomic DNA in Studies I-III was prepared from peripheral blood leucocytes by the Puregene® DNA Purification Kit (Gentra Systems, Inc. Minneapolis, USA), which is based on alcohol and salt precipitation. Variants in *B2AR, B3AR, UCP1, UCP2, UCP3 (rs1800849),* and *LEPR* genes were genotyped by PCR-RFLP methods, which are presented in Table 14. Variants *rs653529*, *rs15763, rs1726745, rs3781907 and rs11235972* of *UCP2* were genotyped by using the custom Golden Gate genotyping reagents and consumables (Illumina Inc, San Diego, CA, USA). Due to technical problems, only 501 (*rs1726745*) or 502 (*rs653529, rs15763, rs3781907, rs11235972*) subjects could be genotyped by Illumina.

### 4.2.5.2. RNA extraction (Study IV)

Total RNA from AT was extracted by using the TRIzol reagent (1 ml/100 mg tissue) (Invitrogen, Carlsbad, CA, USA) followed by further purification with RNeasy Mini Kit columns (Qiagen, Valencia, CA, USA) according to the instructions provided by the manufacturers. The RNA concentration and the $A_{260}/A_{280}$ ratio was measured using NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), the acceptable ratio being 1.9-2.1. Integrity of the RNA was assessed using agarose gel electrophoresis.

Table 14. PCR-RFLP methods used for genotyping of variants in the *B2AR, B3AR, UCP1, UCP2, UCP3,* and *LEPR* genes.

| **Gene**/ variant | Primer (5' to 3') | PCR product size (bp) | Annealing temperature (ºC) | Digestion enzyme | Size of digestion products (bp) | Electrophoresis gel |
|---|---|---|---|---|---|---|
| ***B2AR*** | | | | | | |
| *Gln27Glu* | F GCCCCTAGCACCCGACAAGC<br>R GCCGTGACGCACAGCACATC | 524 | 66 | Fnu4HI | 250/ 196 | 2% agarose |
| ***B3AR*** | | | | | | |
| *Trp64Arg* | F CGCCCAATACCGCCAACAC<br>R CCACCAGGAGTCCCATCACC | 210 | 63 | BstNI | 158/ 97/ 61 | 2% agarose |
| ***UCP1*** | | | | | | |
| *A(-3826)G* | F CCAGTGGTGGCTAATGAGAGAA<br>R GCACAAAGAAGAAGCAGAGAGG | 278 | 60 | BclI | 157/ 121 | 3% agarose |
| ***UCP2*** | | | | | | |
| *G(-866)A* | F CACGCTGCTTCTGCCAGGAC<br>R AGGCGTCAGGAGATGGACCG | 363 | 65 | MluI | 291/ 72 | 2% agarose |
| *Ala55Val* | F GGGAGTCTTGATGGTGTCTAC<br>R ATCACACCGCGGTACTGGGCGTTG | 201 | 56 | HincII | 178/ 23 | 3% agarose |
| *3'UTR Del/Ins* | F GGCTCCCTTCTGAGCCTC<br>R CTTTCCAAGGGACGGGAC | 272/ 317 | 57 | - | - | 2% agarose |
| ***UCP3*** | | | | | | |
| *C(-55)T* | F GGATAAGGTTTCAGGTCAGGC<br>R AAGGGATGAGGGAGGAGAAA | 194 | 62 | Hae III | 110/ 64/ 20 | 9% polyacrylamide |
| ***LEPR*** | | | | | | |
| *Lys109Arg* | F TATCCAATTACTCCTTGGAG<br>R AAACTAAAGAATTTACTGTTGAAACAAATGGC | 283 | 56 | BsuRI | 252/ 31 | 2.5% agarose |
| *Gln223Arg* | F TGTTATTCCTGATGATTAACC<br>R AGAAGCCACTCTTAATACCC | 554 | 57 | MspI | 364/ 190 | 2% agarose |
| *3'UTR Del/Ins* | F ATAATGGGTAATATAAAGTGTAATAGAGTA<br>R AGAGAACAAACAGACAACATT | 114/ 119 | 55 | RsaI | 114/ 90 | 9% polyacrylamide |

#### 4.2.5.3. Probe preparation for microarray (Study IV)

RNA samples from ten subjects from both the WR and control groups were chosen for microarray analyses based on their success in the intervention (weight loss ≥5% in the WR group and weight unchanged in the control group) and the purity of the tissue (avoidance of blood contamination) and RNA. One microarray chip for each tissue sample taken at both baseline and at the 33 weeks' timepoint from the same individual was used for hybridization. Due to the technical problems with one microarray chip, the microarray data from one subject had to be excluded. Thus, the final number of subjects in the WR group was nine.

Synthesis of biotin labelled complementary RNA (cRNA), hybridization to DNA microarrays (Affymetrix HG-U133 Plus 2.0 GeneChip) and detection of hybridised cRNA were performed as recommended by the manufacturer (Affymetrix Inc., Santa Clara, CA, USA). Briefly, 2 µg of total RNA was used to generate double-stranded cDNA by reverse transcription using the One-cycle cDNA Synthesis Kit. Labelled cRNA was prepared from the double-stranded cDNA by in vitro transcription using the IVT Kit. Biotinylated cRNA was fragmented and added to the hybridization cocktail. Two hundred µl of this cocktail was used for hybridization of HG-U133 Plus 2.0 array at 45°C for 16 hours in a hybridization oven at 60 rpm, followed by washings using the GeneChip Fluidics Station 400. The arrays were stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA), incubated with biotinylated anti-streptavidin IgG (Vector Laboratories, Burlingame, CA, USA) and stained again with streptavidin-phycoerythrin.

#### 4.2.5.4. Quantitative real-time PCR (Study IV)

Quantitative real-time PCR (QPCR) was used to confirm the microarray gene expression results. QPCR analyses were performed with TaqMan chemistry based assays according to the instructions provided by the manufacturer in the ABI Prism 7500 analyser (Applied Biosystems, Carlsbad, CA, USA). The analysis for the relative quantity of a specific gene before and after the intervention in 27 subjects of the WR group and in 18 subjects of the control group was analysed in triplets. A standard curve with the points of 0.025, 0.075, 0.3, 0.9, 1.8 ng/µl of cDNA, respectively, and calibrator at a concentration of 0.3 ng/µl were used on every plate. The relative quantity was analysed using ABI Prism 7500 SDS software. Quantities on each plate were first corrected by the calibrator on the plate. The ratio of the amount per plate to the corresponding values of endogenous control was then calculated. The endogenous

control was chosen using Human Endogenous Control Kit (Applied Biosystems). With respect to the 11 possible candidates, cyclophilin A1 was the best choice for human AT.

## 4.2.6. Statistical analyses

### 4.2.6.1. Statistical analyses of the clinical data

The data were analysed using the SPSS/WIN program versions 11.0, 11.5, and 14.0 (SPSS, Chigago, IL, USA). The normality of distributions of study variables was tested with the Kolmogorov-Smirnov test with Lilliefors' correction, and appropriate transformation was used when necessary. For variables with a skewed distribution, Kruskal-Wallis or Mann-Whitney test was used to compare means among groups. Univariate analysis of variance was used to compare the effect of the gene variants on continuous variables (Studies I-III), or to test the difference in the baseline characteristics and in the changes among the groups (Study IV). Adjustment for age, gender and baseline BMI/weight was done, when appropriate. In addition, serum lipid concentrations were adjusted for the use of cholesterol lowering medication (Study III). Paired samples T-test was used for comparing the baseline and endpoint measurements within the study group (Study IV). Chi square test was used in the comparison of categorical variables (Studies I-III). Correlation analyses were done using Pearson's method (Study IV). Partial correlation analysis with adjustment for baseline weight and gender was used when appropriate (Study IV). The relative changes in parameters were calculated as follows: $[(\text{parameter} - \text{parameter}_{\text{baseline}})/ \text{parameter}_{\text{baseline}}] \times 100\%$ (Studies I-III). Longitudinal changes were examined by general linear model (GLM) for repeated measures. Homogeneity of variances was tested using Levene's test. Logistic regression (Studies I-II) or Cox regression (Study III) analysis was performed to evaluate if the gene variants predict the development of T2DM. Adjustment was made for the study group, baseline weight, weight change (from baseline to 3-year measurement or to the last measurement) and baseline fasting plasma glucose.

Haplotype frequencies in Study II were estimated by using the EH programme. Linkage disequilibrium (LD) statistics were calculated by using the expectation maximization algorithm (2LD programme) (Study II) or by Haploview software (422) (Study III). Haplotype analysis (including haplotype frequencies) in Study III was done by THESIAS 3.1 (http://ecgene.net/genecanvas), which is based on the stochastic-EM algorithm (423). Haplotype analyses of the quantitative variables were adjusted for age, gender and BMI, when appropriate. The survival analysis for haplotypes was adjusted

for the study group, baseline weight, weight change and baseline fasting plasma glucose.

