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Doctoral Thesis

Susceptibility variants for obesity and type 2 *diabetes mellitus* in the population of Latvia

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ABSTRACT

Obesity and type 2 *diabetes mellitus* (T2D) are common multifactorial diseases with linked pathogenesis and displaying rapid increase in prevalence promoted by modern lifestyle in interaction with genetic factors. Different study approaches have highlighted numerous susceptibility loci for both traits. The aim of the doctoral thesis was to investigate the role of genetic variation within loci comprising Agouti related protein homolog (*AGRP*), adiponectin, C1Q and collagen domain containing (*ADIPOQ*), transcription factor 7 like 2 (*TCF7L2*), fat mass and obesity associated (*FTO*) and transmembrane protein 18 (*TMEM18*) genes in susceptibility to obesity and T2D in the population of Latvia. The studies presented in the doctoral thesis demonstrate novel association of intronic variant rs11575892 in *AGRP* with increased body mass index (BMI). Although possible functional impact of this variant is unclear, it may lead to the altered splicing or expression of the gene. On the contrary, there was no correlation between single nucleotide polymorphisms (SNPs) within *ADIPOQ* locus with BMI or T2D suggesting that genotyped SNPs unlikely represent significant risk factors for these traits, although they may have an impact on pathogenesis of cardiovascular diseases. We further evaluated contribution of SNPs in *TCF7L2* to T2D susceptibility in our population. Polymorphisms rs12255372, rs7903146 and rs7901695 were significantly associated with T2D with stronger effects observed in non-obese, which agrees with findings in other populations. Investigation of obesity related variants located in the 1th intron of *FTO* and near *TMEM18* confirmed the association with T2D and in the case of *FTO* it retained significance after correction for BMI. Polymorphism rs57103849 in the 4th intron of *FTO* and rs7561317 near *TMEM18* were correlated with younger age at diagnosis of T2D independently of BMI. Together obtained results support the evidence that variants representing *FTO* and *TMEM18* loci may increase T2D risk through the mechanisms other than those triggered by secondary effects of obesity.

KOPSAVILKUMS

Aptaukošanās un 2. tipa cukura diabēts (T2D) ir multifaktoriālas slimības ar saistītu patofizioloģiju, kuru prevalences straujo pieaugumu ir veicinājušas izmaiņas dzīvesveidā mijiedarbībā ar ģenētiskajiem faktoriem. Dažādas pētnieciskās pieejas ir veicinājušas daudzu ar dotajām pazīmēm saistītu lokusu atklāšanu. Doktora darbā apkopoto pētījumu mērķis bija noskaidrot Agouti radniecīgā proteīna homologa (*AGRP*), adiponektīna (*C1Q and collagen domain containing, ADIPOQ*), transkripcijas faktoram 7 līdzīgā 2 (*TCF7L2*), ar tauku masu un adipozitāti saistītā (*fat mass and obesity related, FTO*) un transmembrānas proteīna 18 (*TMEM18*) gēnos esošo ģenētisko variantu nozīmi saistībā ar aptaukošanos un T2D Latvijas populācijā. Iegūtie pētījumu rezultāti parāda līdz šim neaprakstītu saistību starp variantu rs11575892 *AGRP* gēna otrajā intronā un palielinātu ķermeņa masas indeksu (ĶMI). Lai gan šī varianta funkcionālā ietekme nav skaidra, iespējams tas izraisa izmaiņas splaisinga vai gēna ekspresijas regulācijā. Savukārt *ADIPOQ* lokusā esošie viena nukleotīda polimorfismi (SNP) būtisku saistību ar ĶMI vai T2D neuzrādīja, norādot, ka šīs izmaiņas nav nozīmīgi T2D vai ĶMI ietekmējoši faktori, bet, iespējams, ietekmē sirds asinsvadu slimību attīstības risku pētāmajā paraugkopā. Izvērtējot *TCF7L2* lokalizēto variantu ietekmi uz T2D, tika parādīts, ka, līdzīgi citās populācijās novērotajam, rs12255372, rs7903146 un rs7901695 būtiski saistās ar T2D, spēcīgāk izpaužoties pacientiem ar normālu svaru. Savukārt, *FTO* pirmajā intronā un blakus *TMEM18* esošie polimorfismu pētījumi apstiprināja to asociāciju ar T2D, pie tam *FTO* gadījumā saistība saglabājās, veicot korekciju pēc ĶMI. Polimorfisms rs57103849 *FTO* ceturtajā intronā un rs7561317 blakus *TMEM18* asociējās ar agrāku T2D diagnosticēšanas vecumu neatkarīgi no ĶMI. Kopumā iegūtie dati apstiprina iespēju, ka *FTO* un *TMEM18* lokusos esošie varianti paaugstina T2D risku neatkarīgi no aptaukošanās izraisītas negatīvas sekundāras ietekmes uz T2D attīstību.

