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JARNO RUTANEN

Genetic Regulation of Energy Homeostasis and Obesity

Studies on Genes Encoding *Sirtuin 1*, *Melanocortin Receptors 3* and *4* and *Melanin-Concentrating Hormone Receptor 1*

Doctoral dissertation

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Abstract

Prevalence of obesity is rapidly increasing all over the world. Even though the explosion of obesity epidemic is a result of environment that favours unhealthy diet and sedentary lifestyle the genes contribute to individual's susceptibility to obesity. High energy intake and low energy expenditure can lead to obesity. The melanocortin system, located in the hypothalamus, is an important central regulator of energy intake, and potentially also energy expenditure. Rare mutations in the *MC4R* gene leading to impaired melanocortin signalling can cause severe obesity. On the other hand, activation of energy expenditure by increased mitochondrial biogenesis in mice can prevent insulin resistance and diet-induced obesity, suggesting that impaired mitochondrial energy expenditure may cause obesity. In this thesis we examined mechanisms affecting energy expenditure and/or energy intake in a group of healthy offspring of subjects with type 2 diabetes and in a group of healthy subjects together with family members of patients with familial combined hyperlipidemia. Insulin sensitivity was measured with the hyperinsulinemic euglycemic clamp and energy expenditure with indirect calorymetry. First, we demonstrated that insulin-stimulated increase in energy expenditure was strongly associated with insulin sensitivity in humans. Furthermore, we showed that adipose tissue silent information regulator 1 (SIRT1) mRNA and the expression of several other genes regulating mitochondrial function correlated with energy expenditure (EE) and insulin sensitivity during hyperinsulinemia. These findings support the possibility that molecules activating SIRT1, enhancing mitochondrial biogenesis and energy expenditure can potentially be used to treat metabolic disease. Our results that the Val103Ile polymorphism of the melanocortin receptor-4 (*MC4R*) associates with energy expenditure support the view that the central melanocortin system and in particular MC4R regulate not only energy intake but also energy expenditure. In addition, we observed that common inactivating polymorphisms of melanocortin receptor-3 gene (*MC3R*) were associated with substrate oxidation and first-phase insulin release. These results are in line with animal studies suggesting that defects in the MC3R signalling lead to impaired lipid oxidation. Polymorphisms of melanin concentrating hormone-1 gene (*MCHR1)* were also investigated but they did not have any significant associations with obesity or metabolic parameters. In this work we present the close association between energy expenditure, genes regulating mitochondrial function and insulin sensitivity, and an association between gene polymorphisms in the melanocortin system and energy expenditure. However, the exact mechanisms how these genes can predispose to obesity remains to be determined.

National Library of Medicine Classification: QU 125, QU 475, QU 500, WD 210, WK 810, WK 880
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“Use the Force, Luke”
Obi-Wan Kenobi – Yedi Master

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Kuopio, July 2009

Jarno Rutanen

Abbreviations

ACTH	adrenocorticotrophic hormone
ADP	adenosine diphostate
ATP	adenosine triphosphate
AGRP	agouti-related peptide
AMPK	AMP-activated protein kinase
ARC	arcuate nucleus
BDNF	brain derived neurotrophic factor
BMI	body mass index
CART	cocaine-amphetamine related transcript
CB1	endocannabinoid receptor-1
CB2	endocannabinoid receptor-2
CCK	cholecystokinin
CNS	central nervous system
CRH	corticotrophin releasing hormone
db/db	leptin receptor deficient mouse
DNA	deoxyribonucleic acid
DPP-4	dipeptidyl peptidase-4
EE	energy expenditure
ESRRα	estrogen related receptor α
FFAs	free fatty acids
FOXO	forkhead-O transcription factor
FTO	fat and obesity related gene
GHS-R	growth hormone-receptor secretagogue receptor
GIP	glucose-dependent insulinotrophic polypeptide
GK	glucokinase
GLP-1	glucagon like peptide-1
GWA	genome wide association
HDL	high-density lipoprotein
INSIG-2	insulin induced gene-2
K_{ATP}	ATP sensitive potassium channel
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IVGTT	intravenous glucose tolerance test
MAF	minor allele frquency
MC3R	melanocortin 3-receptor
MC3R-KO	melanocortin 3-receptor knock-out
MC4R	melanocortin 4-receptor
MC4R-KO	melanocortin 4-receptor knock-out
MCH	melanin concentrating hormone
MCHR-1	melanin concentrating hormone receptor-1
NGT	normal glucose tolerance
LBM	lean body mass
LD	linkage disequilibrium
LDL	low-density lipoprotein
LEPR	leptin receptor
mRNA	messenger ribonucleic acid
α-MSH	α-melanocyte stimulating hormone
mTOR	mammalian target of rapamycin
NPY	neuropeptide Y
NTS	nucleus of solitary tract
ob/ob	leptin deficient mouse
OGTT	oral glucose tolerance test
PGC-1α	peroxisome proliferating receptor coactivator-1α
PC-1	pro-hormone convertase-1
PCR	polymerase chain reaction
POMC	pro-opiomelanocortin
PI3K	phosphatidylinositol 3-kinase
PYY	peptide tyrosine tyrosine
RFLP	restriction-fragment length polymorphism
RBP4	retinol binding protein-4
RNA	ribonucleic acid
ROS	reactive oxygen species
Sir2	silent information regulator 1 (yeast)
SIRT1	silent information regulator 1 (mammalian homologue for Sir2)
SNP	single nucleotide polymorhism
STAT3	signal transducer and activator of transcription 3
TGs	triglycerides
TRH	tyreotropin releasing hormone
TrkB	receptor for brain derived neurotrophic factor
WBGU	whole body glucose uptake
WHO	World Health Organization

List of original publications

This thesis is based on the following original publications, which will be referred by Roman numerals in the text:

I Rutanen J, Pihlajamäki J, Vänttinen M, Itkonen P, Kainulainen S, Yaluri N, Yamamoto H, Lagouge M, Sinclair DA, Elliott P, Westphal C, Auwerx J, and Laakso M. SIRT1 mRNA Expression Is Associated with Energy Expenditure and Insulin Sensitivity. *Submitted.*

II Rutanen J, Pihlajamäki J, Karhapää P, Vauhkonen I, Kuusisto J, Moilanen Mykkänen L, Laakso M 2004 The Val103Ile polymorphism of melanocortin-4 receptor regulates energy expenditure and weight gain. Obes Res 12(7):1060-1066

III Rutanen J, Pihlajamäki J, Vänttinen M, Salmenniemi U, Ruotsalainen E, Kuulasmaa T, Kainulainen S, Laakso M 2007 Single nucleotide polymorphisms of the melanocortin-3 receptor gene are associated with substrate oxidation and first-phase insulin secretion in offspring of type 2 diabetic subjects. J Clin Endocrinol Metab 92(3):1112-1117

IV Rutanen J, Pihlajamäki J, Vänttinen M, Salmenniemi U, Ruotsalainen E, Kuulasmaa T, Kainulainen S, Kuusisto J, Laakso M 2007 Single nucleotide polymorphisms of the MCHR1 gene do not affect metabolism in humans. Obesity (Silver Spring) 15(12):2902-2907

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1 Introduction

Obesity is a growing public health concern all over the world. World Health Organization estimated that 1.6 billion adults were overweight and 400 million obese in the year 2005 (http://www.who.int/topics/obesity/en/). Although the prevalence of obesity has exploded in Western Countries, it is noteworthy that obesity is rapidly increasing also in developing countries causing a major threat for their healthcare systems. The cause for the epidemic of obesity is the imbalance between energy consumed and energy ingested. The availability of inexpensive, energy dense and highly palatable food, together with a trend towards physical inactivity and sedentary lifestyle have created an environment that promotes obesity. In changed environment our ability to regulate energy balance and maintain normal weight is challenging. Obesity leads to harmful health consequences by increasing the risk for many obesity-associated diseases e.g. type 2 diabetes, cardiovascular disease, musculoskeletal disorders and some cancers (1).

Rapidly changing environment is the key element for worsening obesity epidemic. However, different individuals living in the same environment differ in their susceptibility to develop obesity. The important determinant of this susceptibility is heredity. The classical twin study Stunkard et al. (2) showed that BMI of identical twins who had reared apart had high correlation (r=0.70), whereas childhood environment had little or no influence. Indeed, family, twin and adoption studies have shown that weight is almost as highly hereditary trait as is height (3). Although rare gene mutations that cause serious form of early-onset obesity have been identified (4), our knowledge about the risk genes of common polygenic obesity have been limited until recently.

The regulation of balance between energy intake and energy expenditure is a complex interaction between central nervous system (CNS) and peripheral tissues, which are both under complex but strict control to maintain stable weight. Although the mechanisms regulating food intake and energy expenditure are insufficiently known, disturbances in the CNS causing increased food intake are able to induce significant changes in body's energy balance. Indeed, all mutations causing monogenic obesity in humans, discovered so far, mediate their effects via the CNS (4). On the other hand, in animal models impaired or enhanced energy expenditure in peripheral tissues can result in metabolic disease (5) or ability to resist diet induced obesity (6), respectively.

The key player in the CNS regulating energy balance is the melanocortin system of the hypothalamus (7). In the hypothalamic level melanocortin system includes anorexigenic neurons expressing pro-opiomelanocortin/cocaine amphetamine related transcript (POMC/CART) and

orexigenic neurons expressing agouti related peptide/neuropeptide Y (AGRP/NPY). These neurons receive input from our environment and peripheral tissues, integrate this information, and project to brainstem and areas of higher cognitive functions to regulate energy expenditure in peripheral tissues and our eating behavior. An essential downstream factor mediating the effects of the melanocortin system is melanocortin-4 receptor (MC4R). Melanocortin system has broad effects on energy metabolism since it regulates energy intake and energy expenditure, but also substrate oxidation (8) and the activity of the autonomic nervous system (9).

Regarding energy expenditure in peripheral tissues, increasing evidence have evolved that factors regulating function of mitochondria contribute to energy expenditure and metabolic disease. For example, genes regulating oxidative phosphorylation are down-regulated in subjects with insulin resistance (10, 11). Moreover, pharmacological activation of silent information regulator 1 (SIRT1), that modulates cellular metabolism to correspond nutritional status, has shown to induce mitochondrial biogenesis protecting mice from diet induced obesity.

In the present study we investigated the relationship of energy expenditure, insulin sensitivity and SIRT1 mRNA expression, and the association of obesity and common polymorphisms in genes encoding MC4R, melanocortin-3 receptor (MC3R) and melanin concentrating hormone receptor-1 (MCHR1).

2 Review of the literature

2.1 General aspects of obesity

2.1.1 Definition and epidemiology of obesity

The most commonly used parameter to measure obesity is body mass index (BMI), that is determined by weight (kg) divided with height (m) squared. The definitions of overweight and obesity have changed over the years complicating the comparisons of epidemiological data in different populations in different times. Currently the World Health Organization (WHO) and National Institutes of Health (NIH) define overweight as BMI 25.0 - 29.9 kg/m^2, obesity 30.0 - 39.9 kg/m^2 and morbid obesity higher than 40 kg/m^2 (12, 13).

The epidemic of obesity is a major public health concern. In the United States (US) the prevalence of obesity (the proportion of individuals of the entire population whose BMI > 30 kg/m^2) increased in 1960-1980 about 0.1 % per year. However, starting in 1980's the rate increased rapidly by 5-10 fold, and the proportion of obese individuals increased with 0.5 - 1.0 % per year (14). Epidemic of obesity affected all segments of society, including all age groups, men and women and ethnic backgrounds. Later epidemiological surveys have shown that an increase in body weight is continuing in the US. In 2003-2004 17.1 % of children and adolescents were overweight and 32.2 % of adults were obese (15). Similar trends have been observed in the European populations (16). Latest surveys reporting the prevalence of obesity range from 4 - 36 % in Europe with a considerable geographical variation, highest rates in Southern and Eastern Europe and lowest rates in Western and Northern Europe (17). Increasing prevalence of obesity is not a threat only in Western countries but also low income countries (18).

2.1.2 Obesity-related diseases

Obesity is associated with many diseases that can be attributable to two different etiologies (1). The excess fat mass causes metabolic changes in the body increasing the risk of cardiovascular disease, type 2 diabetes, hypertension, polycystic ovary syndrome in women, and some cancers. Obesity can cause diseases directly, because of increased weight and fat mass, e.g. osteoarthritis and sleep apnea, and obese subjects often suffer from psychiatric disorders, even though the causal association is under debate.

2.1.2.1 Diseases caused by increased fat accumulation in the body

Adipose tissue can be considered as an active endocrine organ. In obesity increased fat mass results especially from hypertrophia (enlarged) of fat cells (19). Enlarged fat cells secrete increased amounts of free fatty acids, adipokines and inflammatory peptides, which cause metabolic changes in the body favoring the development of type 2 diabetes and atherosclerosis. Especially the distribution of fat is important for its activity. Fat that accumulates intra-abdominally (visceral obesity, so called "apple obesity"), which is typical for men, is more harmful. In contrast, subcutaneous adipose tissue that accumulates on hips (so called "pear obesity"), which is typical for women, is less hazardous. In addition, obesity leads to harmful ectopic fat accumulation for example in liver, skeletal muscle and pancreas resulting to insulin resistance and lipotoxicity in these organs (20).

Type 2 diabetes is tightly associated with overweight and obesity. It has been estimated that 65 % of diabetes cases are attributable to overweight and the risk increases dramatically with the degree, duration and more central distribution of obesity in both genders (21, 22). A 14-year follow-up of The Nurses' Health Study showed that in the US women the risk of type 2 diabetes started to increase at BMI levels above 22 kg/m$^{2.}$ Subjects with BMI 35 kg/m^2 or more during the follow-up, had up to a 93-fold increase in the risk of diabetes compared to those who had BMI < 22 kg/m^2 (22). In the Health Professionals Follow-Up Study, men whose BMI was 35 kg/m^2 or more had a 42-fold risk of developing type 2 diabetes during a 5-year follow-up than men whose BMI was < 23 kg/m^2 (21). Furthermore, overweight or obese subjects who are gaining weight were at higher risk of developing diabetes than those whose body weight had remained stable during the last years (23).