A $p$-value <0.05 was considered statistically significant. Correction for multiple hypothesis testing in Study III was performed with the false discovery rate (FDR) using Q-value 1.0 software. $\pi_0$ was estimated with bootstrap method (424) using $\lambda$ range from 0 to 0.9 by 0.05. Due to the distribution of $p$-values, the $\lambda$ was set to 0 for correcting the results of Cox regression. In the text, $q$ stands for FDR, and is reported for each $p$<0.05 and should be interpreted as the minimum FDR that is incurred when calling that test significant. Data are given as means ± SD, unless otherwise indicated.

#### 4.2.6.2. Array data extraction and analysis (Study IV)

The arrays were scanned using HP GeneArray Scanner 3000 (Affymetrix Inc.). Primary data extraction was performed with Affymetrix GeneChip Operating Software. The software produces a one-cell intensity file that contains probe-level intensities for each chip. The detection calls were calculated using the Affymetrix detection algorithm (Affymetrix, Statistical algorithms description document, 2002). Microarray data analysis was performed with dChip (www.dchip.org) software. All chips were normalised by using Invariant Set Normalization (425). Model based expression indexes (MBEI) (426) were calculated to summarise expression levels. A perfect match/mis-match difference model was used in the MBEI calculation, and outlier detection and correction was applied. After preprocessing steps, only genes that were called 'Present' in more than 50 percent of the replicates in at least one of the two time points were selected for further analysis. Differentially expressed genes were identified by using paired $t$-test with $p$<0.01 producing slightly different FDR values for the WR and control group. Differential expression of genes was examined within the groups. The set of differentially expressed genes was clustered with dChip's hierarchical clustering function. Before clustering, redundant probe sets were removed. Correlation was used as distance metric and the centroid linkage method was applied. Finally, the differentially expressed genes were grouped by their Gene Ontology (GO) annotations (version update 3/2007).

### 4.2.7. Approvals of the Ethics Committees

The study procedures were approved by the Ethics Committee of the National Public Health Institute in Helsinki, Finland (Studies I-III), and the Ethics Committee of the District Hospital Region of Northern Savo and Kuopio University Hospital (Study IV). All participants gave their written informed consent.

# 5. RESULTS

## 5.1. Association studies (Studies I-III)

### 5.1.1. Genotype frequencies

Genotype and minor allele frequencies of the variants in *UCP1, UCP2, UCP3, B2AR, B3AR,* and *LEPR* genes studied are presented in Table 15, together with the minor allele frequencies in the reference population CEU (Utah residents with ancestry from Northern and Western Europe) of the HapMap database (116). All the frequencies were consistent with the Hardy-Weinberg equilibrium, and did not differ between the study groups. With respect to *B2AR* and *B3AR,* we analysed combinations of genotypes; their frequencies are shown in Table 16. The three variants in *LEPR* were in strong LD (Table 17), as well as the variants in the *UCP2-UCP3* gene cluster (Study III: Table 1).

Table 15. Genotype and allele frequencies of the variants studied in DPS.

| **Gene**/ variant | Rs number | Genotype frequencies (%) [a] | | | Minor allele frequency | Minor allele frequency (n) in ref. population [b] |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | | |
| ***B2AR*** | | | | | | |
| *Gln27Glu* | *rs1042714* | 37 | 47 | 16 | 0.40 | 0.47 (120) |
| ***B3AR*** | | | | | | |
| *Trp64Arg* | *rs4994* | 86 | 13 | 1 | 0.08 | 0.21 (184) |
| ***UCP1*** | | | | | | |
| *A(-3826)G* | *rs1800661* | 60 | 35 | 4 | 0.22 | 0.08 (116) |
| ***UCP2*** | | | | | | |
| *G(-866)A* | *rs659366* | 36 | 47 | 17 | 0.40 | 0.33 (120) |
| *Ala55Val* | *rs660339* | 30 | 46 | 24 | 0.47 | 0.37 (120) |
| *3'UTR Del/Ins* | - | 50 | 41 | 9 | 0.30 | - |
| ***UCP3*** | | | | | | |
| *C(-55)T* | *rs1800849* | 38 | 49 | 13 | 0.38 | 0.19 (120) |
| *GA* | *rs11235972* | 39 | 48 | 13 | 0.37 | 0.18 (120) |
| *AG* | *rs3781907* | 44 | 46 | 10 | 0.33 | 0.28 (120) |
| *GA* | *rs1726745* | 36 | 47 | 17 | 0.40 | 0.48 (100) |
| *GA* | *rs15763* | 62 | 33 | 5 | 0.22 | 0.27 (116) |
| *AG* | *rs653529* | 35 | 47 | 18 | 0.42 | 0.37 (120) |
| ***LEPR*** | | | | | | |
| *Lys109Arg* | *rs1137100* | 41 | 46 | 13 | 0.36 | 0.34 (120) |
| *Gln223Arg* | *rs1137101* | 19 | 47 | 34 | 0.57 | 0.55 (120) |
| *3'UTR Del/Ins* | - | 70 | 28 | 2 | 0.16 | - |

[a] 0, homogyzous for the major allele; 1, heterozygous; 2, homozygous for the minor allele

[b] Reference population CEU (Utah residents with ancestry from Northern and Western Europe), representing one of the populations studied in the International HapMap project. n= number of alleles studied.

Table 16.Distribution of genotypes defined by the *Gln27Glu* variant of the *B2AR* gene and the *Trp64Arg* variant of the *B3AR* gene.

| n (%) | *Trp64Arg / B3AR* | | | |
|---|---|---|---|---|
| | *Trp64Trp* | *Trp64Arg* | *Arg64Arg* | Total |
| *Gln27Glu / B2AR* | | | | |
| *Gln27Gln* | 155 (31.6) | 22 (4.5) | 2 (0.4) | 179 (36.5) |
| *Gln27Glu* | 196 (40.0) | 33 (6.7) | 2 (0.4) | 231 (47.1) |
| *Glu27Glu* | 69 (14.1) | 10 (2.0) | 1 (0.2) | 80 (16.3) |
| Total | 420 (85.7) | 65 (13.3) | 5 (1.0) | 490 |

The grey area represents the protective genotype combination for T2DM.
n= number of subjects

Table 17. Pairwise linkage disequilibrium between the variants in the *LEPR* gene is shown as D' and $r^2$ values.

| | | D' | | |
|---|---|---|---|---|
| | variant | *Lys109Arg* | *Gln223Arg* | *3'UTR Del/Ins* |
| | *Lys109Arg* | - | 0.98 | 0.99 |
| $r^2$ | *Gln223Arg* | 0.40 | - | 0.79 |
| | *3'UTR Del/Ins* | 0.11 | 0.16 | - |

The *rs*-numbering of genetic variants became common during this work. However, in order to prevent confusion with the original studies where *rs*-numbering was not used (Studies I-II), *rs*-numbering is used only in Study III in this work. Furthermore, it should be noted that deletion-insertion polymorphisms do not have *rs*-numbers.

We were able to analyse haplotypes for *UCP2, UCP3* and *LEPR* genes, because several variants were studied in these genes. For *LEPR*, six haplotypes were observed (Study II: Table 1) with the four most common haplotypes accounting for 98% of all observed haplotypes. The most common haplotype (frequency 0.36) included the *Arg109, Arg223, and 3'UTR Del* alleles of *LEPR*.

For combined *UCP2* and *UCP3* gene cluster, three haploblocks were formed based on solid spine of LD; block 1 (*DelIns* and *rs660339* of the *UCP2* gene), block 2 (*rs659366, rs653529, rs15763* and *rs1726745* of the *UCP2* and *UCP3* genes), and block 3 (*rs3781907, rs11235972* and *rs1800849* of the *UCP3* gene) (Study III: Figure 1, Table 4). Haplotype analysis was performed separately for blocks 1, 2 and 3. Only the haplotypes with frequency ≥0.05 were included in the analysis; thus, block 1 consisted 3 haplotypes (DelC, InsT, DelT), block 2 consisted 4 haplotypes (GAGA, GAGG, AGAG, AGGG) and block 3 consisted 3 different haplotypes (AGC, GAT, AAT).

### 5.1.2. Associations with anthropometric measurements

Body weight differed in longitudinal analysis according to genotypes of the *UCP1 A(-3826)G* and *LEPR 3'UTR Del/Ins* variants. The *G(-3826)G* homozygotes of the *UCP1* and the *3'UTR Del/Del* homozygotes of the *LEPR* had the highest weight during the 3-year follow-up ($p$=0.029 and 0.020, respectively) (Study I: Figure 1). When the study groups were analysed separately, the trend was significant in the intervention group (p=0.046) and nonsignificant in the control group (Figure 6). No significant associations were seen when the absolute (kg) or relative (%) body weight changes were studied among the different genotypes (Study II: Table 3).