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ABBREVIATIONS

A2BP1	ataxin binding protein 1 gene
ABBC8	ATP-binding cassette, sub-family C(CFTR/MRP), member 8 gene
ACC	acetyl-CoA carboxylase
Acyl-CoA	acyl coenzyme A
Acetyl-CoA	acetyl coenzyme A
ACTH	adrenocorticotropic hormone
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9 gene
ADCY5	adenylate cyclase 5
Add	additive
ADIPOQ	adiponectin, C1Q and collagen domain containing gene
ADIPOR1	diponectin receptor 1
ADIPOR2	diponectin receptor 2
ADP	adenosine diphosphate
AGEs	advanced glycation endproducts
AGPAT2	acylglycerol-3-phosphate O-acyltransferase 2 gene
AGRP	Agouti related protein
AGRG	Agouti related protein homolog (mouse) gene
Akt	protein kinase B (PKB)
AMPK	AMP-activated protein kinase
APPL1	adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1
ARC	arcuate nucleus
ARL15	ADP-ribosylation factor-like 15 gene
ASK1	apoptosis signal-regulating kinase 1
ATF4	activating transcription factor-4
ATF6	activating transcription factor-6
ATP	adenosine triphosphate
BAD	BCL2 agonist of cell death
BCL2	B-cell CLL/lymphoma 2
BDNF	brain derived neurotrophic factor
BiP/GRP78	chaperone/glucose regulated protein 78
BMI	body mass index
BSCL2	Berardinelli-Seip congenital lipodystrophy 2 (seipin) gene
CADM2	cell adhesion molecule 2 gene
cAMP	cyclic adenosine monophosphate
CAP	CBL associated protein
CAPN10	calpain-10 gene
CART	cocaine and amphetamine regulated transcript
CaSR	calcium sensing receptors
CB1	cannabinoid receptor 1
CBL	Cbl proto-oncogene
CCK	cholecystokinin
CCK1R	cholecystokinin 1 receptor
CDC123/CAMK1D	cell division cycle 123/calcium/calmodulin-dependent protein kinase ID genes
CDH13	cadherin 13 gene
CDKAL1	CDK5regulatory subunit associated protein 1-like 1 gene
CDKN2A/B	cyclin-dependent kinase inhibitor 2A/B gene
CDP138	138 kDa C2 domain-containing phosphoprotein
CEU	Utah residents with ancestry from northern and western Europe
CHD	coronary heart disease
CHOP	CCAAT/enhancer-binding protein homologous protein
CI95%	confidence interval 95%
CISD2	CDGSH iron sulfur domain 2 gene
CLIP	corticotropin-like-intermediate lobe peptide
CMIP	c-Maf inducing protein gene
CNR1	endocannabinoid receptor 1 gene
CNS	central nervous system
CNV	copy number variants
CRH	corticotropin releasing hormone
CRK	v-crk avian sarcoma virus CT10 oncogene homolog
	transforming sequence homologue
CUX1	cut-like homeobox 1
DAG	diacylglycerol
DAPK	death-associated protein kinase
ddNTP	dideoxyribonucleotide
DDR2 and *4*	dopamine receptor 2 and 4 genes
DEND	developmental delay, epilepsy and neonatal diabetes
dNTP	deoxyribonucleotide
ddNTP	dideoxyribonucleotide
DMN	dorsomedial hypothalamic nucleus
Dom	dominant
DUSP9	phosphatase 9 gene
4E-BP1	eIF4E-binding protein 1
EIF2AK3	immediate early response 3 interacting protein 1 gene
ENCODE	The Encyclopedia of DNA elements project
ENPP1	ctonucleotide pyrophosphatase/ phosphodiesterase 1 gene
Epac2A	exchange protein activated by cAMP 2A
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FOXO1	transcription factors forkhead box O1
FTO	fat mass and obesity associated gene
G6P	glucose-6-phosphatase
Gab1	Grb2 associated binding protein 1
GAD2	glutamic acid decarboxylase 2 gene
GALNT10	polypeptide N-acetylgalactosaminyl-transferase 10 gene
GCK	glucokinase (hexokinase 4)
GCKR	glucokinase (hexokinase 4) regulator Gene
GI	glycemic index
GIP	gastric inhibitory polypeptide
GIPR	gastric inhibitory polypeptide receptor gene
GLIS3	GLIS family zinc finger 3 gene
GLM	general linear model
GLO1	glyoxalase 1 gene
GLP1	glucagon-like peptide 1
GLUT	glucose transporter
GPCR	G-protein coupled receptors
GPR120	G-protein coupling receptor 120