Cardiovascular diseases are the major concern for health of overweight and obese subjects. In the Nurses' Health Study the risk to develop coronary heart disease was 3.3-fold in women with BMI 29 kg/m^2 or greater compared to women with BMI < 21 kg/m^2. (24). Furthermore, weight gain increases this risk significantly, regardless of initial BMI (25). The mechanisms behind increased cardiovascular risk in obesity are closely related to the metabolic syndrome, insulin resistance, and type 2 diabetes (26). Metabolic syndrome favors the development of atherogenic lipid triad that is characterized by high serum triglycerides (TGs), low high density lipoprotein (HDL) cholesterol and increased concentrations of small dense low density lipoprotein (small dense-LDL) cholesterol. In addition, low grade inflammation in the metabolic syndrome enhances atherosclerosis, as well as hyperglycemia in full developed diabetes. Moreover, hypertension is more common in obese subjects (27) further increasing the cardiovascular risk.

Liver diseases commonly associate with obesity. Non-alcoholic fatty liver disease (NAFLD) and its more serious form non-alcoholic steatohepatitis (NASH), are closely related to the metabolic syndrome and type 2 diabetes. NAFLD is characterized by hepatomegaly, elevated liver enzymes and steatosis of liver tissue, that can progress to steatohepatitis, fibrosis and cirrhosis ultimately leading to liver failure (28). In addition, cholelithiasis is more common in obese subjects than in normal weight subjects (29).

Some cancers are related to obesity. These include cancers of colon, kidney, prostate cancer in men, endometrium and breast cancer in women (30, 31). The pathological mechanisms how obesity can cause cancer are not well known, but the large diversity of obesity related cancers suggests that multiple factors are involved, possibly insulin resistance, insulin-like growth factors, sex steroids, inflammation and increased oxidative stress.

Polycystic ovary syndrome (PCOS) is a common cause of infertility among women (32). The cause of PCOS is poorly known but insulin resistance, related to obesity, is considered to be a key pathophysiological abnormality resulting in compensatory hyperinsulinemia (33). These metabolic changes together with inappropriate gonadotropin release and increased concentration of androgens result in anovulation, irregular menorrhea, hirsutism and infertility. However, lean women may also have PCOS suggesting that hormonal background of this syndrome is complex.

2.1.2.2 Diseases caused directly by increased body mass

Obesity can cause or worsen disease directly because of increased body mass. For example, obesity has deleterious effect on osteoarthritis. This is likely attributable to the excess body weight that puts more pressure on knees and hips (34). Furthermore, a moderate weight loss, about 5 %, improves physical disability caused by osteoarthritis (35). Pulmonary function is also impaired in obesity because of abdominal pressure on diaphragm reducing residual lung volume. This may lead to obesity hypoventilation syndrome, characterized by dyspnea, chronic hypercapnia and sleep disordered breathing (36). In addition obesity causes sleep apnea because increased fat depots in the pharyngeal area can obstruct the airways.

2.1.3 Risk factors of obesity

There is agreement that changes in our environment are the driving force of obesity epidemic (37). However, heritability obviously contributes to individual differences in body weight in our changing

environment (2). In other words, our environment favors the development of obesity but some individuals appear to be genetically more vulnerable to weight gain than others.

2.1.3.1 Environmental risk factors

Our environment has changed substantially during the last century. Rapid industrial development has moved human kind from hunter-gatherers to society of highly efficient agriculture and industrial food production. Earlier risk of famine has changed to unlimited supply of convenient, inexpensive, energy-dense food. Together with physical inactivity this has hazardous consequences to our health.

It is likely that increased energy intake has had a major impact on the development of obesity. In Western countries a call for inexpensive, energy-dense food have created competition between food industry companies who as a marketing strategy increase portions and aim their products for families, children and adolescents. This is supported by the findings showing that increased food availability and consumption in the US in 1980's corresponds tightly with the prevalence of overweight and obesity, that expanded at the same time in all segments of society (38).

At the same time when caloric intake has increased, the physical activity and energy expenditure have decreased. Although physical inactivity plays a role in obesity epidemic, there are arguments that it is not as important as changes in energy intake. This view is supported by the fact that the major part of the energy expenditure is non-modifiable basal thermogenesis, and many human populations entail less volitional physical activity, contrary to energy intake (38). On the other hand, if energy intake and expenditure do not match this causes an "energy gap" resulting in obesity. For example, it has been estimated that at the population level this "energy gap" could be reversed with only a 15 minutes walk each day (39).

Fetal and childhood growth are also related to the risk of obesity and metabolic diseases later in life. Helsinki Birth Cohort Study showed that adulthood obesity was associated with high birth weight and high BMI at all ages from 6 months to 12 years (40). It is noteworthy that growth trajectories of obesity and type 2 diabetes differ from each other. Subjects who later develop insulin resistance, type 2 diabetes and coronary heart disease follow trajectory characterized by premature birth or low birth weight, low weight at early childhood and rapid increase in body weight from 4 to 12 years of age (41, 42).

2.1.3.2 Genetic risk factors

Although weight is highly hereditary trait our genes have not changed during the last few generations when epidemic of obesity has evolved. Therefore, the change in our environment is considered as a driving force for epidemic of obesity (43). However, there is a considerable variation in weight among individuals living in the similar environment, showing that some individuals are more susceptible to develop obesity than are others. Indeed, family, twin and adoption studies have shown that genes are the key players determining individual's susceptibility to weight gain (2, 3). Therefore, obesity is a result of a complex interaction between environment, lifestyle and genes.

1962 James Neel presented "thrifty gene hypothesis" to explain the relationship of environment and heredity (44). This concept assumes that regulatory systems of energy balance and weight have been under intense selective pressure. "Thrifty genes" were possibly beneficial for ancient populations that lived in the environment where food was available only sporadically and efficient mechanism to store energy as fat helped to survive through famine. This could explain why our protection mechanisms for obesity are weak, whereas human body can efficiently resist starvation, by down-regulating energy expenditure (45). "Thrifty gene hypothesis" achieved wide acceptance but it has received also criticism and it has been recently challenged (46, 47). The critics have presented the notion that the periods of famine in the past did not have enough selective pressure to select "thrifty genes", and if they had, then all individuals would be obese. An alternative hypothesis is that the genes favoring the development of obesity are a result from a genetic drift after human ancestors were released from predation and selective pressure was disappeared ("drifty gene" hypothesis, or predation release hypothesis).

2.2 Pathophysiology of obesity

To maintain the stable energy balance and weight, the CNS receives signals from peripheral tissues about body's energy status and environment, integrates this information, and coordinates appropriate response to food intake and energy expenditure. Disturbances in this sensing – integration – response pathway may lead to inappropriately high food intake with low energy expenditure, and ultimately obesity.

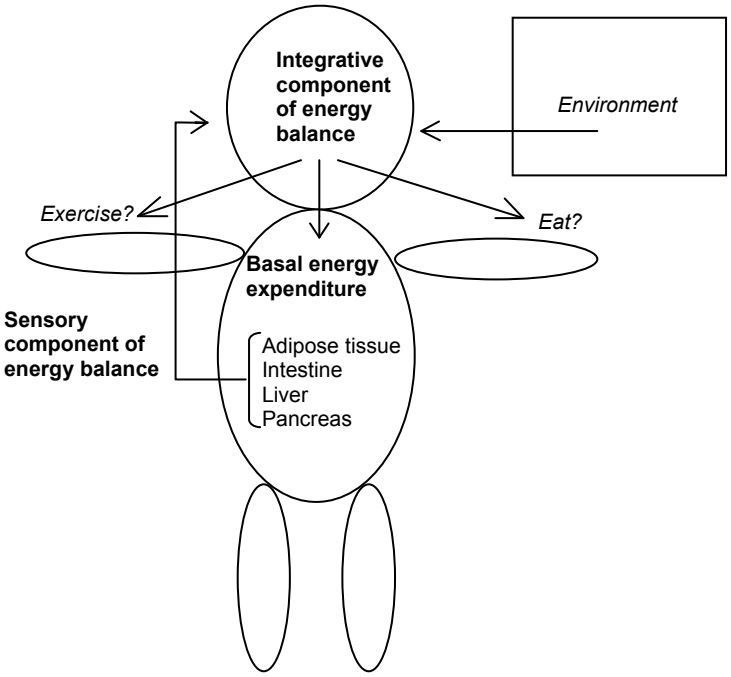


Figure 1. The central nervous system works as an integrative component of energy balance. It receives signals from other organs and senses the current energy status, together with information received from environment the central nervous system regulates behavior and energy expenditure.

2.2.1 Sensory component of energy balance

To maintain the stable weight it is essential to recognize the current metabolic condition and therefore the CNS senses several neuroendocrine factors from different peripheral organs that mediate information about body's energy status.

2.2.1.1 Adipokines

Adipose tissue secretes several adipokines that can cross the blood brain barrier in the arcuate nucleus of the hypothalamus. The first discovered and the best known adipokine mediating information about energy homeostasis from adipose tissue to brain is leptin (48-51). Leptin is secreted in relation to fat tissue and it inhibits food intake and increases energy expenditure (52). Leptin mediates its effects on energy balance by increasing the activity of the melanocortin system in the CNS via leptin receptor that is densely expressed in the arcuate nucleus of the hypothalamus (53-55). In the hypothalamic level melanocortin system includes anorexigenic POMC neurons and orexigenic NPY/AGRP neurons. These neurons project to other areas of the CNS regulating food

intake, thermogenesis, lipid oxidation and insulin sensitivity in peripheral organs (7). Leptin can activate many metabolic pathways, important mediators of leptin's functions down-stream from the leptin receptor are corticotropine releasing hormone (CRH) and tyreotropin releasing hormone (TRH). Leptin is also known to indirectly regulate the expression of orexigenic melanin concentrating hormone (MCH) expression in the CNS.

Genetic defects in the leptin signalling pathway have been recognized to be rare causes of monogenic early onset obesity. Indeed, leptin deficiency leads to obesity in children and sub-cutaneous treatment with leptin has dramatic and sustained beneficial effects on phenotypic abnormalities related to leptin deficiency (56).

Although the discovery of leptin was a remarkable breakthrough in obesity research, it was disappointing to discover that it was not a solution for common polygenic obesity. In contrast, obese animals and humans have hyperleptinemia attributable to leptin resistance, i.e. leptin is unable to mediate its anorexigenic effects (57). These findings suggest that leptin cannot protect humans from obesity. Instead, the lack of leptin appears to protect from starvation.

Another important adipokine is adiponectin (58). Adiponectin is exceptional adipokine since its concentration in blood correlates negatively with obesity (59). Low levels of circulating adiponectin are especially related to visceral obesity and metabolic syndrome (60, 61). A recent study has shown that adiponectin also has a central effect and it stimulates food intake and decreases energy expenditure with a direct effect on the CNS (62).

2.2.1.2 Gut hormones

Gastrointestinal tract is recognized to work as an endocrine organ that secretes several gut peptides which regulate satiety and short-term energy balance. In addition, gut peptides are known to affect glucose homeostasis by regulating insulin and glucagon release from the pancreas.

Cholecystokinin (CCK), secreted in response to food ingestion, is involved in the regulation of gut motility, secretion of exocrine pancreas, and contraction of the gallbladder (63). CCK induces satiety and exogenous CCK administration reduces the meal size in animals and humans (64). However, continuous intra-peritoneal infusion of CCK did not reduce the food intake, since smaller meal size was compensated with more frequent eating in rats, emphasizing the role of CCK in the regulation of short term eating behavior. The mechanisms how CCK regulates satiety are not known. However, the finding that central effects of CCK can be blocked by vagotomy suggest a vagal contribution (65). There is also evidence that CCK mediates its effects, at least partly, through the

melanocortin system since MC4R-KO mouse has attenuated response to anorexigenic effect of CCK (66).

Ghrelin is an endogenous growth hormone-secretagogue, which acts on the growth hormone-secretagogue receptor (GHS-R) and it is expressed and secreted predominantly from the stomach (67). Ghrelin is secreted between the meals and stimulates food intake in animals (68) and humans (69) whereas eating suppresses ghrelin secretion suggesting a role for ghrelin in particular in the initiation of eating. Peripherally administrated ghrelin causes the activation of orexigenic NPY/AGRP neurons through GHS-R:s of the hypothalamic arcuate nucleus (70, 71) demonstrating an interaction between ghrelin and the melanocortin system.

So called incretins include glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP). GLP-1 is secreted from the L-cells and GIP from the K-cells of the intestine (72). The half life of incretins is less than two minutes in the circulation and they are cleaved by dipeptidyl peptidase-4 (DPP-4) (73). GLP-1 and GIP enhance insulin secretion after a meal. This incretin effect was found when study subjects were given oral or intravenous glucose and insulin levels after an oral administration were considerably higher than after an intravenous administration (74) even though the amount of glucose was similar. This results from a rapid release of GLP-1 and GIP after glucose or fat ingestion, which enhance glucose-dependent secretion of insulin (72) and inhibit the secretion of glucagon (75) from pancreatic islets. It is not fully known how L-cells recognize ingested lipid and glucose since GLP-1 is secreted very rapidly after ingestion (within minutes), although nutrients have not reached the L-cells. Therefore, it has been suggested that the autonomic nervous system recognizes the ingested meal and mediates the information to intestinal cells that secrete GLP-1 (76) via the CNS and the vagus nerve (77). In addition to pancreatic effects, GLP-1 has direct anorexigenic effect in the CNS to eating behaviour. Intra-cerebroventricular administration of GLP-1 reduces eating and drinking in animals (78, 79) and in humans subcutaneous GLP-1 receptor mimetics promote satiety, decrease appetite and lead to weight loss in healthy, diabetic and obese individuals. Weight reducing effects may be attributable to the inhibition of gastric emptying leading to reduced food intake (80). The findings that incretin effect is attenuated in subjects with type 2 diabetes (81), have given background for the development of a new class of type 2 diabetes drugs. These drugs enhance incretin effect with exogenous incretin mimetics or preventing the disappearance of endogenous incretins with DPP-4 inhibitors (82). The effect of incretins was also demonstrated by a study where obese subjects underwent gastric bypass surgery and consequently their incretin secretion improved together with

weight loss. A total of 69 % of those who initially had type 2 diabetes developed remission whereas in the obese control group without weight loss, the rate was only 16 % (27).

Peptide Tyrosine Tyrosine (PYY) is a member of pancreatic polypeptide family and secreted from the pancreas. Peripherally given PYY_{3-36}, the circulating form of PYY, has been reported to inhibit food intake and to reduce body weight (83-85). This observation was suspected to be mediated by increased anorexigenic levels of POMC mRNA in neurons of the arcuate nucleus (86). However, the role of PYY has remained contradictory since other studies have not been able reproduce the its effect on body weight (87).