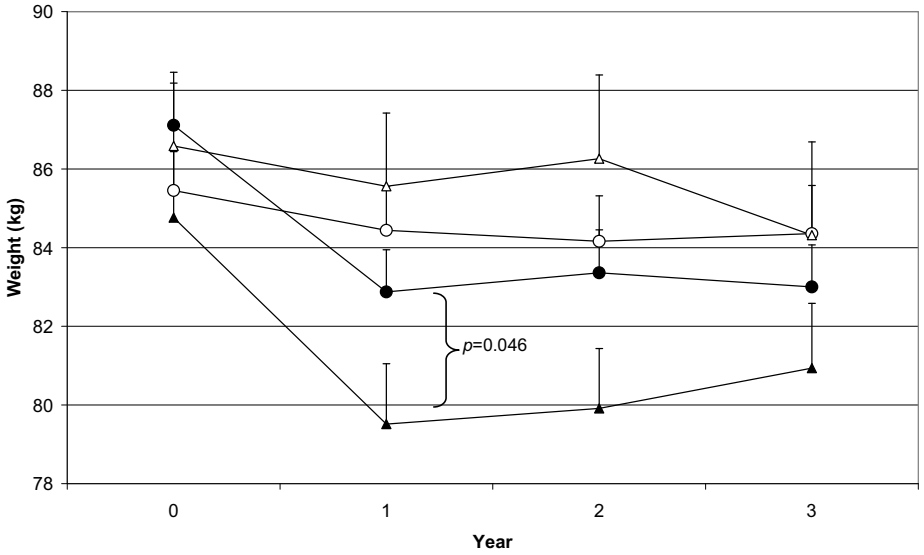



Figure 6. Changes in weight (kg) during the 3-year follow-up according to the *3'UTR Del/Ins* variant of the *LEPR* gene. Data are mean + SEM. Black figures, intervention group; white figures, control group; circles, *Del/Del* genotype; triangles, *Ins* allele.

For the *UCP2* and *UCP3* genes, four variants in the haploblock 2 associated with indices of abdominal obesity: WC, waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR). *Rs659366, rs653529, rs15763* and *rs1726745* associated with WHR at baseline ($p$=0.048, 0.009, 0.018 and 0.031, respectively), as subjects with *rs659366-AA, rs653529-GG*, *rs15763-AA* and *rs1726745-GG* genotypes had the lowest WHR (Study III: Table 2). Similar, although less consistent, associations were seen between these variants and WC and WHtR. WC was associated with *rs659366, rs653529* and *rs1726745*, whereas WHtR was associated with *rs653529, rs15763* and *rs1726745*

(Study III: Table 2). Also the *DelIns* variation in *UCP2* associated with WHR, with subjects with the *DelDel* genotype having the highest WHR at baseline ($p$=0.017).

Longitudinal analysis of WHR showed that subjects with *rs653529-GG*, *rs15763-AA* and *rs1726745-GG* genotypes had the lowest WHR throughout the years 0-3 ($p$=0.025, 0.039 and 0.035, respectively) (Study III: Figure 2). Longitudinal analyses of WC and WHtR showed similar results, WC associating with *rs15763* ($p$=0.030) and WHtR associating with *rs15763* and *rs1726745* ($p$=0.009 and 0.040, respectively).

### 5.1.3. Associations with glucose and insulin metabolism

For the *UCP2* gene, fasting and 2-h plasma glucose levels differed according to genotypes of *rs660339* ($p$=0.006 and 0.025, respectively) and *rs659366* ($p$=0.007 and 0.025, respectively). Specifically, the *rs660339-CC* and *rs659366-AA* homozygotes had the highest fasting glucose concentrations and the *rs660339-CC* and *rs659366-GG* homozygotes had the highest 2-h plasma glucose concentrations. In addition, *rs653529* associated with 2-h plasma glucose level, *AA* homozygotes having the highest value ($p$=0.028). However, none of the associations with glucose was dependent on the allele dosage.

With respect to the *B2AR* gene, there were differences in fasting and 2-h serum insulin levels among the genotypes ($p$=0.018 and 0.009, respectively). The *Gln27Glu* heterozygotes had the highest values, and the *Glu27Glu* homozygotes had the lowest values; thus, the associations were not dependent on the allele dosage.

Insulin secretion was studied by HOMA-IS. No differences at baseline or at 3-year were seen among the genotypes. However, the 3-year change in HOMA-IS (unadjusted) was associated similarly with three *UCP2* variants (*DelIns, rs660339, rs659366)*, the intergenic region variant *rs653529* and *UCP3* variant *rs15763*, showing increased values for wild-type subjects, intermediate for heterozygous and decreased values for homozygous subjects ($p$=0.004, 0.016, 0.037, 0.018 and 0.016, respectively) (Figure 7). When adjusted by age, gender and baseline BMI, the results remained significant for *DelIns* and *rs660339*, whereas they were no longer significant for *rs659366, rs653529* and *rs15763* ($p$=0.047, 0.029, 0.095, 0.098 and 0.168, respectively).

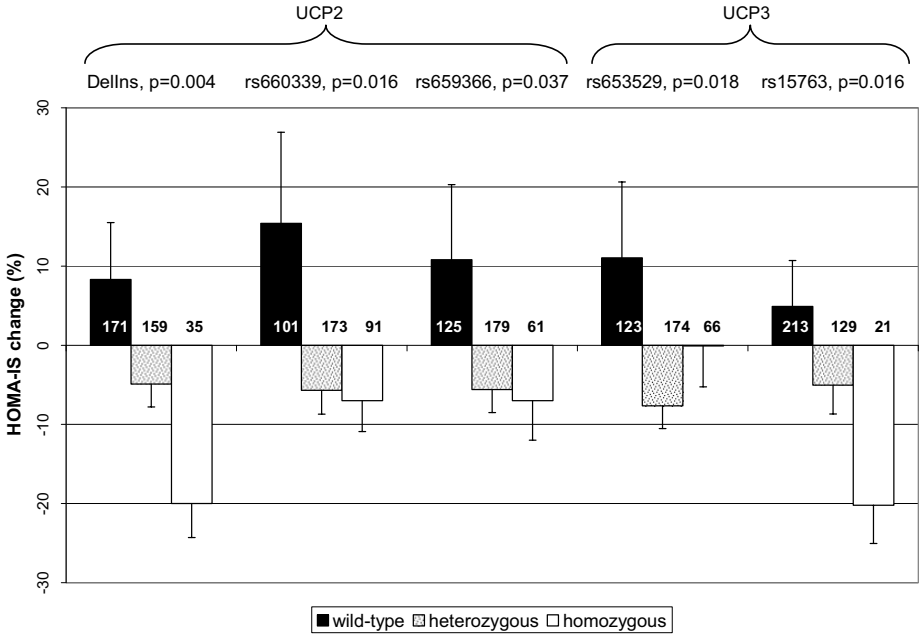



Figure 7. Three-year change in HOMA-IS (%, mean ± SEM) according to the variants of the *UCP2* and *UCP3* genes, analysed by Kruskal-Wallis test. Number of the subjects are shown in the corresponding bars.

### 5.1.4. Associations with serum total and lipoprotein lipids

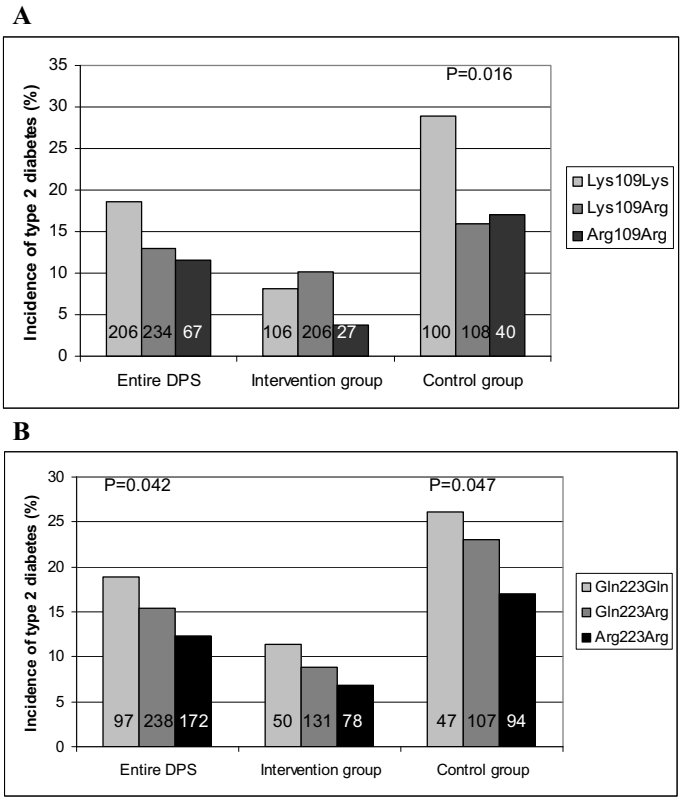
*Rs1726745, rs3781907, rs11235972* and *rs1800849* of the *UCP3* gene associated with serum total cholesterol and LDL-cholesterol levels. Subjects with *rs1726745-GG*, *rs3781907-GG*, *rs11235972-AA* and *rs1800849-TT* genotypes had both the highest serum total cholesterol ($p$=0.022, 0.005, 0.032 and 0.050, respectively), and serum LDL-cholesterol concentrations ($p$=0.005, 0.004, 0.045 and 0.071, respectively). Moreover, the total cholesterol-to-HDL-cholesterol ratio was highest for the subjects with *rs3781907-GG* genotype (p=0.045) (Study III: Table 3).