GPR40	G-protein coupling receptor 40
GPRC5B	G protein-coupled receptor, class C, group 5, member B gene
GRB14	growth factor receptor bound protein 14 gene
GRB2	growth factor receptor bound 2
GPX1	glutathione peroxidase 1
GSIS	glucosestimulated insulin secretion
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GWA	genome wide association
HHEX	hematopoietically expressed homeobox gene
HIF1α	hypoxia-inducible factor-1α
HMG20A	high mobility group 20A gene
HNF1A	hepatocyte nuclear factor 1 homeobox A gene
HNF1α	hepatocyte nuclear factor 1α
HNF4α	hepatocyte nuclear factor 4α
HNF4A	hepatocyte nuclear factor 1 homeobox A gene
HNF1B	hepatocyte nuclear factor 1 homeobox B gene
HNF1β	hepatocyte nuclear factor 1β
HOMA-B	homeostasis model assessment of beta cell function
HOMA-IR	homeostasis model assessment of insulin resistance
HOXB5	homeobox B5 gene
HPA	hypothalamic-pituitary-adrenal axis
HPT	hypothalamic-pituitary-thyroid axis
HWE	Hardy-Weinberg equilibrium
ICD-10	International Classification of Diseases
icv	intracerebroventricular
IGF1	insulin-like growth factor 1 gene
IGF2BP2	insulin-like growth factor 2 mRNA binding protein 2 gene
IKKβ	inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta
IL10	interleukin 10
IL6	interleukin 6
INS	insulin gene
INSR	insulin receptor gene
IP3	inositol triphosphate
IR	insulin receptor
IRE1	inositol-requiring transmembrane kinase/endoribonuclease 1
IRS	insulin receptor substrate
IRX3	iroquois homeobox 3 gene
JAK2	Janus activated kinase 2
JAZF1	JAZF zinc finger 1 gene
JNK	c-Jun N-terminal protein kinase
K_{ATP}	ATP sensitive potassium
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11 gene
KCNQ1	KQT-like subfamily, member 1 gene
KLF11	Krüppel-like factor 11 gene
KNG1	kininogen 1 gene
LD	linkage disequilibrium
LEP	leptin
LEPR	leptin receptor
LGDB	Latvian Genome Data Base
LHA	lateral hypothalamic area
logBMI	logarithmically transformed BMI
LSRPD	Latvian State Research Program Database
LYPLAL1	lysophospholipase-like 1 gene
MAF	minor allele frequency
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAP2K3	mitogen-activated protein kinase kinase 3 gene
MAP2K4	mitogen-activated protein kinase kinase 4
MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4
MAPK	mitogen activated protein kinase
MC1R	melanocortin 1 receptor
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MCP-1	monocyte chemotactic protein-1
MEK	MAPK and ERK kinase
MIDD	maternally inherited diabetes and deafness
MIR148A/NFE2L3	microRNA 148a/nuclear factor erythroid 2-like 3 genes
MNK	MAPK-interacting kinases
MODY	maturity onset diabetes of the young
MRAP2	melanocortin 2 receptor accessory protein 2 gene
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormones
MSK	mitogen and stress activated kinases
MTNR1B	melatonin receptor 1B gene
mTORC	mammalian target of rapamicin complex
MTTL1	mitochondrially encoded tRNA leucine 1 gene
n	number
NAMPT	nicotinamide phosphoribosyltransferase or visfatin gene
NDM	neonatal *diabetes mellitus*
NEGR1	neuronal growth regulator 1 gene
NEUROD1	neuronal differentiation 1
NF-kB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NOTHC2	notch 2 gene
NPY	neuropeptide Y
NRXN3	neurexin 3 gene
ns	non-significant
NTRK2	neurotrophic tyrosine kinase receptor type 2 gene
NTS	nucleus of the solitary tract
OGTT	oral glucose tolerance test
OLFM4	olfactomedin 4 gene
OR	odds ratio
MAPK	p38 mitogen-activated protein kinase
PAX4	paired box 4 gene
p-perm	p - values derived performing permutation tests
PCR	polymerase chain reaction
PCSK1	proprotein convertase subtilisin/kexin type 1
PCSK2	proprotein convertase subtilisin/kexin type 2
PDE3B	phosphodiesterase 3B
PDK1	phosphoinositide-dependent kinase 1
PDX1	pancreatic and duodenal homeobox 1
PEPCK	phosphoenolpyruvate carboxykinase
PEPD	peptidase D gene