2.2.1.3 Glucose and long-chain fatty acids

Circulating nutrients regulate the appetite and energy expenditure indirectly through i.e. insulin, adipokines and gut hormones (88, 89). However, nutrients may have direct effects on the regulation of energy metabolism in the CNS. The brain is dependent on a constant supply of glucose and it has specialized neurons to monitor and respond to the availability of glucose (90). These neurons include glucose-excited and glucose-inhibited neurons. Glucose is transported through blood-brain barrier and taken into the cells by high-capacity high-affinity glucose transporter 3 (GLUT3). Intracellular glucose metabolism increases the ratio of ATP to ADP causing ATP to bind to the ATP-sensitive K^+ (K_{ATP}) channel closing it and depolarizing cell membrane resulting in the influx of calcium through voltage dependent calcium channels, and ultimately increased neuronal activity. Although K_{ATP}-channel is important in glucose sensing (91) it is not the only determinant of neuronal glucose sensing since K_{ATP}-channel is present in many neurons without glucose sensing capability. Instead, there is evidence that glucokinase (GK) could be the primary regulator of glucose sensing in neurons since the function of glucose-excited and glucose-inhibited neurons can be considerably changed with GK blockade (92-94). Although the function of glucosensing neurons is clearly established, their significance to obesity and metabolic diseases is not clear, and some POMC-neuron specific glucose sensing manipulation models have revealed contrary phenotypes (95-97).

The significance of long-chain fatty acids for energy sensing was shown in an elegant series of studies (98-100). Intacerebroventricular administration of long-chain fatty acids directly down-regulated the expression of orexigenic NPY decreasing food intake and hepatic glucose production in rats (98).This effect is induced by increased intracellular long-chain fatty acid coenzyme A in hypothalamic neurons. Indeed, a similar phenotype can be induced by blocking the carnitine

palmitoyl transferase-1 enzyme that also leads to long-chain fatty acid accumulation into the cytosol (99). At least in animals the disruption of this nutrient sensing pathway is able to contribute to obesity (100). However, its relevance to human obesity has not yet been established.

2.2.1.4 Insulin

Insulin can cross blood brain-barrier in proportion to serum insulin levels (101, 102) and reach insulin receptors that are expressed widely in the CNS. Although neurons are able to get glucose without insulin receptor, insulin is an important signal for the CNS about body's energy homeostasis and a regulator of appetite. Intracerebroventricular infusion of insulin reduces food intake and weight (103, 104), whereas mice with the CNS specific insulin receptor knockout (NIRKO-mouse) develop diet-sensitive obesity, increased adipose mass, hyperleptinemia and insulin resistance (105), proving evidence that impaired insulin signalling in the CNS results in positive energy balance and metabolic diseases.

The anorexigenic effect of insulin is, at least in part, mediated through its inhibitory effect on orexigenic AGRP/NPY neurons and increasing expression of anorexigenic POMC (104, 106, 107). These effects of insulin on hypothalamic neuropeptides are analogous to the effects of leptin. However, the molecular signalling cascades are distinct. Insulin mediates its effects on POMC and AGRP expression through PI3K and forkhead-O transcription factor (FOXO1), whereas leptin also activates STAT3 phosphorylation. Both of these cascades results in increased POMC and decreased AGRP expression leading to reduced appetite (108). Central effects of insulin are not restricted to the regulation of the appetite because efferent feed back loops from the CNS also regulate hepatic glucose production. This was shown in a study where intracerebroventricular injections of insulin mimetics diminished hepatic glucose output (109). This effect appeared to be mediated via K_{ATP}-ion channels rather than melanocortin neurons since the blocking of K_{ATP}-channels prevented the central effects of insulin on hepatic glucose production whereas the blocking of melanocortin receptors did not affect hepatic glucose production.

Studying insulin actions in CNS is challenging since in addition to neuropeptide expression insulin may affect also synaptic plasticity of the neuronal pathways (110), and electrical activity of POMC and AGRP neurons that will ultimately determine neuropeptide/neurotransmitter release (111, 112).

2.2.2 CNS - Integrative component of energy balance

2.2.2.1 Melanocortin and NPY systems (CNS)

Arcuate nucleus (ARC) of the hypothalamus and nucleus of the tractus solitarius (NTS) of the brainstem, are key anatomical areas in the CNS to control appetite and energy expenditure. The ARC lies near the bottom of the third brain ventricle and has neuronal connections with NTS, which receives vagal input from the autonomic nervous system and gastrointestinal tract (113, 114). The ARC is also connected to many other hypothalamic nuclei and areas that control higher cognitive functions. In addition, many humoral factors can cross blood-brain barrier in the ARC. This framework provides a large amount of neurohumoral signals from our body and environment to the ARC (115, 116), where satiety and adiposity signals integrate resulting in appropriate balance between anabolic and catabolic neuronal pathways. Furthermore, the anabolic and catabolic neuronal pathways, which are both expressed in the ARC, interact with other neuronal circuits. This results in appropriate eating behavior and peripheral energy metabolism by efferent neurons that send connections also to peripheral tissues e.g. liver, skeletal muscle and adipose tissue.

The melanocortin system of the CNS is a target of afferent vagal and humoral signals e.g. insulin, adipokines, long-chain fatty acids and ghrelin (7, 117, 118). Melanocortin neurons located in the ARC include two types of neurons. First, neurons that express pro-opiomelanocortin (POMC) and cocaine-amphetamine related transcript (CART). These POMC/CART neurons work as an anorexigenic pathway (melanocortin agonists with catabolic effect) that inhibit appetite and increase energy expenditure via activation of MC4Rs. POMC/CART neurons are neuroendocrine cells where pre-hormone POMC is further cleaved to α-melanocyte stimulating hormone (α-MSH), β-MSH and γ-MSH. α-MSH is a potent MC4R agonist inhibiting food intake and increasing energy expenditure. Second, the ARC contains neurons that express agouti-related peptide (AGRP) and neuropeptide Y (NPY) which are potent antagonists of the MC4R. These AGRP/NPY neurons increase appetite and decrease energy expenditure (melanocortin antagonists with anabolic effect). POMC/CART and AGRP/NPY neurons have projections to other areas of the CNS for example NTS of the brain stem and spinal cord (119). The balance between anorexigenic and orexigenic neurotransmitters determines eating behavior and energy expenditure.

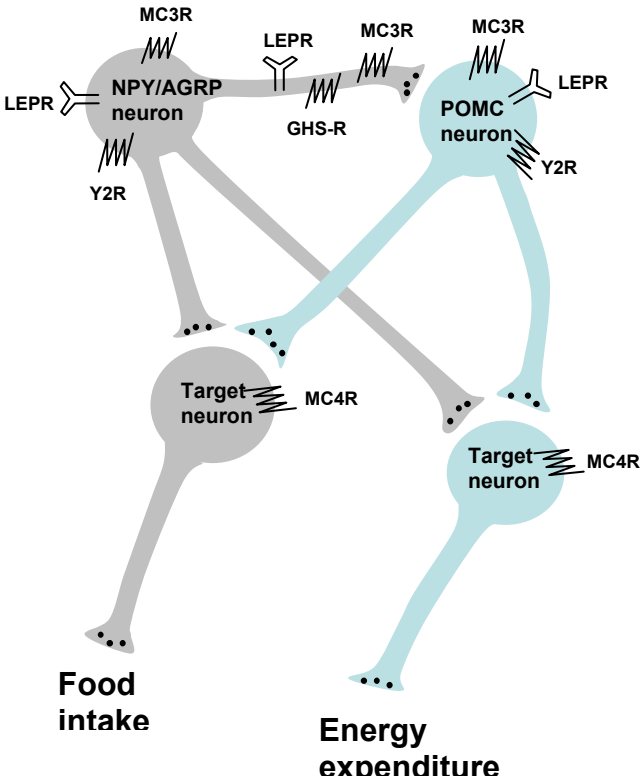


Figure 2. Schematic presentation of the melanocortin system. The net effect on energy balance results from the balance between orexigenic NPY/AGRP and anorexigenic POMC neurons. Note the crucial location of melanocortin-4 receptor to mediate the effects of the melanocortin system. MC3R = melanocortin-3 receptor, MC4R = melanocortin-4 receptor, LEPR = leptin receptor, Y2R=neuropeptide Y2 receptor GHS-R = ghrelin receptor.

Blocking the activation of the melanocortin system leads to obesity. For example, MC4R-KO mice are characterized by obesity syndrome with hyperphagia, hyperglycemia and hyperinsulinemia (120), whereas MC3R-KO mice have normal weight but increased adiposity, possibly due to inability to increase lipid oxidation (121). POMC deficiency leads to the absence of all melanocortins, including α-MSH and ACTH, resulting in early-onset of obesity and adrenal insufficiency and altered pigmentation (122). In addition, transgenic overexpression of AGRP leads to obesity due to chronic MC4R and MC3R antagonism (123). In contrast, hyperactivating the melanocortin system with the ablation of AGRP neurons in adult mice leads to starvation (124, 125).

Genetic defects in MC4R are the most common causes of monogenic early-onset obesity in children (126, 127). POMC-deficiency in humans leads to a rare syndrome of early onset of obesity, adrenal insufficiency and red hair phenotype (128). Missense mutation in the coding region of

POMC derived peptide β-MSH leads to obese phenotype in humans, indicating that α-MSH is not the only significant POMC cleaved melanocortin regulating energy metabolism (129).

Upstream from the melanocortin system. Leptin is an anorexigenic regulator of hypothalamic melanocortin neurons, which also express leptin receptors (LEPR). Leptin crosses blood-brain barrier and up-regulates POMC expression and excitability of neurons, whereas AGRP/NPY expression and excitability of neurons is down-regulated. Indeed, the significance of hypothalamic melanocortin neurons for anorexigenic effect of leptin was demonstrated in a study with leptin receptor deficient mice (*db/db*). These mice are obese and hyperleptinemic because of leptin resistance due to a defect in leptin sensing. However, transgenic specific restoration of leptin receptor into hypothalamic neurons can normalize the expression of POMC, AGRP and NPY and rescue the mice from obesity and diabetes (130).

Leptin activates many intra-cellular signalling cascades in melanocortin neurons. One of the best known mechanisms is the activation of LEPR associated Janus kinase 2 (Jak2) tyrosine kinase, resulting in Jak2 autophosphorylation and phosphorylation of intracellular tyrosine residues on LEPR (131-133). This leads to phosphorylation of STAT3 (signal transducer and activator of transcription 3), a transcription factor regulating POMC gene expression (131, 133-135). Although STAT3 is crucial for leptin's anorexigenic effects (134), melanocortin neurons are activated by leptin even in STAT3-KO mouse (136). Therefore, leptin is able to mediate its effects to POMC neurons also by Jak2-STAT3 independent intracellular cascades, such as phosphatidylinositol 3-kinase (PI3K) that is shown to mediate leptin-dependent acute depolarization of POMC neurons. Blunting PI3K in mice prevents leptin to mediate its anorexigenic effect. However, also these mice are able to maintain normal weight (137, 138). These examples emphasize the remarkable ability of the CNS to activate compensatory signalling cascades if one fails. Indeed, leptin regulates also AMPK and mTOR pathways in hypothalamic neurons. In addition, cell specific knockout of leptin receptor from POMC neurons in mouse results in only mildly obese and hyperleptinemic phenotype (139), suggesting that the melanocortin system is not the only target for leptin.

An interesting pathophysiological phenomenon in obesity is leptin resistance, i.e. leptin's inability to mediate its anorexigenic effects (140). Obesity can induce leptin resistance at least with two different mechanism, blood-brain barrier changes more impenetrable for leptin and leptin's ability to activate its signalling cascades impairs (141). High fat diet in mouse has been shown to produce reversible leptin resistance by both mechanisms, ultimately leading to inability to down-regulate AGRP and NPY expression in the hypothalamus (141, 142). Recently it was suggested that

perturbations in post-translational processing of proteins in endoplasmic reticulum, so called endoplasmic reticulum stress, is a major factor for leptin resistance (143). Moreover, treatment with chemical chaperons that improve the function of endoplasmic reticulum could decrease the stress and worked as leptin sensitizers. This finding may give opportunities to overcome leptin resistance in the future.

Downstream from the melanocortin system. MC4R is a seven transmembrane G-protein coupled receptor and the far most important downstream receptor for mediating the effects of melanocortin neurons on appetite and energy expenditure (7). MC4R is blocked by orexigenic AGRP and activated by anorexigenic α-MSH. Although appetite regulation appears to be the most important mechanism to control energy balance in the melanocortin system, studies with MC4R-KO mice have revealed that also energy expenditure is regulated by MC4Rs (144). Interestingly, melanocortin pathways have functional divergence in the control of appetite and energy expenditure. To regulate appetite POMC/CART and AGRP/NPY neurons of the ARC projects to MC4Rs in the paraventricular hypothalamus and amygdala, whereas energy expenditure regulating neurons project elsewhere to the CNS (145). At intra-cellular level the anorexigenic effects of MC4R activation are possibly mediated by the inhibition of the activity of AMP-activated protein kinase (AMPK), whereas MC4R antagonists induce AMPK activity and increase in appetite (146). MC4R agonist have been shown to up-regulate brain-derived neurotrophic factor (BDNF) that is considered to be a factor that participates in the MC4R downstream signalling and control of energy balance (147).

In addition to energy balance, the central melanocortin system regulates substrate metabolism in peripheral tissues together with insulin. This was shown when intracerebroventricular infusion of melanocortin agonist potentiated the insulin's effect on glucose uptake in the liver and adipose tissue, whereas melanocortin antagonist exerted opposite effects (148). From anatomical point of view, the central melanocortin system has been shown to send efferent neurons polysynaptically via multiple CNS nuclei to brown adipose tissue in mice (149), giving background for the CNS and adipose tissue interaction. Indeed, the connections of the melanocortin system and adipose tissue were also confirmed in a study where MC3R and MC4R blockade promoted lipid uptake, triglyceride synthesis and lipid accumulation into white adipose tissue. In contrast, MC3R and MC4R activation results in lipid mobilization and enhancement in insulin sensitivity (8).