Longitudinal changes in serum cholesterol levels associated with *rs3781907* of the *UCP3* gene, with *AA* homozygotes having the lowest total cholesterol ($p$=0.020) and LDL-cholesterol concentrations ($p$=0.010) throughout the years 0-3 (Study III: Figure 3). The HDL-cholesterol level was lowest in the subjects with *rs3781907-GG* genotype in the intervention group ($p$=0.041). Furthermore, the total cholesterol-to-HDL-cholesterol ratio was highest in those subjects with the *rs3781907-GG* genotype in the entire DPS ($p$=0.015).

### 5.1.5. Conversion to T2DM

During the 3-year follow-up, 73 individuals (22 in the intervention group and 51 in the control group) developed diabetes (98). During the extended follow-up time of 7 years (4 years of intervention and 3 years of post-intervention follow-up) 185 individuals (75 in the intervention group and 110 in the control group) developed T2DM (111).

The conversion to T2DM during the 3-year follow-up differed between the three genotypes of the *Gln223Arg* in *LEPR* gene (Figure 8, Table 18) [OR=2.01 (95% CI 1.03-3.93)]. In addition, when the study groups were analysed separately, an association was found for the *3'UTR Del/Ins* in the *UCP2* gene in the intervention group [OR=5.37 (95% CI 1.06-27.21)], and *Lys109Arg* in the *LEPR* gene in the control group [OR=2.38 (95% CI 1.18-4.81)] (Figure 8). Furthermore, a risk genotype combination was found in the two variants of the *B2AR* and *B3AR* genes [OR 1.91 (95% CI 1.09-3.33)] in the entire DPS, *Glu27+Trp64Trp* (for genotype frequencies see Table 16) being the protective combination (Table 18).



**C**

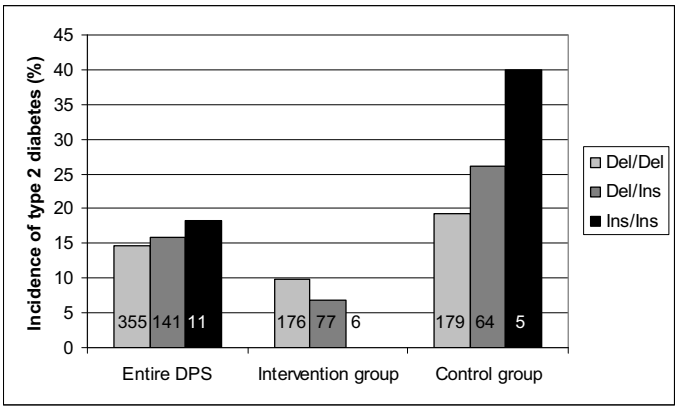



Figure 8. Three-year incidence of T2DM by group according to the *Lys109Arg* (A), *Gln223Arg* (B) and *3'UTR Del/Ins* (C) variants in *LEPR* [% (number of subjects who developed T2DM/ total number of subjects)]. Numbers in the bars represent the genotype frequencies. *P*-values indicate the logistic regression analysis; *P*= NS, unless otherwise shown in the figure.

When the follow-up time was extended to a median of 7 years (Table 18), subjects with the *rs3781907-G* allele of the *UCP3* gene were at risk for T2DM when compared to subjects with *AA* genotype, with HR of 1.48 (95% CI 1.09-2.00) ($p$=0.011). When the study groups were analysed separately, a similar HR was seen in control group [HR 1.58 (95% CI 1.06-2.33), $p$=0.024], whereas no risk was seen in intervention group. The percentage of subjects with *AA* genotype converting to T2DM was 32.6%, whereas it was 38.2% and 47.9% for the subjects with *AG* and *GG* genotypes, respectively ($p$=0.039) (Study III: Table 3).

Subjects in the intervention group with the *UCP2 InsIns* genotype were at a higher risk compared to subjects with *Del* allele, with HR 2.53 (95% CI 1.11-5.73) ($p$=0.027). This risk was not seen in the control group or in the entire DPS (Table 18).

All three variants in the *LEPR* gene (*Lys109Arg, Gln223Arg* and *3'UTR Del/Ins*) associated with T2DM conversion in the control group with HR (95% CI) of 1.53 (1.04-2.24), 1.92 (1.21-3.05) and 1.57 (1.06-2.34), respectively. With respect to the *3'UTR Del/Ins,* the association was seen in the entire DPS as well [HR 1.41 (1.03-1.92)], with subjects with the *Ins* allele being at higher risk (Table 18).

Table 18. Conversion to T2DM during the 3-year (OR) and 7-year (HR) follow-up by group according to the statistically significant variants in *UCP2, UCP3, B2AR, B3AR*, and *LEPR*.

| **Gene**/ variant | Risk genotype/ Subjects (n) with the risk genotype[a] | Odds ratio (OR) Hazard ratio (HR) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Intervention group | $P^b$ | Control group | $P^b$ | Entire DPS | $P^c$ |
| ***B2AR+B3AR*** [d] | combination[e] | | | | | | |
| | 216 | 2.34 | 0.112 | 1.73 | 0.102 | **1.91** | **0.023** |
| | 225 | 1.16 | 0.536 | 1.11 | 0.610 | 1.13 | 0.408 |
| ***UCP2*** | *Ins/Ins* | | | | | | |
| *3'UTR Del/Ins* | 42 | **5.37** | **0.042** | 1.77 | 0.269 | 2.35 | 0.052 |
| | 45 | **2.53** | **0.027** | 0.82 | 0.560 | 1.12 | 0.670 |
| ***UCP3*** | *G* allele | | | | | | |
| *rs3781907* | 265 | 1.19 | 0.749 | 1.75 | 0.125 | 1.54 | 0.148 |
| | 281 | 1.26 | 0.344 | **1.58** | **0.024** | **1.48** | **0.011** |
| ***LEPR*** | | | | | | | |
| *Lys109Arg* | *Lys109Lys* | | | | | | |
| | 194 | 0.88 | 0.804 | **2.38** | **0.016** | 1.69 | 0.069 |
| | 206 | 0.92 | 0.735 | **1.53** | **0.029** | 1.26 | 0.124 |
| *Gln223Arg* | *Gln223Gln* | | | | | | |
| | 90 | 1.55 | 0.468 | **2.33** | **0.047** | **2.01** | **0.042** |
| | 97 | 0.83 | 0.571 | **1.92** | **0.006** | 1.39 | 0.080 |
| *3'UTR Del/Ins* | *Ins* allele | | | | | | |
| | 144 | 0.96 | 0.941 | 1.63 | 0.192 | 1.32 | 0.379 |
| | 152 | 1.27 | 0.353 | **1.57** | **0.026** | **1.41** | **0.031** |

[a] Upper line, number of subjects in logistic regression analysis (3-year follow-up); lower line, number of subjects in Cox regression analysis (7-year follow-up)
[b] Adjusted for baseline body weight, weight change, and baseline FPG
[c] Adjusted for baseline body weight, weight change, baseline FPG, and study group
[d] Adjustment made without baseline FPG
[e] Glu27+Trp64Trp is the protective combination, whereas other combinations are at risk

### 5.1.6. Haplotype analysis

Haplotype analysis was performed for *LEPR* gene (only the survival analysis) and *UCP2-UCP3* gene cluster, including nine variants. For *UCP2-UCP3,* haploblocks 1, 2 and 3 were analysed separately. Only those haplotypes with a frequency ≥0.05 were included.

No haplotypic associations for the studied baseline variables or 0-3 year change in variables were found in block 1 of *UCP2-UCP3*. However, in block 2, the haplotype *AGAG* had lower WHR at baseline when compared to the reference haplotype *GAGA* ($p$=0.050) (Study III: Table 4). In block 3, the haplotype *GAT* had higher serum total cholesterol ($p$=0.006) and LDL-cholesterol ($p$=0.024) at baseline when compared to the reference haplotype *AGC*. Moreover, the haplotype *AAT* showed a greater decrease (0-3 years) in LDL-cholesterol level compared to the reference haplotype *AGC* (p=0.037).

The risk of T2DM was estimated by haplotypic survival analysis. None of the studied haplotypes in the *UCP2-UCP3* gene cluster exhibited any association with conversion from IGT to T2DM. However, with respect to the *LEPR* gene, two risk haplotypes for T2DM were found (Table 19), thus confirming the original results in Study II.

All the main findings from the association studies (both individual gene variations and haplotypic analyses) in DPS are summarized in Table 20.