PERK	double stranded RNA activated protein kinase (PKR) like endoplasmic reticulum kinase
PGC1α	peroxisome proliferative activated receptor-γ coactivator 1α
PI3K	phosphatidylinositol 3-kinase
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (also GRB1) gene
PKA	protein kinase A
PKC	protein kinase C
PLAGL1	pleiomorphic adenoma gene-like 1 gene
PLC	phospholipase C
PNDM	permanent neonatal *diabetes mellitus*
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PPARG	peroxisome proliferator activated receptor gamma gene
PPARγ	peroxisome proliferator activated receptor gamma
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3 gene
PSMA6	proteasome (prosome, macropain) subunit, alpha type, 6 gene
PTF1A	pancreas specific transcription factor 1a
PTP1B	protein tyrosine phosphatase 1B
PTPN1	protein tyrosine phosphatase, non-receptor type 1 gene
PVN	paraventricular nucleus of hypothalamus
PWS	Prader-Willi syndrome gene
PYY	peptide tyrosine-tyrosine
QPCTL/GIPR	glutaminyl-peptide cyclotransferase-like/gastric inhibitory polypeptide receptor genes
RAF	risk allele frequency
Raf	1v-raf1 murine leukemia viral oncogene homolog 1
RBMS1	RNA binding motif, single stranded interacting protein 1 gene
Rec	recessive
RFLP	restriction fragment length polymorphism
RND3	Rho family GTPase 3 gene
ROI	region of interest
ROS	reactive oxygen species
RSK	p90 ribosomal S6 kinase
S6K1	ribosomal protein S6 kinase 70-KD 1
SAP	shrimp alkaline phosphatase
SBE	single base extension reaction
SD	standart deviation
SEC16B	SEC16 homolog B (S.cerevisiae) gene
SGLT1	sodium dependent glucose transporter 1
SH2B1 and 2	SH2B adaptor protein 1 and 2
Shc	Src homology 2 containing transforming proteins
SIM1	singleminded homolog 1 gene
SLC30A8	solute carrier family 30 (zinc transporter), member 8 gene
SLC39A8	solute carrier family 39 (zinc transporter), member 8
SLC6A14	amino acid transporter solute carrier family 6 member 14 gene
SNARE	soluble N-ethylmaleimide-sensitive factor activating protein receptor
SNAP25	synaptosomal-associated protein 25kDa
SNORD116	snoRNA C/D box 116-1
snoRNA	small nucleolar RNA
SNP	single nucleotide polymorphism
SOCS3	suppressor of cytokine signalling 3
SOD1	superoxide dismutase 1
SOS	son of sevenless
Sp1	trans-acting transcription factor 1
SPRY2	sprouty homolog 2 gene
SREBP1	sterol regulatory element binding protein 1
STAT3	signal tranducer and activator of transcription 3
T1D	type 1 *diabetes mellitus*
T2D	type 2 *diabetes mellitus*
tagSNP	single nucleotide polymorphism used to capture other SNPs in LD
TBC1D1	TBC1 domain family member 1
TBC1D4	TBC1 domain family, member 4 (or AS160)
TC10	C3G activating ras homolog family member Q (or RHOQ)
TCF4	T cell specific transcription factor 4
TCF7L2	transcription factor 7 like 2 gene
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta) gene
THADA	thyroid adenoma associated gene
TMEM18	transmembrane 18 gene
TNFα	tumor necrosis factor α
TRH	thyrotropin-releasing hormone
TRKB	tyrosine kinase receptor B
TSPAN8/LGR5	tetraspanin 8/leucine-rich repeat containing G protein-coupled receptor 5 genes
UBE2E2	ubiquitin-conjugating enzyme E2E 2 gene
UPR	unfolded protein response
VAMP2	vesicle-associated membrane protein 2 (synaptobrevin 2)
VMN	ventromedial nucleus
WFS1	Wolfram syndrome 1 (wolframin) gene
WHR	waist to hip ratio
WWOX	WW domain containing oxidoreductase gene
XBP1	X-box binding protein 1
Y1R	neuropeptide Y receptor Y1
Y5R	neuropeptide Y receptor Y5
ZFAND3	zinc finger, AN1-type domain 3 gene