The central melanocortin system can regulate lipid metabolism not only in the liver and adipose tissue, but also in skeletal muscle by increasing AMPK phosphorylation (150). AMPK is a central factor of energy metabolism in skeletal muscle that increases β-oxidation, stimulates glucose uptake

and mitochondrial biogenesis (151). In mice with high fat diet-induced leptin resistance, an increase in AMPK activity was shown for a centrally given melanocortin agonist, but not for leptin, suggesting that the leptin signalling cascade is functional downstream from the leptin receptor although leptin resistance prevents leptin to activate the melanocortin system.

The melanin concentrating hormone (MCH) is a neuroendocrine factor that increases appetite and inhibits energy expenditure (152). Melanin concentrating hormone receptor 1 (MCHR1) is expressed widely in the CNS (153) but also in adipocytes (154), skeletal muscle (155) and pancreatic β-cells (156). MCHR1 is an interesting candidate target for anti-obesity drugs (157).

MCH-knockout (KO) mice are lean, hypophagic and have high metabolic rate (158). In contrast, over-expression of MCH in mice leads to obesity and insulin resistance and hyperplasia of pancreatic islets (159). The phenotype of MCHR1- KO mice is similar to that of MCH-KO mice (160). However, MCH-KO mice are hypophagic, whereas MCHR1-KO mice are slightly hyperphagic but still lean (161). The leanness of MCHR1-KO mice is explained by high metabolic rate and increased locomotor activity. Information on MCH and MCHR1 in humans is limited. Obese subjects have higher circulating MCH levels, and fasting also increases peripheral MCH levels (162). However, the relevance of these findings on obesity and metabolic disease remains to be determined.

2.2.2.2 AMPK and mTOR in the CNS

AMP-activated protein kinase (AMPK) is ubiquitously expressed evolutionarily conserved regulator of cellular pathways. AMPK is a downstream component of a kinase cascade that is activated when ATP is degraded and AMP/ATP ratio in the cell increases, for example during exercise. Activation of AMPK leads to ATP production (163). In addition, AMPK takes part in appetite regulation in melanocortin neurons and its activation in the hypothalamus is increased during fasting and inhibited by feeding (146). AMPK mediates the effects of leptin, adiponectin, insulin, fatty acids and glucose in the CNS (164-166). However, in the hypothalamus AMPK is not a general energy sensor. The net effect of AMPK activation or inhibition on energy balance is dependent whether it happens in anorexigenic or orexigenic neurons of the hypothalamus. This was shown in a study where blocking of AMPK in POMC neurons resulted in obese phenotype with impaired energy expenditure and dysregulated food intake, whereas the blocking of AMPK in AGRP expressing neurons resulted in a lean phenotype (95).

Another molecule integrating peripheral signals in the CNS is mammalian target of rapamycin (mTOR). Like AMPK, mTOR is ubiqitously expressed conserved protein kinase that controls cell growth, transcription and translation, and cell cycle. Proximal energy signal for the activation of mTOR is a decrease in the AMP/ATP ratio of the cell (167). In the hypothalamus mTOR regulates food intake and the expression of mTOR in the hypothalamus is down-regulated by fasting and up-regulated by feeding (168). Up-regulation of mTOR mediates anorexigenic effects of leptin and also some nutrients like leucine and hormones can affect mTOR activity and appetite (168).

2.2.2.3 Other neurotransmitters with effects on weight

Many neurotransmitters, expressed in hypothalamic nuclei or elsewhere in the brain, take part in weight regulation. Indeed, several pharmacological substances that enhance or inhibit these neurotransmitters are known to affect appetite and weight. Some of these are tried to be used, used or under development to treat obesity. However, their usage is challenged by their psychiatric or autonomic nervous system side effects.

Table 1. Neurotransmitters, their effects and their enhancers and inhibitors.

Neurotransmitter	**Effect**	**Enhancers/Inhibitors that are known to affect weight**
Endocannabinoids	Anabolic	Δ^9-tetrahydrocannabinol / rimonabant
Serotonin	Catabolic	tesofensine, sibutramine, fenfluramine, SSRIs / --
Noradrenalin	Catabolic	tesofensine, sibutramine, amphetamine / olanzapine
Dopamine	Catabolic	tesofensine / olanzapine

For reviews see (169-171).

2.2.3 Energy expenditure in peripheral tissues

Energy production from nutrients and energy expenditure of a cell are strictly controlled by mechanisms and organelles that are ubiquitously expressed in all tissues. Energy expenditure is traditionally considered to form the other half of the equation contributing to energy balance. Recently, interest towards molecular mechanisms of energy expenditure has increased, since studies have suggested that low energy expenditure does not contribute only to positive energy balance and obesity but impaired energy expenditure could be the primary cause of metabolic diseases.

Mitochondria are the "power plants" of a cell. Cell uses ATP for energy, that is produced by processing carbohydrates in the citric acid cycle and mitochondrial respiratory chain, where oxidative phosphorylation happens. Lipids are first degraded in β-oxidation, in mitochondria and then processed in the citric acid cycle and respiratory chain. Converting dietary calories into energy produces also reactive oxygen species (ROS) as a toxic side product. ROS is hypothesized to be attributable to many aging-related disorders in the cells (172).

2.2.3.1 Caloric restriction studies

Caloric restriction increases mitochondrial activity, increases the life span in yeast, the worm *Caenorhabditis elegans* and fruit fly *Drosophila* (173). In humans, moderate caloric restriction (25 % of needed energy), results in decreased oxygen consumption and energy expenditure. Genes encoding proteins involved in mitochondrial function are up-regulated and oxidative stress reduced, suggesting that caloric restriction induces biogenesis of "more efficient" mitochondria that are possibly able to produce more energy from less nutrients with reduced oxygen demand (45).

2.2.3.2 SIRT1-silent information regulator 1

Calorie restriction leads to increased lifespan and studies in unicellular yeast revealed that calorie restriction can be mimicked by activating silent information regulator protein 2 (Sir2) (174). Later a similar effect has been observed in mice by activating SIRT1, a mammalian homologue of Sir2 (6). Therefore, SIRT1 is considered to mediate positive effects of calorie restriction. Indeed, the expression of SIRT1 increases in the fasting state in several rodent and human tissues (175, 176). SIRT1 over-expressing mice are leaner, more glucose tolerant and metabolically more active than their littermate controls (177)**.**

At molecular level SIRT1 is NAD^+ -dependent deacetylase. Some of the SIRT1 deacetylace substrates are PGC1α (178), FOXO (179) and NF-κB (180). Thus, SIRT1 can regulate the activity of many transcription factors. Regarding metabolic diseases the activating effect of SIRT1 on PGC1α is especially interesting since deacetylation of PGC1α leads to the expression of genes that are required for oxidative phosphorylation and fatty acid oxidation. This will lead to the improvement in mitochondrial function and ultimately, to better insulin sensitivity, aerobic fitness and resistance to diet induced obesity (6).

2.2.3.3 Mitochondria in obesity and insulin resistance

Impaired mitochondrial function is linked to several diseases that are related to aging, including type 2 diabetes and obesity (172). Insulin resistance, an early hallmark of type 2 diabetes, has been shown to be associated with impaired fasting lipid oxidation in skeletal muscle (181). Muscle biopsies from patients with type 2 diabetes have exhibited reduced activity of the respiratory chain and decreased level of citrate synthase, suggesting impaired mitochondrial capacity (182). Moreover, non-invasive nuclear magnetic resonance spectroscopy studies have showed that healthy but insulin resistant elderly subjects have impaired mitochondrial activity, together with increased fat accumulation in skeletal muscle and liver, suggesting that intra-cellular lipid accumulation is possibly caused by impaired mitochondrial oxidative phosphorylation capacity (183). Similarly, studies of insulin resistant offspring of patients with type 2 diabetes have also reported impaired mitochondrial function and increased intra-myocellular lipid content in these individuals (184). Therefore, inherited or aging-related defect in mitochondrial oxidative phosphorylation is likely to cause the accumulation of intra-cellular lipid, such ad fatty acyl CoAs and diacylglycerol, ultimately leading to impaired insulin signalling (185) and insulin resistance. In humans the expression of genes that control mitochondrial activity have been down-regulated in insulin resistant states (10, 11). Rats selected for over 11 generations according to low aerobic capacity and thus reflecting impaired mitochondrial function, have a phenotype with increased cardio-metabolic risk factors (5).

Physical activity and weight loss which improve insulin sensitivity stimulate mitochondrial biogenesis in sedentary subjects (186). Similarly, pharmacological activation of mitochondria with resveratrol, a SIRT1 activator, induced mitochondrial biogenesis and protected mice from metabolic disease and diet-induced obesity (6).

Although several studies have proven the close relationship of mitochondrial impairment and metabolic diseases, their causative or compensatory nature is still a matter of debate. Some studies have also suggested that impairment in oxidative phosphorylation capacity may have beneficial metabolic effects on metabolism, proposing that mitochondrial impairment would be compensatory mechanism for insulin resistance and obesity (187).

2.3 Genetics of obesity

Indirect evidence, provided by family, twin and adoption studies, has suggested that considerable portion, about 40-70 %, of variation in BMI is explained by heritability (2, 188). Although obesity

does not develop without environment that favors positive energy balance, genetic factors have a strong influence on the susceptibility to develop obesity. Thus, weight is almost as heritable trait as is height (3).

2.3.1 Monogenic obesity

The identification of genes causing severe early-onset obesity has been the major progress in the genetics of obesity during the last years. Although these rare monogenic cases represent only a small fraction of obesity at the population level, their impact on the understanding of mechanisms behind general obesity is important (4).

2.3.1.1 Mutations in leptin and leptin receptor genes

Soon after the discovery of leptin, a study on two severely obese Pakistanian cousins was published. These cousins were homozygous for a frameshift mutation in the gene encoding leptin, which resulted in undetectable levels of serum leptin due to a truncated protein that was not secreted (189). These subjects were characterized by obesity, hyperphagia and increased food seeking behavior. Treatment with subcutaneous recombinant leptin dramatically ameliorated the condition of these patients with a decrease in body weight and normalization of endocrine and immunological functions (56, 190). Although leptin treatment in these rare cases was beneficial, supra-physiological doses of peripherally administered leptin could only slightly decrease body weight in obese patients suffering from "common" polygenic obesity without leptin deficiency (191). This demonstrates the difficulty to overcome leptin resistance and achieve clinically significant benefit with exogenous leptin treatment in patients with a common form of obesity.

A rare mutation also in the leptin receptor, causing early onset obesity, has been described (192). Mutations in the leptin receptor gene are more common than are mutations in the leptin gene, and they cause similar phenotype, which is not so severe as is leptin deficiency (193).

2.3.1.2 Mutations in the gene encoding POMC and prohormone converartase-1 (PC-1)

Homozygous mutations in the POMC gene may cause deficiency of all POMC derived peptides like α-MSH, resulting to absent MC4R activation, hyperphagia and early onset of obesity. These patients suffer also hypocortisolemia because of ACTH deficiency (128). Characteristic for these patients is pale skin and red hair consistent with a known role for POMC derived peptides in skin pigmentation. Heterozygous mutation in *POMC* predisposes to obesity suggesting that also milder

forms of obesity are possible if function of *POMC* is partially impaired (194). Similar phenotype has been described in prohormone converartase-1 (PC1) deficiency. PC-1 cleaves POMC to its active peptides. Heterozygous mutations leading to obesity, hypocortisolemia, hypogonadism and hypoglycemia have been described (195).

2.3.1.3 Mutations in the gene encoding MC4R

First heterozygous mutations in *MC4R* associating with obesity were reported in 1998 (196, 197). The prevalence of mutations in *MC4R* are estimated to vary from 6 % in patients with severe childhood obesity (127) to 1 - 2,5 % in adult subjects with BMI > 30 kg/m^2 (198). Usually, functional mutations in *MC4R* result in intracellular retention of the receptor and therefore their signalling is blunted. The main clinical feature leading to obesity in MC4R deficiency is hyperphagia, which starts during the first year of life. These children also have accelerated linear growth that may be a consequence of hyperinsulinemia related to obesity.

Although there is no specific treatment for MC4R deficiency, some recommendations suggest that screening for *MC4R* mutations could be beneficial for children who present very obese phenotype from the first years of their life. Recognizing *MC4R* mutation carriers may emphasize the importance of controlling the feeding behavior of these children. In addition, obesity drugs that are aimed to activate the melanocortin system independent from MC4R, and possibly are available in the future, may represent specific treatment for these individuals.

2.3.1.4 Mutations in genes encoding the brain derived neurotrophic factor (BDNF) and its receptor TrkB

BDNF is likely a downstream effector of MC4R and regulates energy balance and feeding behaviour (147). Mice that lack *BDNF* develop obesity due to increased food intake (199). In humans, mutations in the gene encoding BDNF or in the gene encoding its receptor TrkB are reported to lead to early-onset of obesity. These patients also develop complex syndrome characterized by impaired cognitive function, impaired short term memory and retarded development with disturbances in nociception. These finding demonstrate that BDNF is connecting the regulation of energy balance with higher cognitive function.

2.3.2 Polygenic obesity

Although some rare genetic mutations causing monogenic obesity have been found (4), only very recently common risk genes have been identified causing polygenic obesity at the population level. The difficulty to identify risk genes for polygenic obesity are demonstrated by promising reports about SNPs associated with obesity, e.g. polymorphisms located near genes encoding GAD2 (200, 201), ENPP1 (202, 203) and INSIG-2 (204-206). However, these studies have not been widely replicated in other populations and therefore the significance of these polymorphisms have remained unclear.

The development of genome-wide association (GWA) studies has been a breakthrough in the genetics of polygenic diseases (207). GWA technique uses large population samples to screen SNPs most strongly associated with a certain clinical trait. SNPs with the strongest association are then genotyped in even larger replication samples, usually tens of thousands subjects, to identify true positive findings. The list of polygenic diseases whose risk genes have been identified in GWA studies is long and includes type 2 diabetes, coronary heart disease, dyslipidemia, cancers, autoimmune diseases and osteoporosis. Typical for risk genes identified by GWAs is that they increase the risk only modestly (1,15 – 1,30 fold).