Table 19. Conversion to T2DM (Hazard Risk Ratio, HRR) during the 7-year follow-up of the entire study population according to the haplotypes in the *LEPR* gene.

| Marker | | | Freq. (%) | Unadjusted model | | Adjusted model [a] | |
|---|---|---|---|---|---|---|---|
| Lys109Arg | Gln223Arg | Del/Ins | | HRR (95% CI) | p | HRR (95% CI) | p |
| Arg | Arg | Del | 36 | 1 (reference) | | 1 (reference) | |
| Lys | Gln | Del | 28 | | NS | | NS |
| Lys | Arg | Del | 20 | 1.45 (1.11-1.89) | 0.006 | 1.35 (1.04-1.77) | 0.027 |
| Lys | Gln | Ins | 14 | 1.42 (1.03-1.94) | 0.030 | 1.62 (1.17-2.24) | 0.004 |

[a] Adjusted by baseline weight, weight change, fasting plasma glucose and study group

Table 20. Summary of the significant associations of the variants studied in DPS. The risk genotypes or alleles are indicated.

| **Gene**/ variant | Rs number | Weight | Waist circumference | Waist-to-hip ratio | Waist-to-height ratio | Total cholesterol | LDL cholesterol | HDL cholesterol | Total cholesterol/ HDL cholesterol | Baseline plasma glucose | 2 h plasma glucose | Serum insulin | HOMA-IS | Risk of diabetes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***B2AR*** | | | | | | | | | | | | | | |
| *Gln27Glu* | *rs1042714* | | | | | | | | | | | *GlnGlu* | | *GlnGln*[a] |
| ***B3AR*** | | | | | | | | | | | | | | |
| *Trp64Arg* | *rs4994* | | | | | | | | | | | | | *Arg*[a] |
| ***UCP1*** | | | | | | | | | | | | | | |
| *A(-3826)G* | *rs1800661* | *GG* | | | | | | | | | | | | |
| ***UCP2*** | | | | | | | | | | | | | | |
| *G(-866)A* | *rs659366* | | *G* | *G* | | | | | | *AA* | *GG* | | *GG* | |
| *Ala55Val* | *rs660339* | | | | | | | | | *AlaAla* | *AlaAla* | | *AlaAla* | |
| *3'UTR Del/Ins* | *-* | | | *DelDel* | | | | | | | | | *DelDel* | *InsIns* |
| ***UCP3*** | | | | | | | | | | | | | | |
| *C(-55)T* | *rs1800849* | | | | | *TT* | *TT* | | | | | | | |
| *GA* | *rs11235972* | | | | | *AA* | *AA* | | | | | | | |
| *AG* | *rs3781907* | | | | | *GG* | *GG* | *GG* | *GG* | | | | | *G* |
| *GA* | *rs1726745* | | *A* | *A* | *A* | *GG* | *GG* | | | | | | | |
| *GA* | *rs15763* | | *G* | *G* | *G* | | | | | | | | *GG* | |
| *AG* | *rs653529* | | *A* | *A* | *A* | | | | | | *AA* | | *AA* | |
| ***LEPR*** | | | | | | | | | | | | | | |
| *Lys109Arg* | *rs1137100* | | | | | | | | | | | | | *LysLys* |
| *Gln223Arg* | *rs1137101* | | | | | | | | | | | | | *GlnGln* |
| *3'UTR Del/Ins* | *-* | *DelDel* | | | | | | | | | | | | *Ins* |

[a] *Glu27 (B2AR) + Trp64Trp (B3AR)* is the protective genotype combination, whereas other combinations are at risk.
Associations, which have been confirmed by haplotype analysis, are shown in grey background. Associations with glucose or insulin concentrations were not dependent on the allele dosage. LDL, low density lipoprotein; HDL, high density lipoprotein; HOMA-IS, homeostasis model assessment for insulin secretion.

## *5.2. Gene expression studies (Study IV)*

### 5.2.1. Clinical characteristics

Subjects in the WR group (n=28) and control group (n=18) did not differ in any of the clinical variables at baseline (Study IV: Table 1). The 33–week intervention resulted, however, in a significant reduction of body weight ($p<0.001$), BMI ($p<0.001$), WC ($p<0.001$), lean body mass ($p<0.01$), FPG ($p<0.001$) and serum leptin concentration ($p<0.01$) in the WR group, whereas no changes were seen in control group, showing a significant difference between the groups (Study IV: Table 2). The slight improvement of $S_I$ in the WR group associated with weight change ($r=-0.44$, $p=0.026$).

Dietary fat intake decreased as intended in the WR group ($p<0.05$), whereas the fiber content of the diet clearly decreased in the control group ($p<0.01$) (Study IV: Table 3).

For the microarray analyses we wanted to include ten subjects from both WR and control groups. In order to select those subjects most suitable for the microarray analyses, the WR group was divided into those who lost ≥ 5% of their body weight (n=11, Group 1) and those who lost < 5% (n=17, Group 2). We chose the ten subjects with most reduced body weight, but due to some technical problems with one microarray chip, the microarray data from one subject had to be excluded. Thus, 9 (4 males/5 females) subjects from the Group 1 were included in the microarray analyses. They had lost more weight than the rest of the WR group (7.8 ± 2.9% vs. 3.3 ± 3.3%, $p=0.002$). They also had increased $S_I$ and decreased fasting serum insulin and 2-hour glucose concentrations along with improvements in measures of body adiposity when compared to those selected for microarray analysis from the control group (n=10; 4 males/6 females) (Study IV: Table 4).

### 5.2.2. Gene expression in adipose tissue

The expression of 105 genes (FDR=13%) in the WR group changed during the intervention (Study IV: Table 5). The expression of 82 % (86/105) of genes was down-regulated in the WR group. Overall, the changes were modest, with fold changes ranging from 0.67 to 1.68. When GO clusters for biological processes were assessed for enrichment of genes into each cluster with $p<0.001$, seven clusters were found. These clusters were associated with the function of the ECM and cell death. The expression of only 62 genes (82% of which were up-regulated) changed in the control group (FDR=13%) and no clusters were formed. None of the genes were the same as those

detected in the WR group. To verify the results of the microarray analysis, seven genes showing either up- or down-regulation in the microarray analysis were confirmed by QPCR analysis (Study IV: Figure 2, Table 6).

One of the genes that showed the most pronounced change during the intervention was the *TNMD* gene. The expression level of the *TNMD* in AT in women before and after the intervention was about twice as high as in men in both study groups (p=0.014-0.005 for gender difference within the group). This is in an agreement with the fact that the *TNMD* is located in X-chromosome. Interestingly, the expression of the *TNMD* gene in AT correlated significantly with fasting serum insulin, $S_I$, body fat mass and lean body mass, both before and after the intervention (Study IV: Figure 3).

# 6. DISCUSSION

## *6.1. Methodological considerations*

### 6.1.1. Subjects

The study subjects in DPS were overweight with IGT, and thus at high risk of developing T2DM. Seventy-five percent of the cohort also had the MetS at the onset of the study (427). The advantages of this population are i) homogenous, carefully selected and phenotyped subjects, ii) the prospective study design with extensive longitudinal follow-up data on key variables, iii) confirmation of new T2DM diagnosis in two subsequent OGTTs, and iv) Finnish population with only few founders (428). On the other hand, one drawback for genetic analyses in DPS is the limited power due to the relatively small sample-size, particularly if the analyses are carried out in smaller sub-groups. In particular, for a genetic variant with very low minor allele frequency, it is difficult to undertake statistical testing with adequate power. However, the sample-sizes in previous association studies rarely exceed the sample-size in DPS, as can be seen in tables 7-12. Moreover, the thorough longitudinal data is a major advantage in DPS. Other research groups investigating longitudinal lifestyle intervention studies in individuals with IGT (99, 100, 104-106) have not carried out genetic association studies, except for the DPP Study, where several genes (none of which is overlapping with the genes in the present series of studies) have been studied in 3548 subjects (429-435). Furthermore, since there are separate groups for lifestyle intervention and control subjects in DPS, it is possible to analyse interactions between the group and genotypes and this way acquire knowledge on gene-environment interactions (436-438). Nevertheless, in this series of studies such interactions were not found.

The study subjects in Genobin had either IGT or IFG and two additional features of the MetS and were therefore also at high risk of converting to overt diabetes. In many aspects, these subjects were similar to the study subjects in DPS. Also here, as in DPS, the subjects were Finnish, middle-aged and overweight volunteers. The proportion of women (57%) was higher than that of men, similarly to DPS (67%).

### 6.1.2. Selection of candidate genes

The candidate genes *B2AR, B3AR, UCP1, UCP2* and *UCP3* in Studies I and III relate to EE and lipid and glucose metabolism, being therefore good choices as obesity candidate genes. The *LEPR* gene examined in Study II is related to long-term weight regulation,

satiety and eating behavior through CNS. Monogenic human obesity due to mutations in leptin or leptin receptor genes is extremely rare. A great number of studies have been carried out, to search for a possible common genetic variation in these candidate genes associating to common human obesity. However, at that time this study was designed, studies on Finnish (439-441), Italian (442), or French Caucasian (443) subjects found no associations between common *LEP* gene variants and obesity; therefore we decided to concentrate merely on *LEPR* gene, which had been shown to be highly polymorphic and associate with obesity traits in several populations (374, 375, 378, 382, 384-386, 393, 394). All the candidate genes in this series of studies have been included in the Human Obesity Gene Map (120, 444-448).

Gene variants for each gene were selected on the basis of earlier reported association studies by our group and by other research groups (Studies I-III) and later by using the International HapMap database (116) and Tagger software (449) (Study III). It should be noted that at that time that this work was started in 2002, it was common to study only one variant, usually a missense variation, in one gene, instead of studying multiple variants covering the genetic information of the entire gene, which has later become virtually a requirement for genetic studies of polygenic diseases. This kind of progress can be seen in Studies I-III where there were increasing numbers of gene variants included in the studies.