INTRODUCTION

Obesity and type 2 *diabetes mellitus* (T2D) are complex metabolic diseases with linked pathogenesis representing a major health burden today. The rapid increase in prevalence of these diseases is thought to be promoted by relatively recent changes in lifestyle, characterised by higher consumption of energy rich foods and decreased physical activity, providing necessary background for genetic susceptibility to manifest as an unfavourable phenotype.

Studies of monogenic forms of the diseases and biologically plausible candidate genes have provided first insight in the pathogenic mechanisms of the both complex traits and genetic factors contributing to the development of disease. Among the genes, discovered by linkage and candidate gene approaches harbouring susceptibility variants for common type obesity were those coding for factors relevant to central regulation of energy homeostasis like leptin and members of melanocortin system including melanocortin 4 receptor (MC4R) and Agouti related protein (AGRP). The search for genetic causes of T2D in linkage studies have led to the discovery of one of the most significant T2D susceptibility genes known to date, transcription factor 7 like 2 (*TCF7L2*), as well as highlighted other loci such as one associated with multiple components of metabolic syndrome comprising gene coding for adiponectin (*ADIPOQ*). However, the most successful approaches in terms of discovery of novel susceptibility loci for common traits have been genome wide association (GWA) studies. So far GWA studies have revealed more than 30 obesity related loci and approximately 60 risk loci for T2D. Examples of newly discovered susceptibility loci represent fat mass and obesity associated (*FTO*) and transmembrane 18 (*TMEM18*) genes. Common variants within the *FTO* and near *TMEM18* are among the most highly associated obesity risk variants. Apart from that, there is evidence towards their impact on T2D risk.

However, known disease susceptibility loci explain only small proportion of variation in each of the traits attributable to heritable factors. Factors contributing to "missing" heritability may be risk variants found below detection threshold of currently used methods, loss of effects strength due to incomplete correlation between marker and causal variant or existence of additional independent associated variants within the same loci masked by the strongest SNPs. Considering the evidence indicating association of obesity susceptibility loci with T2D risk, it could be speculated that apart from being linked on physiological level both traits could have a shared genetic component. Yet, it is still not clear whether observed associations could not be explained solely by confounding effects of increased adiposity.

The aim of the research was to investigate the role of genetic variation representing risk genes described by association studies in susceptibility to obesity and type 2 *diabetes mellitus* in the population of Latvia. To achieve the proposed aim of the study following tasks were set:

1. Assess genetic variation within *AGRP* gene and its flanking regions and estimate the correlation between the variants present in the locus and body mass index;
2. Investigate the role of common polymorphisms representing promoter and coding regions of *ADIPOQ* gene in the determination of body mass index and risk of type 2 *diabetes mellitus*;
3. Evaluate significance of *TCF7L2* intronic variation in context of increased risk of type 2 *diabetes mellitus* in the population in Latvia;
4. Estimate the impact of common variants within the obesity related loci comprising *FTO* and *TMEM18* genes on type 2 *diabetes mellitus*.