The first widely replicated SNPs to contribute obesity in adults and children were SNPs located near the fat and obesity associated gene (*FTO*) (205, 208). The risk allele of *FTO* increases BMI by ~0,36 kg/m^2 per allele in adults and risk being obese (BMI >30 kg/m^2) is 1,3 fold in a general adult population. The risk allele of *FTO* causes global obesity in the subcutaneus tissue rather than in the visceral tissue (209) and is associated also with type 2 diabetes that is found to be a consequence for obesity. Mechanisms how FTO predisposes to obesity are not known, but FTO is strongly expressed in the hypothalamic nuclei and it is proposed to take part in nucleic acid demethylation. Moreover, levels of FTO expression are regulated by fasting and feeding giving more evidence that the CNS is likely to be the main target of FTO (210).

After discovery of FTO, more polymorphisms related to obesity have been identified. Interestingly, common polymorphisms located 110-190 kB from the coding sequence of *MC4R* are also associated with polygenic obesity (211, 212) showing that the same gene contributes to rare monogenic form of obesity and common polygenic obesity. The important role of the CNS in polygenic obesity is emphasized by the latest GWA studies that have identified several obesity risk loci located near genes encoding transmembrane protein 18 (*TMEM18*), potassium channel tetramerisation domain containing 15 (*KCTD15*), SH2B adaptor protein 1 (*SH2B1*), glucosamine-6 phosphate deaminase 2 (*GNPDA2*) and neuronal growth regulator 1 (*NEGR1*) that are expressed at

high levels in brain and hypothalamus. The other identified loci near mitochondrial carrier homolog 2 (*MTCH2*) and brain-derived neurotrophic factor (*BDNF*) are also expressed in the brain. The exact mechanisms how these genes contribute to obesity remains to be determined, however, multiple possible effects are proposed like regulation of appetite, energy expenditure and behavioral aspects (213, 214).

3 Aims of the study

This study was undertaken to investigate the association of energy expenditure, insulin sensitivity and SIRT1 expression, and to investigate the effects of polymorphisms of genes regulating appetite and energy expenditure with obesity and metabolic disturbances. Our primary focus was in genes that regulate melanocortin system.

The specific aims of the study were the following:

1. To investigate the association of energy expenditure and insulin sensitivity with SIRT1 expression in adipose tissue.

2. To investigate the effect of the most common genetic variant Val103Ile of *MC4R* on energy expenditure and other metabolic traits.

3. To investigate the metabolic effects of common polymorphisms of *MC3R*.

4. To investigate the association of common polymorphisms of *MCHR1* with obesity and metabolic traits.

4 Subjects and methods

4.1 Study populations

4.1.1 Non-diabetic offspring of patients with type 2 diabetes (Study I, III and IV)

The subjects included healthy non-diabetic offspring of patients with type 2 diabetes (1-3 from each family). Exclusion criteria for the selection of the offspring were diabetes mellitus or other chronic disease that could potentially interfere with glucose metabolism, diabetes mellitus in both parents, pregnancy and age under 25 or over 50 years. Offspring with NGT, IFG and/or IGT were included into study. The diabetic patients (probands) were randomly selected among type 2 diabetic subjects living in the region of the Kuopio University Hospital. The number of offspring participants in Studies were from 216 to 247.

Metabolic studies were performed on three different visits, 1-2 months apart. On the first visit, subjects were interviewed regarding their medical history and life style and anthropometric measurements were done. Blood samples were collected to measure plasma glucose, insulin, C-peptide and lipids after 12-hour fast followed by an oral glucose tolerance test (OGTT). On the second visit, indirect calorimetry was performed after a 12-hour fast followed by an intravenous glucose tolerance test (IVGTT) and 2-hour hyperinsulinemic euglycemic clamp. Indirect calorimetry was repeated during the last 30 minutes of clamp. On the third visit, a CT scan was performed to measure abdominal fat volume and distribution.

4.1.2 Healthy control subjects and family members of patients with familial combined hyperlipidemia (Study II)

Study 2 included subjects who had undergone the hyperinsulinemic euglycemic clamp in our previous studies (215). This group consists of two subgroups. A healthy control subjects (Group 1A, n=124), and family members of patients with familial combined hyperlipidemia (Group 1B, n=105). All subjects had a normal glucose tolerance according to the World Health Organization criteria (1985), normal liver, kidney and thyroid function tests, no history of excessive alcohol intake, and no severe chronic diseases. In Group 1A subjects did not have hypertension, symptoms or signs of coronary heart disease or permanent drug treatment.

4.1.3 Elderly subjects (Study II)

This study group (Group 2, n=1013) was taken from a population-based study of elderly subjects (216, 217). A total of 1298 elderly subjects were a random sample of inhabitants from Kuopio, aged 65-74 years at the baseline study in 1986-1988. Altogether 1054 subjects participated in the follow-up study in 1990-1991, and DNA was taken during this visit. Blood samples were collected at both visits in the fasting state to measure plasma glucose, insulin and lipids followed by an OGTT. DNA was available for 1013 subjects, of whom 146 had type 2 diabetes and 867 did not. The mean follow-up period was 3.5 years (range 2.7-5.2 years).

4.1.4 Metabolic syndrome in men, a population based study (Study IV)

Participants were drawn from an ongoing population-based cross-sectional study of men, aged from 45 to 70 years. A total of 1455 men were a random sample of inhabitants living in Kuopio.

4.2 Methods

4.2.1 Clinical and laboratory measurements

4.2.1.1 Anthropometric measurements

Blood pressure (BP) was measured in a sitting position after a 5-min rest with a mercury sphygmomanometer. Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Waist (at the midpoint between the lateral iliac crest and lowest rib) was measured to the nearest 0.5 centimeter. Body composition was determined with bioimpedance.

4.2.1.2 Laboratory measurements

Blood glucose was measured by the glucose oxidase method (Glucose & Lactate Analyzer 2300 Stat Plus, Yellow Springs Instrument Co., Inc, Ohio), and plasma insulin and C-peptide by radioimmunoassay (Phadeseph Insulin RIA 100, Pharmacia Diagnostics AB, Uppsala, Sweden, and 125J RIA kit, Incstar Co., Stillwater, MN., respectively). Cholesterol and triglyceride levels from the whole serum and from lipoprotein fractions were assayed by automated enzymatic methods (Roche Diagnostics, Mannheim, Germany) (218). Serum FFAs were determined by an enzymatic method from Wako Chemicals GmbH (Neuss, Germany). Nonprotein urinary nitrogen was measured by automated Kjeldahl method.

4.1.2.3 Oral glucose tolerance test

2-hour OGTT (75g of glucose) was performed after overnight fasting. Blood samples to determine glucose and insulin levels were drawn at 0 and 120 minutes in all studies. Additional blood samples were drawn at 30, 60 and 90 minutes depending on the study protocol in different study populations.

4.1.2.4 Intravenous glucose tolerance test (Studies I, III and IV)

IVGTT was performed to determine the first phase insulin release (219) (Studies 1, 3 and 4). After an overnight fast an intravenous cannula was placed into the left antecubittal vein and another cannula into the dorsum of the right hand which was placed in a heated box for arterialization of venous blood. Glucose was infused (300mg/kg in a 50% solution) within 30 seconds and blood samples were collected at -5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50 and 60 minutes to determine glucose and insulin levels.

4.1.2.5 Hyperinsulinemic euglycemic clamp and indirect calorimetry (Studies I - IV)

The degree of insulin sensitivity was evaluated with the hyperinsulinemic euglycemic clamp and indirect calorimetry. Euglycemic clamp was performed after a 12-hour fast (Study II) or after IVGTT (Studies I, III and IV). Priming dose of insulin (Actrapid 100 IU/ml, Novo Nordisk, Gentofte, Denmark) was administered during the initial 10 minutes to raise plasma insulin concentration quickly to the desired level, where it was maintained by a continuous insulin infusion of 40 or 80 mU/min/m² body surface area. Under these study conditions hepatic glucose production is completely suppressed in nondiabetic subjects. Blood glucose was clamped at 5.0 mmol/l for the next 120 minutes by the infusion of 20 % glucose at varying rates according to blood glucose measurements performed at 5-minute intervals. The mean rates of glucose infusion during the last hour of the clamp were used to calculate the rates of insulin stimulated whole body glucose uptake (WBGU).

Indirect calorimetry was performed with a computerized flow-through canopy gas analyzer system (Deltatrac, Datex, Helsinki, Finland). Gas exchange was measured for 30 minutes after a 12-hour fast and during the last 30 minutes of the euglycemic clamp. The rates of glucose and lipid oxidation were calculated according to Ferrannini (determined by indirect calorimetry during the last 20 minutes of the euglycemic clamp) (220). The rates of nonoxidative glucose disposal during the euglycemic clamp were estimated by subtracting glucose oxidation rate from the rates of WBGU.

4.2.2. Genotyping

4.2.2.1 DNA extraction

Genomic DNA was extracted from peripheral blood leucocytes by the proteinase K-phenol-chloroform extraction method (221).

4.2.2.2 *Polymerase chain reaction and genotyping*

In Study II polymerase chain reactions (PCR) were performed with thermocyclers (PTC-100, Programmable Thermal Controller, MJ-research Inc, Watertown, MA, USA) and genotyping with Restriction Fragment Length Polymorphism (RFLP). PCR products carrying the site of Val103Ile polymorphism of MC4R gene were digested overnight with restriction enzyme Hinc II. This restriction site cuts amplified PCR product into two fragments if the G allele is present. The fragments were resolved on 9% polyacrylamide gel electrophoresis, and visualized by staining with ethidium bromide under ultraviolet illumination.

In Studies III – IV genotyping was performed using the Taqman allelic Discrimination Assays (Applied Biosystems). Genotyping reaction was amplified on a GeneAmp PCR system 2700 (95°C for 10 min, followed by 40 cycles of 95°C 15 s and 60°C 1 min), and fluorescence was detected on an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

4.2.3. Statistical analyses

Statistical analyses were performed with the SPSS/Win programs (version 9.0, 11.0 or 14.0, SPSS Inc. Ill.). Data are expressed as mean ± standard deviation (SD), unless indicated otherwise. Variables with skewed distribution (glucose when diabetics were included, insulin, TGs, FFAs, subcutaneous and intra-abdominal fat) were logarithmically transformed for statistical analyses. A P-value equal or less than 0.05 was considered statistically significant.

The differences between the two or three groups were tested using the analysis of variance (ANOVA) for continuous variables or the analysis of covariance (ANCOVA) using age, and body mass index as covariates, when appropriate. Interaction analyses, when done, were calculated with the ANCOVA. The χ^2 test was used to test differences in non-continuous variables. Linear mixed model analysis was applied to adjust for confounding factors. For mixed model analysis we included the pedigree (coded as a family number) as a random factor, the genotype and gender as fixed factors, and BMI and age as covariates. Linear regression was used to calculate the

correlations between continuous variables. The incremental area under the insulin curve in an IVGTT was calculated by the trapezoidal method.

In Studies III and IV, Haploview software (222), available at http://www.broad.mit.edu/mpg/haploview/, was used to calculate the LD statistics. Haplotype estimation from unrelated individuals was performed by using the SNPHAP, available at http://www-gene.cimr.cam.ac.uk/clayton/software/. I genetic studies the allele frequencies were in Hardy-Weinberg equilibrium.

In Study IV, power calculations were used to estimate the power to detect minimal statistically significant differences (power = 0.8 and $p < 0.05$) under the dominant model with Java applets for power and sample size, available at http://www.cs.uiowa.edu/~rlenth/Power/.

4.3 Approval of the Ethics Committee

Written informed consent was obtained from all study participants. The study protocols were approved by the Ethics Committee of the Kuopio University and Kuopio University Hospital.

5 Results

5.1 SIRT1 mRNA expression, energy expenditure and insulin sensitivity (Study I)

5.1.1 Energy expenditure and insulin sensitivity

Energy expenditure (EE) during the clamp positively correlated with insulin sensitivity (r=0.375, P < 0.001; Figure 3). Even stronger correlation was found between ΔEE (defined as EE during the clamp - EE in the fasting state) and insulin sensitivity (r=0.602, P < 0.001). In contrast, fasting EE was not correlated with insulin sensitivity (r = -0.004).

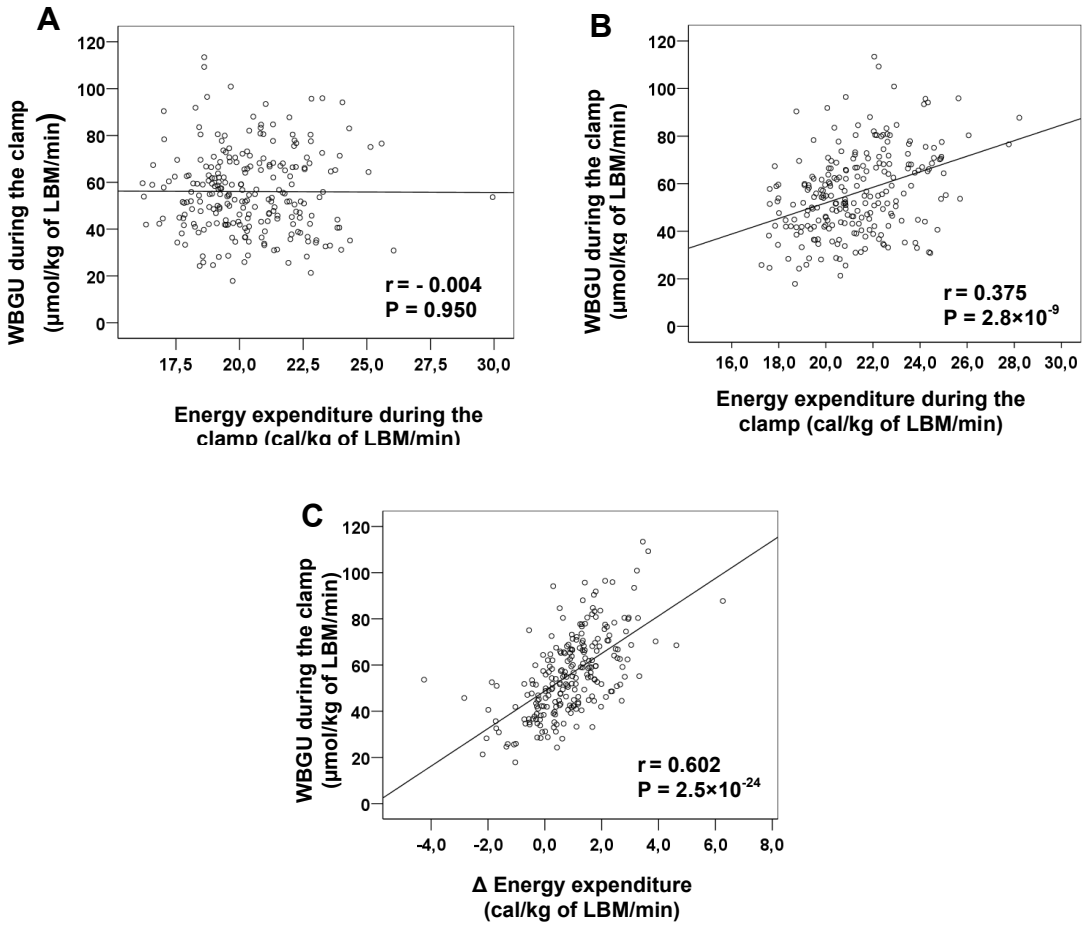


Figure 3. (A) Correlation between the rates of whole body glucose uptake (WBGU) and fasting energy expenditure (univariate linear regression). **(B)** Correlation between the rates of WBGU and energy expenditure during the hyperinsulinemic clamp and **(C)** Correlation between the rates of WBGU and Δ energy expenditure (defined as energy expenditure during the clamp - energy expenditure in the fasting state).