### 6.1.3. Genotyping methods

Methods in genotyping have evolved with rapidity during this work. Initially, the variants were genotyped with laborious PCR-RFLP methods with many hands-on steps, whereas the last variants in the *UCP3* gene were genotyped with Illumina's high-throughput oligo ligation method. When there are many manual steps, the sources of error are numerous when compared to automated techniques. However, with the traditional methods, all the minor allele homozygous samples in addition to ~10% of all samples were routinely confirmed by a second analysis to guarantee that the results were uniform. Control samples were included in the analysis as well. The amount of digestion enzymes was optimized so that no partial digestion occurred. Furthermore, as the genotype frequencies are compared to the reference population CEU, it is possible to see that differences occur either using RFLP or Illumina method.

### 6.1.4. Haplotype analyses

At present, the haplotypic associations have not been studied as extensively as the individual variants, and furthermore, SNP selection rarely matches from one study

design to another. Even if the SNP selection would match, different LD statistics can produce different haploblocks. Therefore it is challenging to draw comparisons between the studies and certain 'risk haplotypes'.

### 6.1.5. Statistical analyses

During the present series of studies, the statistical methodology changed so that T2DM conversion was first studied by logistic regression with 3 years' follow-up, and later on by survival analysis (Cox regression) with 7 years' follow-up. Hence, the possible genetic risk factors were finally reanalysed such that both methods were used for all gene variants. As expected, the results differed slightly from each other both due to the length of the follow-up time and the different statistical analysis method. Nonetheless, we regard the survival analysis as being more reliable, because it has a longer follow-up time and is capable of dealing with the drop-outs, thus increasing the number of individuals included in the analysis.

The progress of the statistical procedures can also be seen with the use of FDR. As the number of genetic association studies increased, the number of type I errors, i.e. false positive findings, undoubtedly increased as well, so that the publication criteria became more stringent, demanding more accurate statistical methods. Since the use of Bonferroni correction might be too conservative for many purposes, thus increasing the amount of type II errors, i.e. false negative findings, we ended up using FDR to correct for multiple testing in the Study III. Results with significant $p$-values and high $q$-values must be interpreted with caution, since they might represent false positive findings. FDR is widely used in microarray studies with large data sets, also in Study IV.

### 6.1.6. Microarray studies

From the results of GO clustering in Study IV, it can be seen that most of the expression alterations in SAT were noted in genes of ECM and cell death. Surprisingly, no GO clusters directly referring to insulin signaling, lipid/ carbohydrate metabolism or immune responses were seen, although an evident amelioration in insulin sensitivity due to weight reduction was observed in the WR subgroup 2. We do not know the reasons for this phenomenon, but it can be speculated that the changes in gene expression might have occurred during the 12-week intensive weight reduction period, after which a new state of balance had been attained. More frequent AT sampling, which was not carried out in this study due to ethical reasons, might had revealed this possibility. Additionally, the changes in gene expression are most probably modest, due to moderate results of the intervention, and thus, might not be detected with this kind of robust microarray

technology covering all the genes in the human genome. However, the intervention resembles the clinical practice and thus, gives new information on the changes of gene expression during every day clinical practice instead of those under more artificial experimental settings.

Furthermore, this was also a question of data handling, for which no well-established rules existed, and a wide variety of different methods have been used in the field. If we had chosen less stringent statistical criteria when analysing the huge amount of microarray data in this study, we would have ended up with >300 genes with >10 clusters involving different aspects of the immune response or its regulation. The statistical boundaries must be clearly defined in order to determine the reliable results inside such a massive dataset. In the present study, we applied stringent statistical criteria (FDR 13%, $p<0.01$) in order to deal with interindividual variation, which is always present in human studies, no matter how homogeneous the study population. Today, the methodology for the statistical analyses of transcriptomic data is becoming more standardized and it is rather well established which procedures should be performed during the handling of data from the raw data until the final clustering steps.

Moreover, our study was focused on SAT, thus, we do not know the magnitude of gene expression alterations that have taken place in VAT, muscle, liver or pancreas, for example. However, recent gene expression studies in peripheral blood mononuclear cells (PBMC) in the Genobin Study have detected that the downregulation of the genes involved in NFκB activation after weight loss is associated with the improvement of insulin sensitivity (450). Moreover, weight reduction resulted in a decrease of the expression of IL-1β, IL-1 receptor antagonist, and TNFα and an increase of expression of IL-6 and IL-8 in PBMC (451). Further gene expression studies on PBMC and AT are ongoing in the Genobin Study.

## 6.2. General discussion

When compared to traditional methods in genetic studies of polygenic diseases, such as association studies, the novel GWAS strategy seems to be very effective and powerful. It can be regarded as a major advance in the methodology, providing researchers with highly significant novel candidate genes. However, the traditional candidate gene studies will remain important for replication and there will always be a need for more in-depth information on any novel findings. One recent GWAS examining a very large population has discovered new insights into complex diseases of major public health importance, such as T2DM, CAD, hypertension and type 1 diabetes among others

(117). Six new genes for T2DM and one for obesity have been found by GWAS, located on chromosomes 3 (*IGF2BP2*), 6 (*CDKAL1*), 8 (*SLC30A8*), 9 (*CDKN2B*), 10 (*HHEX-IDE*), and 16 (*FTO*). Additionally, three 'old' candidate genes locating in chromosomes 3 (*PPARγ)*, 10 (*TCF7L2*), and 11 (*KCNJ11*) were also confirmed by GWAS. None of the candidate genes studied in this series of studies associated with T2DM risk or adiposity in GWAS, which is not surprising due to the totally different study settings.

### 6.2.1. Association studies on *B2AR, B3AR* and *UCP1* genes

In Study I, the combination of *Gln27Gln* variant in the *B2AR* gene and the *Arg64* allele in the *B3AR* gene was associated with an increased incidence of T2DM compared with the other genotypes. Since 46% of the study subjects possessed this high-risk genetic constellation (either *Gln27Gln* genotype or *Arg64* allele or both of them), we conclude that this is a very common genetic factor predisposing to diabetes among the IGT population. This kind of gene-gene interaction in the prevalence of T2DM has been previously reported in a case-control association study (188), where 38% of 237 non-diabetic individuals and 45% of 219 diabetic individuals possessed such a genotype combination. Individuals homozygous for the low-risk alleles (*Glu27* and *Trp64*) had a lower prevalence of diabetes (OR=0.58) than individuals with the other genotype combinations, similarly to the findings of our study (OR=0.52).

Both of the risk genotypes (*Gln27Gln* genotype and *Arg64* allele) individually contributed a trend towards increased incidence of diabetes, although not statistically significant. The *Arg64* variant of the *B3AR* has been suggested to be associated with an earlier onset of T2DM (232, 233, 250, 253), IR (249), decreased AIR and Sg (235). Perfetti et al. (228) have shown that human pancreas is a major site for the expression of the *B3AR* gene and that transfection of the cells with the *Arg64* variant of the human *B3AR* is associated with a decrease in glucose-dependent insulin secretion, and in the ability to secrete insulin in response to the activation of the *B3AR*.

The situation with the *B2AR* gene, seems to depend on the population being studied, i.e. both *Gln27* (188, 219) and *Glu27* (185) alleles have been associated with T2DM incidence. The Swedish study (188) also showed an association between higher fasting insulin and non-esterified fatty acid (NEFA) concentrations and the *Gln27* allele. It is not known, however, whether altered lipolysis and higher NEFA concentrations are genetically determined or result from the obesity common in subjects with T2DM (188). In our study, the fasting insulin concentration was highest for the heterozygous

individuals and lowest for the *Glu27Glu* homozygotes, i.e. it was independent of gene allele dosage.

The T2DM risk in Study I was studied with 3 years' follow-up. However, when the follow-up time was later extended to 7 years, the risk genotype combination no longer existed, and neither of the genes associated with T2DM incidence individually. This certainly reduces the importance of the original findings in Study I.

In Study I, the promoter region variant *A(-3826)G* in the *UCP1* gene associated with anthropometric parameters. We used repeated measures GLM to analyse body weight among the genotypes during the 3-year follow-up. This analysis is more powerful than cross-sectional analyses, since it takes into account the periodic and random deviation. This longitudinal analysis suggested that the individuals homozygous for *GG* have the highest body weight, thus confirming the results from previous studies (166, 266, 283, 290). Esterbauer et al. (286) have shown that the sequence variations in the *UCP1* gene account for 19.3% of the variance in its expression. The lowest expression was seen in obese *G(-3826)G* homozygous individuals and the highest expression detected in obese individuals with the *AA* genotype. They also demonstrated that the *A(-3826)G* variant is not a functional mutation but a marker for a frequent mutation resulting in reduced mRNA expression in AT (286).