To further investigate the association of EE and insulin sensitivity we analyzed the rates of WBGU/LBM during the hyperinsulinemic clamp according to the tertiles of EE. We did not find differences in WBGU among the tertiles of fasting EE, glucose oxidation or non-oxidative glucose disposal (data not shown). In contrast, subjects in the highest tertile of EE/LBM during the hyperinsulinemic clamp had highest WBGU/LBM (49.85 ± 15.43 in the lowest tertile vs. 55.02 ± 15.46 in the middle tertile vs. 63.44 ± 18.76 in the highest tertile, μmol/kg of LBM/min, $P < 0.001$), which was attributable to both high glucose oxidation (19.54 ± 5.42 vs. 20.96 ± 5.62 vs. 22.75 ± 6.11 μmol/kg of LBM/min, $P = 0.007$, respectively) and high non-oxidative glucose disposal (30.31 ± 12.81 vs. 34.06 ± 13.24 vs. 40.68 ± 16.52 μmol/kg of LBM/min, $P < 0.001$). These differences were even more pronounced across the tertiles of ΔEE/LBM, where subjects in the highest tertile had highest WBGU/LBM (43.82 ± 13.25 vs. 55.75 ± 13.64 vs. 67.96 ± 16.31 μmol/kg of LBM/min, $P < 0.001$), attributable to both high glucose oxidation (17.51 ± 4.34 vs. 20.81 ± 5.45 vs. 24.58 ± 5.31 μmol/kg of LBM/min, $P < 0.001$) and high non-oxidative glucose disposal (26.31 ± 12.08 vs. 34.94 ± 12.24 vs. 43.38 ± 15.16 μmol/kg of LBM/min, $P < 0.001$).

5.1.2 Substrate oxidation in the tertiles of ΔEE

Subjects in the highest ΔEE tertile used more glucose for energy production than did subjects in the lower ΔEE tertiles, as indicated by their higher respiratory quotient (RQ) in the fasting state ($P = 0.01$) and during the hyperinsulinemic clamp ($P < 0.001$). Subjects with the highest ΔEE had the lowest lipid oxidation in the fasting state ($P < 0.001$) and during the hyperinsulinemic clamp ($P < 0.001$). In the fasting state, FFA levels were not different among the tertiles ($P = 0.42$), whereas during the hyperinsulinemic clamp subjects with the highest ΔEE had the lowest levels of FFAs (0.05 ± 0.03 vs. 0.04 ± 0.02 vs. 0.03 ± 0.03, mmol/L, $P < 0.001$).

5.1.3 Determinants of the rates of whole body glucose uptake

To evaluate variables associated with the rates of WBGU/LBM during the hyperinsulinemic clamp we performed univariate linear regression analysis. High ΔEE was the best predictor of high WBGU/LBM, followed by low levels of total triglycerides and low intra-abdominal adipose tissue mass. Other significant predictors of WBGU/LBM were low lipid oxidation during the hyperinsulinemic clamp and low subcutaneus adipose tissue mass.

To explore the determinants of insulin-stimulated EE and WBGU/LBM, we measured adipose tissue mRNA expression of SIRT1 and PGC-1α. SIRT1 mRNA expression correlated significantly with EE (r = 0.289, P = 0.010) and with WBGU/LBM (r = 0.334, P = 0.002) during the euglycemic clamp (Figure 4). No statistically significant correlation was found between SIRT1 expression and EE in the fasting state (r = 0.142). The correlation between SIRT1 expression and PGC-1α expression was 0.448 (P < 0.001). PGC-1α expression correlated significantly only with WBGU/LBM (r = 0.387, P< 0.001) but not with EE during the clamp (r = 0.167).

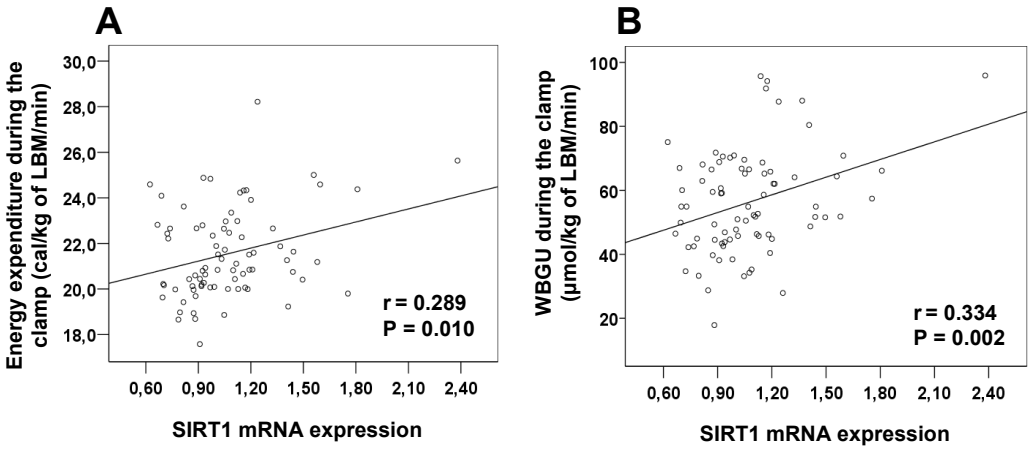


Figure 4. (A) Correlation of adipose tissue SIRT1 mRNA expression level with energy expenditure during the hyperinsulinemic clamp in offspring of type 2 diabetic patients. **(B)** Correlation of adipose tissue SIRT1 mRNA expression level with the rates of whole body glucose uptake in offspring of type 2 diabetic patients.

We also measured adipose tissue mRNA levels of several target genes of SIRT1 and PGC-1α (Table 2). SIRT1 mRNA expression correlated significantly with PGC-1β expression, estrogen-related receptor α, nuclear respiratory factor -1, mitochondrial transcription factor A, and with several genes of the respiratory chain, including NADH dehydrogenase (ubiquinone) 1α subcomplex 2, cytochrome c oxidase subunit IV isoform 1and ATP synthase. SIRT1 mRNA expression also correlated with the expression of soluble superoxide dismutase 1 and catalase. The correlations of mRNA expression of these genes with PGC-1α expression were quite similar, but somewhat weaker. Neither SIRT1 mRNA expression nor PGC-1α mRNA expression correlated with superoxide dismutase 2.

Since we performed gene expression analysis from adipose tissue samples we determined Sirt1 expression in 11 non-diabetic subjects also in skeletal muscle biopsies. We found that Sirt1 mRNA

expression in adipose tissue had a high correlation with skeletal muscle Sirt1 mRNA (r=0.655). Therefore, we believe that our results reflect metabolic changes also in skeletal muscle.

Table 2. Pearson correlations between adipose tissue mRNA expression of SIRT1 and PGC-1α with adipose tissue mRNA expression of genes regulating mitochondrial function (N=81)

	SIRT1	**PGC1α**
PGC-1β	r = 0.358 P = 0.001	r = 0.152 P = 0.179
NRF1	r = 0.286 P = 0.010	r = 0.235 P = 0.036
ESRRA	r = 0.339 P = 0.002	r = 0.260 P = 0.021
TFAM	r = 0.379 P = 0.001	r = 0.213 P = 0.059
NDUFA2	r = 0.392 P = 3.5×10^{-4}	r = 0.273 P = 0.015
CYCS	r = 0.263 P = 0.019	r = 0.159 P = 0.161
COX4I1	r = 0.332 P = 0.003	r = 0.262 P = 0.020
ATP5G1	r = 0.248 P = 0.027	r = 0.196 P = 0.084
SOD1	r = 0.460 P = 2.0×10^{-5}	r = 0.348 P = 0.002
SOD2	r = -0.046 P = 0.689	r = -0.009 P = 0.940
CAT	r = 0.350 P = 0.002	r = 0.422 P = 1.3×10^{-4}

PGC1-β =peroxisome proliferator-activated receptor gamma, coactivator 1 beta; NRF1= nuclear respiratory factor 1; ESRRA=estrogen related receptor alpha; TFAM=transcription factor A, mitochondrial; NDUFA2=NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2; CYCS= cytochrome c, somatic; COX4I1=cytochrome c oxidase subunit IV isoform 1; ATP5G1=ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1; SOD1= superoxide dismutase 1, soluble; SOD2=superoxide dismutase 2, mitochondrial; CAT=catalase. Expressions of all genes were normalized to RPL0 expression

5.2 The Val103Ile polymorphism of melanocortin-4 receptor regulates energy expenditure (Study II)

5.2.1 The allele frequencies

We found the rare 103Ile allele of *MC4R* in eight subjects belonging to Group 1 (allele frequency 0.02). In Group 2 we found 33 heterozygotes for the 103Ile allele and one homozygote for the 103Ile allele (allele frequency 0.02). This homozygous subject was combined with heterozygous

subjects in all statistical analyses. The genotypes were in Hardy-Weinberg equilibrium in both study groups. No significant difference was observed in allele frequencies between subjects with (n=146, allele frequency 0.01) and without (n=867, 0.02) type 2 diabetes in Group 2 (p=0.349).

5.2.2 Association with energy expenditure

In Group 1 we found an effect of the 103Ile allele on energy expenditure. Subjects with the Val103Ile genotype had higher energy expenditure in the fasting state compared to subjects with the Val103Val genotype [63.42 ± 13.40 in subjects with the Val103Ile genotype vs. 59.86 ± 7.33 J/kg/min in subjects with the Val103Val genotype, p=0.007 adjusted for age, sex, BMI and the subgroup (1A/1B), Figure 5], whereas no significant difference was observed during the hyperinsulinemic clamp (67.56 ± 13.52 vs. 66.43 ± 9.29 J/kg/min, p=0.104, respectively). Subjects with the 103Ile allele also had higher rates of glucose oxidation in the fasting state (8.90 ± 6.15 vs. 6.07 ± 4.38 μmol/kg/min, p=0.020) and during the hyperinsulinemic clamp (18.88 ± 4.63 vs. 17.60 ± 3.24 μmol/kg/min, p=0.031).

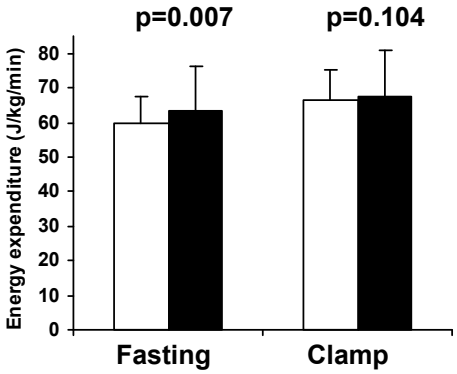


Figure 5. The rates of energy expenditure in the fasting state and during the hyperinsulinemic clamp according to the Val103Val (open bars) and Val103Ile (black bars) genotypes of the melanocortin-4 receptor gene in middle-aged subjects. ANCOVA, adjusted for age, sex, BMI and the subgroup (1A/1B).

No significant differences were observed in the rates of WBGU (53.21 ± 17.49 vs. 53.31 ± 15.39 μmol/kg/min, p=0.290) and non-oxidative glucose disposal (34.33 ± 15.43 vs. 35.60 ± 13.49 μmol/kg/min, p=0.531) during the hyperinsulinemic clamp or lipid oxidation in the fasting state (0.55 ± 0.28 vs. 0.64 ± 0.35 mg/kg/min, p=0.338) and during the hyperinsulinemic clamp (-0.03 ± 0.24 vs. 0.01 ± 0.25 mg/kg/min, p=0.199). However, the subjects with the Val103Ile genotype had

significantly lower levels of FFAs than the subjects with the Val103Val genotype in the fasting state (0.45 ± 0.18 vs. 0.56 ± 0.23 mmol/l, p=0.029), and the same trend was observed during the clamp (0.10 ± 0.05 vs. 0.13 ± 0.12 mmol/l, p=0.057).

5.2.3 Anthropometric measurements

Table 3. Clinical and biochemical characteristics (mean ± SD) in the study groups according to the Val103Ile polymorphisms of the melanocortin-4 receptor gene

	Group 1		Group 2	
	Val103Val n=221	Val103Ile n=8	Val103Val n=979	Val103Ile n=34
Gender (men /women)	142/79	6/2	359/620	9/25
Age (years)	51.0 ± 9.8	56.3 ± 5.9	69.9 ± 2.9	68.7 ± 3.0
Height (cm)	170 ± 9	168 ± 6	161 ± 9	159 ± 9
Weight (kg)	77 ± 15	78 ± 17	71 ± 12	70 ± 12
Waist to hip ratio	0.93 ± 0.08	0.97 ± 0.07	0.93 ± 0.08	0.90 ± 0.09
Body mass index (kg/m²)	26.7 ± 4.4	28.1 ± 7.0	27.4 ± 4.1	27.5 ± 4.2
Systolic blood pressure (mmHg)	134 ± 16	132 ± 15	157 ± 24	156 ± 23
Diastolic blood pressure (mmHg)	85 ± 9	83 ± 6	82 ± 10	84 ± 10
Fasting plasma glucose (mmol/l)	5.5 ± 0.6	5.4 ± 0.5	6.3 ± 2.1	5.8 ± 1.1
Fasting plasma insulin (pmol/l)	62.3 ± 36.2	57.6 ± 26.7	96.0 ± 54.0	97.8 ± 52.2
Total cholesterol (mmol/l)	6.27 ± 1.29	7.03 ± 1.12	6.55 ± 1.28	6.74 ± 1.13
HDL-cholesterol (mmol/l)	1.31 ± 0.28	1.34 ± 0.41	1.27 ± 0.33	1.29 ± 0.25
Total triglycerides (mmol/l)	1.72 ± 1.13	2.33 ± 1.19	1.82 ± 0.92	1.89 ± 0.66
Apolipoprotein B (g/l)	1.12 ± 0.32	1.21 ± 0.23	1.17 ± 0.28	1.17 ± 0.24

All data are presented as mean ± standard deviation. HDL = high density lipoprotein. None of the comparisons between the genotype groups within Group 1 or Group 2 was statistically significant when adjusted for body mass index, age and sex (except weight that was adjusted for age and sex). Comparisons between the genotypes within Group 1 were also adjusted for subgroup (1A/1B).

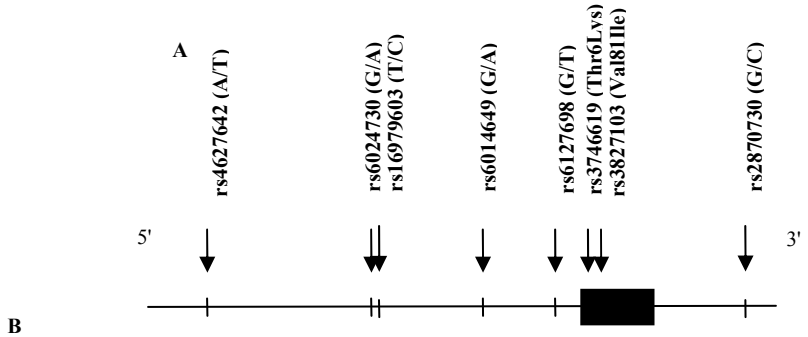
In Group 2 subjects with the103Ile allele gained weight (0.78 ± 3.98 kg) while subjects with the Val103Val genotype lost weight (-0.82 ± 3.98 kg) during the 3.5 year follow-up (p=0.038, adjusted for age, sex and BMI). Similar change was observed in BMI (0.37 ± 1.58 vs. -0.32 ± 1.55, kg/m², p=0.019, respectively).

In Group 1 and Group 2 no significant differences were observed in weight, height, WHR, BMI, systolic or diastolic blood pressure, fasting glucose or insulin, total or HDL-cholesterol, total triglycerides and apolipoprotein B according to the Val103Ile polymorphism (Table 3).

5.3 The polymorphism of melanocortin-3 receptor and substrate oxidation (Study III)

5.3.1 The allele frequencies and location of polymorphisms

The location of eight SNPs of *MC3R*, their minor allele frequencies (MAF) and LD statistics are shown in Figure 6. No carriers of the Ile/Asn 183 mutation were found in our study population.



	SNPs	D'							
		rs4627642	rs6024730	rs16979603	rs6014649	rs6127698	rs3746619	rs3827103	rs2870730
r^2	rs4627642	-	0.985	1.000	1.000	0.980	1.000	1.000	0.525
	rs6024730	0.591	-	0.986	1.000	1.000	0.841	0.829	0.482
	rs16979603	0.826	0.717	-	1.000	0.976	1.000	1.000	0.536
	rs6014649	0.243	0.399	0.294	-	1.000	1.000	0.928	0.885
	rs6127698	0.348	0.220	0.285	0.088	-	1.000	1.000	0.853
	rs3746619	0.278	0.322	0.336	0.875	0.100	-	1.000	0.889
	rs3827103	0.260	0.294	0.315	0.804	0.094	0.937	-	0.891
	rs2870730	0.148	0.205	0.186	0.355	0.141	0.418	0.385	-
	MAF	0.29	0.19	0.25	0.09	0.46	0.10	0.10	0.19

Figure 6. (**A**) Gene map shows SNPs genotyped in *the melanocortin-3 receptor gene*. Coding exon is marked by black box. Genotyped SNPs are shown with NCBI's dbSNP accession numbers. (**B**) Linkage disequilibrium statistics (D', *r*2) and the minor allele frequencies (MAF) are shown among the SNPs of *the melanocortin-3 receptor gene*.

The coding region variants Lys/Thr 6 and Ile/Val 81 substitutions were almost in complete LD with each other, but had a substantially lower LD with non-coding region variants. Altogether 35 subjects had the Lys/Thr 6 genotype and five subjects the Thr/Thr 6 genotype (the frequency of the Thr 6 allele 0.10). The Ile/Val 81 genotype was found in 33 subjects and the Val/Val 81 genotype in

five subjects (the frequency of the Val 81 allele 0.10). The five subjects homozygous for both Thr 6 and Val 81 alleles were combined with heterozygotes in all statistical analyses. Two subjects carried the haplotypes which did not include either the Lys 6 Ile 81 or Thr 6 Val 81 combinations, and therefore they were excluded from all statistical analyses.

5.3.2 The substrate oxidation

We found that lipid oxidation in the fasting state was significantly lower in carriers of the Lys 6 and Ile 81 alleles compared to that of subjects with the Thr/Thr 6 and Val/Val 81 genotypes (0.85 ± 0.38 vs.1.00 ± 0.43, mg/kg of LBM/min, p=0.022, respectively, adjusted for BMI, age, sex and family relationship, Figure 7A). Similar results were obtained during the hyperinsulinemic clamp (0.32 ± 0.41 vs. 0.44 ± 0.34 mg/kg of LBM/min, p=0.021, respectively). Glucose oxidation in the fasting state was significantly higher in carriers of the Lys 6 and Ile 81 alleles compared to subjects with the Thr/Thr 6 and Val/Val 81 genotypes (11.28 ± 4.64 vs. 9.71 ± 4.53 μmol/kg of LBM/min, p=0.031, Figure 7B), and similar, non-significant trend was observed during the hyperinsulinemic clamp. Levels of fasting FFAs were significantly lower in carriers of the Lys 6 and Ile 81 alleles (0.50 ± 0.19 vs. 0.60 ± 0.24 mmol/l, p=0.003, Figure 7C), whereas no differences were found in levels of FFAs during the hyperinsulinemic clamp.

5.3.3 Energy expenditure and obesity

No differences were observed in the rates of energy expenditure in the fasting state or during the hyperinsulinemic clamp. Similarly, no statistically significant differences were observed in BMI, waist, BP, fasting glucose or insulin, subcutaneous or intra-abdominal fat measured by CT with respect to any SNPs screened.

5.3.4 The association with insulin secretion and insulin sensitivity

We did not find differences in the rates of WBGU during the hyperinsulinemic euglycemic clamp between the risk alleles and the common genotypes of the SNPs. However, subjects with the Thr/Thr 6 and Val/Val 81 genotypes had lower first phase insulin secretion (insulin under the curve during the first 10 minutes of the IVGTT) than did subjects with the Lys 6 and Ile 81 alleles (2454 ± 1538 vs. 3220 ± 1765 pmol/L × min, p=0.025, respectively).

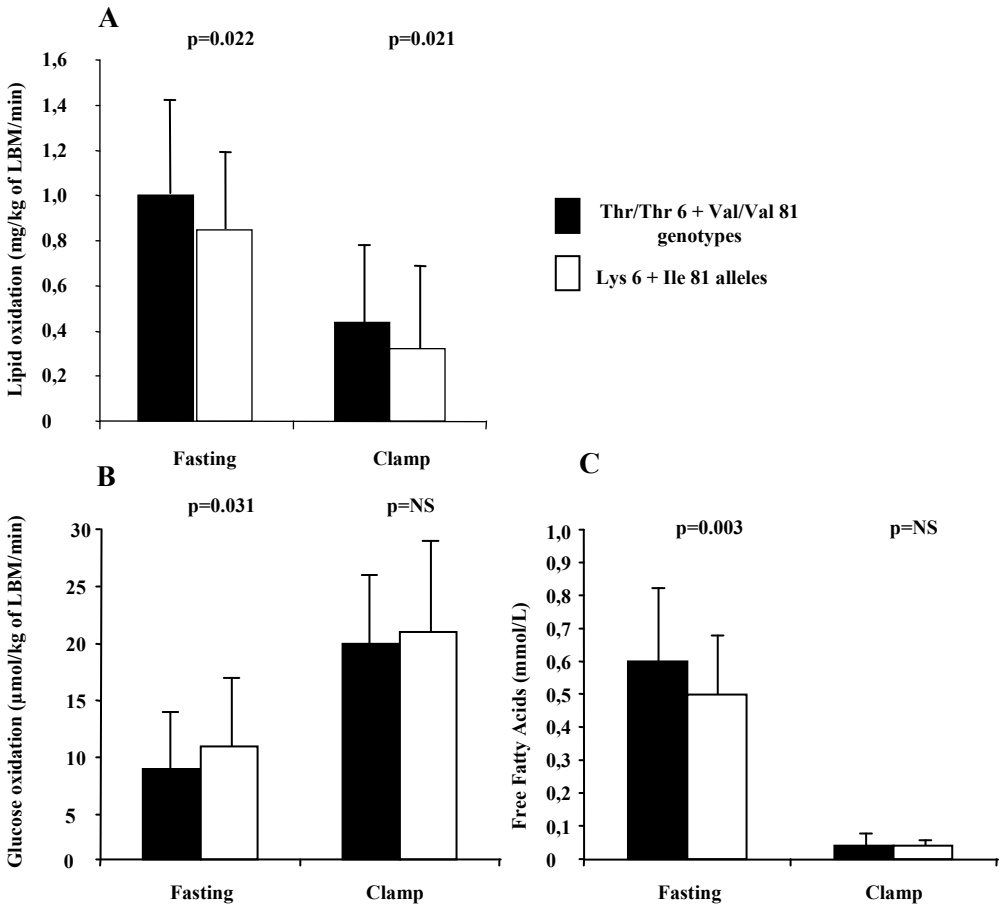


Figure 7. (A) Lipid oxidation, **(B)** glucose oxidation and **(C)** fatty acid levels in the fasting state and during the hyperinsulinemic euglycemic clamp according to the Thr/Lys 6 and Val/Ile 81 polymorphisms of the *melanocortin-3 receptor gene*. Subjects with the Thr/Thr 6 and Val/Val 81 genotypes (black bars, n=176) vs. carriers of the Lys 6 and Ile 81 alleles (open bars, n=38). p-values are adjusted for BMI, age, sex and family relationship (linear mixed model analysis, n=214). LBM = lean body mass.

5.3.5 The haplogenotype analysis

Five haplogenotypes were formed from the three SNPs that were associated with metabolic phenotypes (rs6014649, Thr/Lys 6 and Val/Ile 81), haplogenotype 111/111 (n=176, frequency 0.815), haplogenotype 111/222 (n=27, 0.125), haplogenotype 222/222 (n=5, 0.023), haplogenotype 111/122 (n=6, 0.028) and haplogenotype 111/221 (n=2, 0.009). Subjects with the 111/111 haplogenotype were compared to carriers of the 222 haplotype (haplogenotypes 111/222 and 222/222 combined). In the fasting state the 222 haplotype was associated with lower rates of lipid oxidation than the 111/111 haplogenotype (0.86 ± 0.40 vs. 1.00 ± 0.43, mg/kg of LBM/min, p=0.047) and higher rates of glucose oxidation (11.52 ± 4.82 vs. 9.71 ± 4.53 µmol/kg of LBM/min,

p=0.029). Thus, haplotype analysis did not identify haplogenotypes having an effect beyond those of individual SNPs.

5.4 The polymorphisms of melanin concentrating hormone receptor-1 (Study IV)

We screened six SNPs of *MCHR1* in offspring of type 2 diabetic subjects (Group 1). No significant differences in anthropometric measurements and glucose tolerance, energy expenditure, energy partitioning, insulin secretion, insulin sensitivity or body composition (Group 1) were found. We also generated nine haplogenotypes based on four SNPs (rs133070, rs133072, rs133073 and rs133074) of *MCHR*. The three most common haplogenotypes (the number of subjects at least 16) were not associated with any metabolic parameters.

rs133072 (MAF 0.33), which was in > 0.50 LD with other SNPs and thus covering most of genetic information of other SNPs, was also screened in Group 2 that was taken from a population-based study of 1455 unrelated Finnish men, aged from 45 to 70 years. In this population, we did not find differences in BMI, waist, glucose or in insulin levels during an oral glucose tolerance test (OGTT) between different genotypes (data not shown).

6 Discussion

6.1 Study subjects and methods

6.1.1 Representativeness of the study subjects

Subjects in Studies I, III and IV were non-diabetic offspring of patients with type 2 diabetes who were randomly selected from people living in the Kuopio University Hospital region. Offspring of subjects with type 2 diabetes are known to have inherited insulin resistance (223, 224), and their lifetime risk of diabetes is approximately 40% (225). Therefore, young offspring (mean of our study subjects was 35.1 years) without any confounding co-morbidities are ideal to investigate the development of insulin resistance, type 2 diabetes and related conditions. Our study subjects were accurately phenotyped providing an opportunity to recognize early disturbances in glucose and lipid metabolism. The limitation of this study population is a relatively small size (n=216-247 during this work) for genetic association analysis.

In Study II we investigated the effect of a SNP having a low frequency. To maximize the statistical power we combined two subgroups who had undergone the hyperinsulinemic euglycemic clamp (215). The first group included healthy control subjects (Group 1A, n=124), and the second group family members of patients with familial combined hyperlipidemia (Group 1B, n=105). To further increase the statistical power we investigated elderly subjects (Group 2) who were identified from our large population based follow-up study (216, 217) that was a random sample of inhabitants from the Kuopio town, aged 65-74 years at the baseline study in 1986-1988. Since study protocol and characteristics in Group 2 differed from Groups 1A and 1B the data were analyzed separately.

Second group of participants in Study IV were drawn from ongoing study of metabolic syndrome in men. Importantly study group is population-based and large, including 1455 men from 45 to 70 years of age, making it ideal for genetic analyses.

6.1.2 Measurements

We used the hyperinsulinemic euglycemic clamp in combination with indirect calorimetry to measure insulin sensitivity and energy expenditure in Studies I-IV. This method is the golden standard for the evaluation of insulin sensitivity and whole body energy expenditure. However, it is time consuming and laborious limiting the number of subjects possible to examine.