### 6.2.2. Association studies on *UCP2* and *UCP3* genes

As far as we are aware, Study III is the first study exploring the variants *rs653529, rs15763*, *rs1726745, rs3781907* and *rs11235972* in the *UCP3* gene. Interestingly, subjects with *rs3781907-G* allele experienced a higher risk for T2DM and dyslipidaemia compared to subjects homozygous for the common allele. However, results with significant *p*-values and high *q*-values must be interpreted with caution, since they might be false positive findings. In the case of the *rs3781907-G* association with T2DM, FDR was 0.100, thus representing a finding of borderline significance. Conversion to T2DM and the serum levels of total cholesterol, LDL-cholesterol and total cholesterol-to-HDL-cholesterol ratio increased gene dose-dependently according to *rs3781907* genotypes. The *UCP3* promoter variant *rs1800849,* which is located in the same haploblock and is in LD with *rs3781907,* has been previously shown to associate with increased (324) or decreased (343) risk of T2DM, increased skeletal muscle UCP3 mRNA expression (341), higher total, LDL- (343) and HDL-cholesterol concentrations (353), higher (342) or lower BMI (346, 350, 353), higher WHR (345, 348) and higher fat mass and lean mass (349). In this study, *rs1800849* associated with higher total cholesterol and LDL-cholesterol at baseline, both independently as well as a member of

haploblock 3, in line with earlier studies (343). Interestingly, all the variants in haploblock 3 associated with total and LDL-cholesterol at the baseline, although the most consistent association with various lipid concentrations at baseline and longitudinally was seen for *rs3781907*. FDR was low for these associations, which further provides support for our findings. However, as no previous studies on this variant exist, confirmation of the present results will be needed in other populations.

Although none of the single variants in Study III associated with weight or BMI, variants in haploblock 2 associated with several indices of abdominal obesity at baseline (*rs659366, rs653529, rs15763, rs1726745*) and longitudinally (*rs653529, rs15763, rs1726745*). Although WC and BMI are strongly correlated (452), WC, WHR and WHtR are all more accurate predictors of obesity-related cardiovascular risk than BMI (453), and together with serum lipid measurements, they offer a simple clinical marker of excess visceral fat (452). In this study, the genetic variation in *UCP2-UCP3* gene cluster seems to associate with both serum lipids (haploblock 3) and also with indices of abdominal obesity (haploblock 2). Our results should, however, be confirmed by others.

With respect to the functional *UCP2* promoter variant *G(-866)A* (*rs659366)*, previous studies have found an association with reduced obesity prevalence (306), reduced risk of coronary heart disease (328), reduced (322), but also increased (305) risk of T2DM, lower insulin secretion (307, 321), reduced insulin sensitivity (317), decreased (318) but also increased (306) UCP2 mRNA level in AT, higher oxidative stress and CHD risk (309) and increased TG, total cholesterol and LDL-cholesterol levels (316). In our study, the subjects with the *rs659366-A* allele had lower WC and WHR at baseline, compared to subjects with the *G* allele, both individually as well as a member of haploblock 2. Also the insulin secretion, measured by HOMA-IS, seemed to decrease most in the subjects with the *rs659366-A* allele during the follow-up, although no differences were detected in this allele at the baseline. These findings are in line with previous findings on *rs659366* (306, 307, 321). FDR was low for the associations with indices of abdominal obesity providing some support for the significance of this finding, but high for the associations with HOMA-IS, which naturally weakens its strength. Furthermore, adjustment by age, gender and BMI abolished the statistical significance of the HOMA-IS association.

UCP2 has been said to act as an adiposity angel and diabetes devil (293), whereas increased expression of UCP3 has been suggested to associate with successful weight loss (329). The SNPs locating in the promoter area of the UCP2 and UCP3 genes are known to be associated with gene expression levels of the corresponding genes. Specifically, *rs659366-A* in *UCP2* and *rs1800849-T* in *UCP3* have been shown to

increase their gene expression, and thus these alleles could be protective against obesity. In our study, the *rs659366-A* allele associated with lower WHR and WC at baseline, which is in line with the studies of Esterbauer et al. (306). However, no association between *rs1800849* and obesity was seen in this study. Furthermore, the promoter area variants were not associated with the risk of T2DM. This could be due to the relatively small study population, but also the genetic 'makeup' and environmental factors vary from one population to another.

### 6.2.3. Association studies on *LEPR* gene

In Study II we found that three *LEPR* gene variants associated with body weight or the conversion to T2DM. Different genotypic combinations did not give any additional information, thus, the three variants were analysed individually in the statistical analyses.

The main finding of Study II is that the benefits of a lifestyle intervention on i) the risk of T2DM and ii) changes in body weight can be modified by variants in the *LEPR* gene. Subjects possessing either the *Lys109Lys* or *Gln223Gln* genotype converted more often from IGT to overt diabetes in the control group, whereas the changes in the intervention group were nonsignificant, and also the interaction term between the study group and the genotype was nonsignificant. An increased risk for *Gln223Gln* was seen in the entire DPS as well, whereas it was nonsignificant for *Lys109Lys*. Thus, we can conclude that the exonic variation in the *LEPR* gene is a modulating factor when changing ones lifestyle, suggesting that IGT individuals with *Lys109Lys* or *Gln223Gln* genotype are more amenable to benefit from lifestyle intervention than the other genotypes, in order to prevent T2DM.

When the follow-up time was later extended to 7 years, the results remained somewhat similar. In addition to the risk genotypes *Lys109Lys* and *Gln223Gln* found earlier, subjects with the *3'UTR Ins* allele also had an increased risk of T2DM. This was seen in the entire DPS, and in the control group. This strengthens our previous findings about the *LEPR* gene. The haplotypic survival analysis, which was also done later, further reinforced our findings on the individual variants. In this analysis, two risk haplotypes for T2DM were found (see Results, Table 19). In contradiction to our findings, one previous study claimed the *3'UTR Ins* allele associated with reduced risk of T2DM (375). However, that study was rather small, examining only 122 men included.

In the anthropometric measurements, no differences with respect to different *LEPR* genotypes were seen at baseline or in absolute changes from baseline to the 3-year

examination. However, when body weight was analysed longitudinally, the subjects with *3'UTR Del/Del* genotype had a higher weight than the subjects with the *Ins* allele. Thus, we suggest that the *Del/Ins* variation could be involved in body weight regulation. The *3'UTR Del/Ins* variant may affect mRNA stability and abundance in the cell (374). The *Ins* allele has been associated with lower serum insulin levels in obese individuals (374, 375, 454). None of the earlier studies has shown any association between *3'UTR Del/Ins* variant and body weight, as we demonstrated in Study II. However, none of them included IGT subjects, instead they examined morbidly obese subjects, (374, 454) young healthy men (376) or a population-based cohort (375). As far as we are aware, there is only one previous association study on *LEPR* variants in an IGT population (378), but it did not cover the *Del/Ins* variant. In our study we included only overweight or obese subjects with IGT, and 75% of the cohort also had the MetS (427). Thus, it is not surprising that the results from these different study settings are not directly comparable with each other.

The *Lys109Arg* and *Gln223Arg* variants are within the region encoding the extracellular domain of the leptin receptor and, therefore, amino acid changes affect all isoforms of the receptor, since they all have identical extracellular and transmembrane domains. A meta-analysis has been conducted on the association of these variants with BMI and WC (392). The result was negative, but it did conclude that the effect of these variants could be population-specific, and that the alleles might influence intermediate traits or phenotypes (392). Wauters et al. (378) also studied women with IGT and observed associations of the *Lys109Arg* and *Gln223Arg* variants with glucose and insulin levels. They concluded that the LEPR gene could interact with factors associated with IGT, such as hyperinsulinaemia or IR. In a recent study, Chiu et al. (380) found that the *Arg223* allele is associated with IR, explaining 6-7% of the variability in insulin sensitivity. That study was conducted in 67 healthy Caucasian subjects, and it is consistent with other studies on young and healthy populations (381, 385, 396) but inconsistent with studies on postmenopausal women (378, 384), IGT (378) or overfeeding (394). They speculated that this variant could contribute to the initiation of the events leading to IR in a subset of subjects.

All the associations found in Studies I-III are summarized and compared to findings made in previous studies in Table 21.

Table 21. Comparison of the main findings in Studies I-III with previous studies.

| | | Comparison of the associated trait with other association studies | | | The risk allele[a] | |
|---|---|---|---|---|---|---|
| **Gene/ variant** | **Rs number** | **Similar results** | **Contradictory results** | **No previous data available** | **This study** | **Other studies** |
| ***B2AR*** | | | | | | |
| *Gln27Glu* | *rs1042714* | T2DM | T2DM | | *Gln* | *Glu* |
| ***B3AR*** | | | | | | |
| *Trp64Arg* | *rs4994* | T2DM | | | *Arg* | *Arg* |
| ***UCP1*** | | | | | | |
| *A(-3826)G* | *rs1800661* | Obesity | | | *G* | *G* |
| ***UCP2*** | | | | | | |
| *G(-866)A* | *rs659366* | Central obesity | Insulin secretion | | *G* | *A* |
| *Ala55Val* | *rs660339* | | | Insulin secretion | *Ala* | *Val* |
| *3'UTR Del/Ins* | - | Central obesity | Central obesity | | - | - |
| ***UCP3*** | | | | | | |
| *C(-55)T* | *rs1800849* | Total and LDL cholesterol | | | *T* | *T* |
| *GA* | *rs11235972* | | | Total and LDL cholesterol | *A* | - |
| *AG* | *rs3781907* | | | Total, HDL, and LDL cholesterol T2DM | *G* | - |
| *GA* | *rs1726745* | | | Total and LDL cholesterol Central obesity | - | - |
| *GA* | *rs15763* | | | Central obesity Insulin secretion | *G* | - |
| *AG* | *rs653529* | | | Central obesity Insulin secretion | *A* | - |
| ***LEPR*** | | | | | | |
| *Lys109Arg* | *rs1137100* | | | T2DM | *Lys* | - |
| *Gln223Arg* | *rs1137101* | | | T2DM | *Gln* | - |
| *3'UTR Del/Ins* | - | Obesity | T2DM | | - | *Del* |

[a] The risk allele is shown here if most of the findings are consistent. If the findings are inconsistent, or if no previous data exists, the risk allele is marked as -.

### 6.2.4. Gene expression in adipose tissue

The objective of Study IV was to improve our understanding of the role of interaction between nutrition and gene expression in subjects with features of MetS. Weight reduction -sensitive candidate genes were searched by using microarray technology, which was applied on SAT samples before and after the weight reduction intervention.

The gene expression results in Study IV are well in line with previous microarray studies on human SAT (407, 408, 455), in the respect that the alterations in gene

expression induced by caloric restriction were modest; no fold-changes >1.68 or <0.67 were observed, and the amount of regulated genes was rather limited. This was expected due to a mild, long-term (33 weeks) dietary intervention, during which many compensatory effects may have occurred. On the other hand, a new perspective was achieved with this kind of moderate, easily applicable diet with high compliance, compared to other studies where more dramatic and sometimes experimental interventions with hypoenergetic diets lasting for ten weeks (407, 455) or very low calorie diet (VLCD) lasting for 28 days (408) have been used.

It was shown in Study IV that gene expression in ECM and cell death GO clusters was downregulated. AT has an abundant ECM, with every adipocyte supported by a basement membrane, composed of collagens and other ECM proteins (456). The ECM is a post-natally developed mesenchyme which provides scaffolding and structural support for cells and organs. It is capable of exchanging information with cells and thereby modulates a whole host of processes including development, cell migration, attachment, differentiation, and repair (457). Due to the uncontrolled and chronic injurious stimuli as occurs in MetS and T2DM, there is chronic activation of these above processes resulting in fibrosis, structural derangement, tissue or organ dysfunction, and ultimate failure as a result of loss of structure and function (457). Recent studies have shown that the ECM is involved in inflammation (410, 458), angiogenesis (459, 460) and in the development of cardiovascular dysfunction (457, 461). Excessive synthesis of ECM components in SAT of obese subjects has also been shown to contribute to interstitial fibrosis and tissue deterioration (458). Long-term energy restriction in mice resulted in suppression of the genes associated with ECM (410), whereas an increased formation of ECM constituents was seen in WAT of rats, which were exposed to early-life under-nutrition and subsequently developed visceral obesity (462). Our results are in line with these studies in rodents.

In addition to ECM constituents, also genes associated with cell death were downregulated. Approximately 10% of fat cells are renewed annually at all adult ages and levels of BMI (30). Neither adipocyte death nor generation rate is altered in early onset obesity, pointing to a tight regulation of fat cell number during adulthood (30). However, the production rate of fat cells in obese individuals is higher compared to lean individuals (30). In mice it has been shown that adipocyte death is an early and progressive event in diet-induced obesity, suggesting that there is a homeostatic remodeling program that promotes AT expansion in response to energy surfeit (463). Taking into account these findings, it seems logical that weight reduction resulted in downregulation of genes involved in cell death in our study.

One of the most downregulated genes in this study was the tenomodulin gene with fold change of 0.67 in the WR group (1.06 in the control group). Tenomodulin, a type II transmembrane glycoprotein, is predominantly expressed in tendons, ligaments, and the eye, and is believed to regulate tenocyte proliferation and to be involved in collagen fibril maturation (464). Tendons and ligaments connect the elements of the musculoskeletal system and are composed of densely packed collagen-rich connective tissue. The cellular content of tendons is dominated by tenocytes, which surround the collagen fibrils and create cell-cell and cell-ECM interactions (464). Tenomodulin, similarly to its homolog, chondromodulin, is a putative angiogenesis inhibitor (465), and it should be remembered that abnormal angiogenesis is a common complication in T2DM (466). Subsequently, the genetic studies have shown that this gene associates with obesity and conversion from IGT to T2DM (161), low-grade inflammation (162) and serum lipids (467) in DPS, suggesting this gene may be linked to T2DM through effects on systemic immune mediators.

In addition to the WR group, Study IV provides gene expression data on a control group, also including subjects with features of MetS, but not undergoing dietary intervention. The list of genes with altered expression in the control group included far fewer genes than the WR group, and none of them were overlapping. Moreover, the majority of the genes were up-regulated instead of clearly observed down-regulation in the WR group. Thus, it can be concluded that gene expression changes are occurring also without any lifestyle intervention, possibly due to normal seasonal or daily variation. Thus, the results of the intervention group need to be interpreted in the light of this data. In addition, when interpreting the results, one should bear in mind the huge interindividual variation in gene expression, which is an enormous challenge if one is trying to find clear cut gene clusters among free living individuals.

## *6.3. Concluding remarks*

Obesity and T2DM are consequences of complex interactions among multiple genetic variants and environmental risk factors. As the common-disease-common-variant hypothesis states the genetic predisposition is a result of multiple, relatively common genetic variants with small or modest effects. It has been estimated that ~20 genes are needed to explain 50% of the burden of a disease in the population if the predisposing genotypes are common (≥25%), even though the individual risk ratios are relatively small (RR=1.2-1.5) (468).

Identification of the genetic elements of obesity and T2DM is one of the most important areas of research because discovery of the risk genes would certainly facilitate understanding of the disease, its complications, and its treatment, cure, and prevention. However, there are many obstacles on the way to this goal. Association studies very often end up with inconsistent results due to many confounding factors, of which publication bias is one factor. Positive studies more likely find their way into the literature than negative studies, and therefore the proportion of positive findings, both true-positives and false-positives, is amplified. On the other hand, false-negative findings are also common due to the fact that study designs are often underpowered to detect small differences between groups, which may also have been the case with this series of studies. Inconsistency also derives from differences in the ascertainment scheme and study design, ethnic background, phenotyping and statistical analysis. Even meta-analyses can produce different conclusions. Nonetheless, it is obvious that a balanced, healthy diet, and maintenance of an ideal body weight is beneficial, no matter which genotypic profile the individual has, although the genetic studies could reveal genotypes with high predisposition to obesity and T2DM. However, it would certainly be cost-effective, if the genetic 'fingerprint' could be utilized in directing the high-risk individuals to either a lifestyle (diet and/or physical exercise) or a medical intervention, depending on which effort has been proven to yield the best outcome for this particular genotype-phenotype combination. At the present, this is not the case, yet.

The novel methodology of GWAS represents a totally different basis for studying the genetics of common diseases and complex traits. Research groups are combining their resources, and the high speed of technical advances is also bringing down the huge costs of this technology. Thus, it is now possible to have genome-wide information on both genetic variation at the level of DNA and gene expression patterns at the level of mRNA in a relatively wide range of tissues. When these techniques are combined with appropriate data extraction procedures one can predict that major advances will be made in this research field. Nevertheless, the level of proteomics should not be forgotten, since it is the posttranslationally modified proteome that truly defines the phenotype. Viewed in this more complex and realistic way, the link between genotype and phenotype seems potentially rather remote, especially in the case of complex traits weakly linked to multiple alleles (469). Nonetheless, as all the 'omics' technologies have now become a reality, and data mining tools are available, it is reasonable to assume that some 'light at the end of the tunnel' should soon be seen.

# 7. SUMMARY

Study I:
The combination of the *Gln27Gln* genotype in the *B2AR* gene and the *Arg64* allele in the *B3AR* gene was associated with an increased T2DM incidence in subjects with IGT. However, no risk genotype combinations were seen when the follow-up time was extended. The *G(-3826)G* genotype in the *UCP1* gene associated with greater weight during the follow-up.

Study II:
Two variants in the *LEPR* gene were associated with T2DM incidence in subjects with IGT, with *Lys109Lys* and *Gln223Gln* acting as risk genotypes. These results were further confirmed in the extended follow-up time and haplotype analysis, where in addition to *Lys109Lys* and *Gln223Gln*, also the *3'UTR Ins* allele associated with increased T2DM incidence. The *3'UTR Del/Del* genotype associated with greater weight during the follow-up.

Study III:
Four variants *(rs3781907, rs1726745, rs11235972* and *rs1800849)* in the *UCP3* gene associated with total and LDL-cholesterol levels. The *rs3781907* variant associated also with a higher risk of converting to T2DM in subjects with IGT. Variants *rs659366, rs653529, rs15763* and *rs1726745* in the *UCP2-UCP3* gene cluster associated with measures of abdominal obesity. A risk haplotype for WHR and for serum total and LDL-cholesterol was also found.

Study IV:
Long-term moderate weight reduction in subjects with features of MetS resulted in downregulation of gene expression in AT. Specifically, the expression of genes involved in ECM and cell death was reduced. In general, the changes in gene expression were modest. One of the genes in which the most remarkable changes occurred in was tenomodulin, which is an interesting novel candidate gene which should be investigated in detail in future studies.

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