In Studies IV we also used a sample from an ongoing large population-based study of Finnish men.

Obesity and metabolic syndrome are more common in men than in women and therefore it is likely that genetics of these conditions are easier to investigate in men. The study protocol included anthropometrical measurements, OGTT to measure glucose tolerance and the measurements of blood pressure and lipids and lipoproteins.

6.2 Energy expenditure and insulin sensitivity

SIRT1 is a NAD^+-dependent deacetylase that removes an acetyl group from a protein substrate while NAD^+ is a co-substrate in this reaction. Several transcription factors are substrate proteins for SIRT1 and deacetylation regulates their function and gene transcription activity. In addition, SIRT1 has genome instability suppressing properties through chromatin modification and it participates in DNA damage induced chromatin reorganization altering age-related changes in gene expression (226).

SIRT1 mediates the positive effects of caloric restriction and prolonged life span. Therefore, SIRT1 deficient mice are unable to adapt to the conditions of caloric restriction and achieve these benefits (227). SIRT1 activates PGC-1α by deacetylation which results in increased mitochondriogenesis (228, 229) and improved mitochondrial function. In contrast, impaired mitochondrial function is considered to be a contributing factor to insulin resistance (5, 183). Moreover, insulin resistance in human skeletal muscle has been associated with decreased mitochondrial oxidative capacity and ATP synthesis, and decreased expression of the genes that control mitochondrial activity, including PGC-1α (10, 11). These data demonstrate that energy expenditure and insulin resistance are closely associated. Even though the causality of energy expenditure is yet to be established, the observation that treatment with SIRT1 activator resveratrol improves PGC-1α expression, mitochondrial function and insulin sensitivity in mice (6) suggests that SIRT1 activators can be potentially used to treat obesity and type 2 diabetes.

We investigated the relationship of energy expenditure in the fasting state and during hyperinsulinemia with insulin sensitivity in humans, and observed that insulin-stimulated increase in EE was strongly associated with insulin sensitivity in offspring of patients with type 2 diabetes. In contrast, fasting energy expenditure did not correlate with insulin sensitivity. In addition, adipose tissue SIRT1 mRNA expression correlated with EE and insulin sensitivity during hyperinsulinemia. Moreover, SIRT1 expression correlated with the expression of several genes regulating mitochondrial function. Similar results have been reported with 8-h insulin infusion that increased mitochondrial mRNA transcript levels, mitochondrial protein synthesis, and ATP production (230).

This response was, however, blunted in type 2 diabetic patients. Thus, impaired mitochondrial fitness could be a consequence of impaired insulin action. Alternatively, primary mitochondrial dysfunction could lead to insulin resistance. A possible explanation for this hypothesis is given by observations that impaired mitochondrial function leads to increased intramyocellular lipid metabolites, such as fatty acyl Coentzyme A and diacylglycerol, which in turn leads to defects in the insulin signalling cascade through insulin receptor substrate-1. In contrast, in mice blunting acetyl-CoA carboxylase-2, an enzyme that catalyzes lipid synthesis and inhibits lipid oxidation, enhanced energy expenditure and reduced intracellular diacylglycerol content leading to lean and insulin sensitive phenotype (231). However, given the fact that our human data is cross-sectional we can not determine the causality of the association between impaired energy expenditure and insulin sensitivity.

6.3 Central nervous system and obesity

Discovery of leptin and leptin receptor were significant breakthroughs in obesity research (48, 49). However, it was disappointment that leptin was not the solution to common polygenic obesity. In fact, leptin appeared to protect humans from starvation, not from obesity. Actually obese subjects have high leptin levels but its effect is blunted because of leptin resistance. Nevertheless, leptin studies demonstrated how the inability of the CNS to sense body's energy balance can lead to obesity. Later rare genetic mutations were recognized in the leptin and melanocortin pathways causing impaired energy balance sensing in the CNS and leading to early-onset obesity in humans (4). The melanocortin system, and especially MC4R, is as an important mediator of energy balance downstream from leptin (7). Especially mutations in gene encoding MC4R were found to cause obesity in 3-5 % in child patients presenting severe obesity at young age. Similar results have been presented for mutations in the leptin receptor gene (193). Even though these examples of rare monogenic forms of obesity do not explain obesity at the population level, they give important clues about the mechanisms related to obesity. For example, all mutations causing monogenic obesity discovered so far mediate their effects via the CNS emphasizing the role of the CNS in obesity. These uncommon cases may also provide new insights to the regulation of energy balance. An innovative way to study the effects of leptin was recently demonstrated in leptin deficient patients. These patients were shown pictures of food and functional magnetic resonance imaging was performed to measure their brain responses in leptin deficient state and after leptin treatment (232). In leptin deficient state patients felt figures more attractive and neural circuits governing food intake

were more active. This example demonstrates the significance of emotions (e.g. motivation and rewarding) in the pathogenesis of obesity**.**

Recent development in the genetics have expanded our knowledge and revealed the essential role of the CNS as a regulator of polygenetic obesity (213). The first example was the discovery of *FTO* (208, 233) that is the gene most strongly associated with polygenic obesity discovered so far. FTO is robustly expressed in hypothalamic neurons and it is considered to take part in nucleic acid demethylation (210). Common polymorphisms of *MC4R* are also recognized as risk genes for polygenic obesity (211, 212).

The mechanisms leading to obesity in MC4R-KO mouse are increased energy intake and impaired energy expenditure (144). In humans, a mutation in *MC4R* can cause extreme obesity and babies who are homozygous for the mutation can have a complete loss of MC4R function which can lead to obesity at the age of 3-4 months, whereas heterozygous mutation carriers present milder forms of obesity (127). In contrast, the Val103Ile substitution of *MC4R* (the minor allele frequency is ~1%), appears to protect from obesity (234). We observed that the Val103Ile substitution of *MC4R* was associated with high rates of energy expenditure in the fasting state. This suggests that variants in *MC4R* in humans could regulate also energy expenditure, not only energy intake. Thus, genetic variation in *MC4R* is able to drive energy balance in both directions. Animal studies have revealed that the melanocortin system does not only regulate whole body energy balance but also takes part in peripheral lipid metabolism which may have an important role in the development of metabolic diseases (8). An interesting obesity phenotype is presented by MC3R-KO mouse that has normal weight but increased adiposity possibly due to inability to increase lipid oxidation (121). Indeed, we observed that the carriers of the inactivating (235) Lys 6 and Ile 81 alleles in the coding region of *MC3R* had lower lipid oxidation and higher glucose oxidation compared to those of the Thr/Thr 6 and Val/Val 81 genotypes without a difference in energy expenditure or obesity. Even though the role of MC3R in obesity is not yet fully established it is possible that MC3R has an autoregulatory effect on the activity of AGRP and POMC neurons in the regulation of substrate oxidation. In addition, MC3R is expressed in adipocytes and leukocytes, and it has been shown to affect the immune system and inflammation (236, 237). Therefore, it is possible that the effect of MC3R is mediated peripherally. The melanocortin system affects also the autonomic nervous system, and it has been showed in animals (238) and humans (9) that melanocortin activity leads to higher blood pressure. These effects of the melanocortin system should not be overlooked since they may have a major impact in the treatment of obesity.

MCHR-1 is an interesting regulator of energy balance because unlike many other energy balance regulating receptors of the CNS, antagonism (not agonism) of MCHR1 leads to leanness (158). This

makes MCHR-1 an attractive target for drug development. Indeed, blockers of this receptor could potentially be used to treat obesity (157, 239). However, our knowledge regarding MCHR-1 is mostly based on animal studies and the role of this receptor in humans is limited. In animals antagonists of this receptor have shown to affect also emotions and behaviour demonstrating the complex nature of this system. Our purpose was to examine if SNPs close to *MCHR-1*affect obesity or metabolic traits in humans as previously suggested (240). However, we could not confirm the association with obesity in a group of 1455 middle-aged men or association with metabolic traits in a group of 217 carefully phenotyped offspring of patients with type 2 diabetes. This may reflect insufficient statistical power or true difference between different populations demonstrating the difficulty of studying polygenic diseases such as obesity.

6.4 Concluding remarks

Obesity predisposes to several diseases and especially the prevalence of type 2 diabetes has increased with the obesity epidemic. In the future the treatment of obesity related disease, such as diabetic micro- and macrovascular complications, will consume a growing proportion of health care resources. Therefore, knowledge on the pathophysiology and genetics of obesity is needed to develop new preventive and therapeutic approaches.

Small molecules activating SIRT1 can potentially represent a new class of drugs that can be used to treat metabolic diseases in the future (241). SIRT1 activation is considered to induce more efficient mitochondria that reduce oxidative stress by decreasing production of ROS, the toxic side product of oxidative phosphorylation. Therefore, SIRT1 activation is considered to mimic the positive effects of caloric restriction and lead to healthier metabolic condition and increased lifespan. However, all beneficial effects and possible side effects of these ubiquitously expressed molecules, which work as activators of several transcription factors regulating the expression of a wide spectrum of genes, are yet to be determined.

Increasing knowledge from genetic background of obesity has emphasized the significance of the CNS in control of body weight. The melanocortin system located in the hypothalamus is an important regulator of energy balance (7). These complex neuronal networks are closely related to higher cognitive and emotional functions and autonomic nervous system, forming a major challenge for drug development against obesity. Future studies should be aimed to elucidate these neuronal networks and neurotransmitters that control human behaviour, rewarding and motivation. The development of functional brain imaging may give new insights to this challenging field of neuroscience where feasibility of animal models is limited (232).

Although risk genes identified by GWA studies give important clues to the pathophysiology of polygenic diseases, the risk alleles of eight identified obesity loci account only about 1 % of the entire variation of BMI (213), even though 70 % of variation of BMI is hereditary. Similarly, the risk alleles of 30 loci contributing to dyslipidemia account about 6-8 % of the entire variation, leaving a considerable amount of heritability unexplained (242). These observations have led to search more sophisticated methods beyond the DNA sequence to explore heritability of diseases. Therefore, regulations of gene expression have gained increasing attention in recent studies. Since identified SNPs only rarely are located in the coding sequence of genes, the variation in the promotor or regulatory regions that regulate gene expression and RNA processing (including alternative splicing) are likely to be important factors regulating susceptibility for polygenic diseases. Indeed, preliminary reports have shown that gene expression, that can be considered as quantitative trait (expression quantitative trait loci, eQTL), is inherited and closely related to metabolic diseases like obesity (243, 244). Methods that combine genotype, gene expression and clinical phenotype provide a step forward in recognizing metabolic pathways and causality in the pathophysiology of diseases. In the future, collecting genotype - gene expression databases that include samples from different tissues are needed to make progress in the genetics of complex diseases (245).

The information in DNA sequence is not fully explained by SNPs. Other variations in DNA sequence, such as copy number variants (repeated DNA segments that may range from one kilobase from to several megabases), insertions or deletions may also contain important information. For example, copy number variants have been shown to have significant effects (246). However, copy number variants have been sequenced only in a small number of individuals so far (247), and therefore this remarkable variation in human genome is largely unexplored. In addition, a recent study revealed the first genomic deletion, 45-kb located close *NEGR1*, which predisposes to obesity (213).

The DNA sequence is not the only regulator of gene expression. So called epigenetic factors that are inherited but independent from DNA sequence take also part in regulation of gene expression. Epigenetics include DNA methylation and histone modification. DNA methylation occurs exclusively in cytosine (C) residues of the DNA. In mammals the DNA is globally methylated with the exception of so called CpG islands, which are DNA segments where CG dinucleotide occurs with its expected frequency, in contrast CG dinucleotide is depleted elsewhere in the genome. Even though the roles of methylation are not yet entirely established, it has been speculated that DNA methylation functions to maintain repressed chromatin state and stabilize the genome (248). Therefore, the methylation of promoters has been implicated to have silencing effect on gene

transcription. Given the fact that methylation demonstrates family clustering with intra-individual change during time (249), methylation provides dynamic and hereditary trait to regulate gene expression that still remains unexplored. Histones are important in chromatin structure. Unlike DNA methylation that has long term silencing effect for genes, histone modification is a dynamic process that provides regulation for rapid transcription and repair of DNA (250).

In addition, new methods to study gene-gene and gene-environment interactions are needed. Interactions are important mechanisms that can mask significant genetic associations. If the effect of a gene variant is dependent of other genetic or environmental factor, the net effect in entire study population can be neutral and association can is easily be missed. To identify these complex interactions larger population-based samples are needed. In addition, adequate phenotyping, which is often neglected to enlarge sample size, is necessary to make statistical sub-analyses. New innovative methods are also developed, these include systems biology based approach. These methods are based on the hypothesis that traits are not simply a sum of genetic variations. Instead, certain combinations of variants reflect system networks of environmental and genetic effects that contribute to traits and these networks can be recognized by combining information from several genetic variants, eQTLs and phenotypes (251, 252).

The methodology in genetic studies of obesity has taken huge steps during the last years, but still new methods need to be applied, including methods assessing the roles of epigenetics, mRNA processing and small non-coding RNAs (e.g. miRNAs).. Together with the systems biology approach these methods provide a step forward in recognizing metabolic pathways, their disturbances and causality in the development of disease and help developing new therapeutic options.

7 Main findings of the Studies I-IV

Study I showed that the insulin-stimulated increase in energy expenditure was strongly associated with insulin sensitivity in offspring of patients with type 2 diabetes. Furthermore, adipose tissue SIRT1 mRNA expression correlated with energy expenditure, insulin sensitivity and expression of several genes regulating mitochondrial function. Therefore, compromised mitochondrial function, coordinated by low SIRT1 expression, is likely to contribute to insulin resistance and to type 2 diabetes.

In Study II the Val103Ile substitution of *MC4R* was associated with high rates of energy expenditure in the fasting state and glucose oxidation and with low levels of FFAs. Therefore, the Val103Ile polymorphism of *MC4R* may determine the rates of energy expenditure and substrate oxidation in humans.

In Study III we observed that polymorphisms of *MC3R* affect substrate oxidation and first-phase insulin secretion. The carriers of the Lys 6 and Ile 81 alleles in the coding region of *MC3R* had lower lipid oxidation, lower FFA levels, higher glucose oxidation and higher first phase insulin secretion compared to subjects with the Thr/Thr 6 and Val/Val 81 genotypes.

In Study IV we could not demonstrate an association of SNPs of *MCHR1* with metabolic variables.

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