1 LITERATURE REVIEW

1.1 Regulation of energy homeostasis

Despite obvious changes in daily energy intake body weight in adult animals and humans normally remains relatively stable showing only minor variation throughout life. This stability is ensured by complex system of numerous well coordinated central and peripheral signalling mechanisms responsible for maintenance of energy homeostasis - balance between ingested and expended energy. Processes ensuring energy homeostasis are conducted by central nervous system (CNS). In order to adjust energy intake with metabolic demands brain regions responsible for energy turnover, particularly hypothalamus, receive multiple metabolic, hormonal and neuronal signals carrying information about current energy status of the body from digestive system, adipose tissues and other organs. The CNS uses this information to change behavioural components such as appetite and satiety, as well as metabolism and energy expenditure according to acute and regular fuel requirements of the body (Blundell and Halford, 1994; Benelam, 2009; Harrold et al., 2012; Hill et al., 2012).

1.1.1 Peripheral mediators of energy homeostasis

Initial phases of food intake are stimulated by sensory signals such as sight, smell and taste as well as chewing of food. These signals serve to prepare body for food processing as well as provide buffer against sharp changes in circulating nutrients by activating release of hormones via CNS (Berthoud et al., 1981; Teff, 2000; Just et al., 2008; Harrold et al., 2012).

Peripheral signals triggered during food digestion and postdigestive or postabsorptive phases originate predominantly from gastrointestinal tract, pancreas and adipose tissue. These signals can be mechanosensory stimuli, nutrients absorbed from gut into circulation and hormones acting in paracrine and endocrine manner (Ashcroft, 1968; Geliebter, 1988; Maffei et al., 1995; Cecil et al., 1999; Gribble et al., 2003; Liou et al., 2010; Harrold et al., 2012; Grayson et al., 2013). According to the time scale of their action peripheral regulators of energy turnover can be divided into two categories as short term and long term signals. Both short term and long term signals control amount of consumed food and are involved in stimulation of satiation and satiety, components limiting appetite or desire to consume food. Satiation is feeling of fullness that aids termination of the meal, which is triggered in specific areas of brain by short-lived peripheral mechanical and hormonal signals in response to food being consumed (Blundell and Halford, 1994; Benelam, 2009). Long acting peripheral signals inform the central nervous system about the state of total body energy reserves. These signals contribute predominantly to satiety. Satiety is the period after meal during which further energy intake is suppressed until hunger or a drive to eat reappears (Blundell and Halford, 1994; Benelam, 2009).

1.1.1.1 Short term regulators of food intake

Mechanoreceptors

During meal food entering the stomach and small intestine activates mechanoreceptors of vagal afferent nerves located within mucosa and external muscle layers sensitive to mechanical touch and stretch (Page and Blackshaw, 1998; Phillips and Powley, 2000; Page et al., 2002). Signals regarding particle density and volume of consumed food from gastrointestinal mechanosensors are transmitted to brainstem,

primarily nucleus of the solitary tract (NTS), and further to higher neural centres where processing of this information occurs (Gonzalez and Deutsch, 1981; McIntyre et al., 1997; Huo et al., 2007). Consequently, the command to terminate the meal is distributed. These preabsorptive regulatory mechanisms determine the size of a single meal depending on a volume of consumed food regardless of nutrient composition (Geliebter, 1988; Cecil et al., 1999; Wang et al., 2008). Their impact on food intake dissolves, when gastric emptying occurs and tension of stomach returns to normal (Gonzalez and Deutsch, 1981; Cecil et al., 1999; Huo et al., 2007; Benelam, 2009).

Gastrointestinal hormones

Gastrointestinal chemosensory mechanisms, on their turn, enable recognition of nutrient composition of consumed food. Information on nutrients entering gastrointestinal lumen is transmitted mainly by enteroendocrine cells playing the role of chemosensitive mediators between lumen and vagal afferent nerve terminals as well as circulatory system delivering information to the CNS (Gribble et al., 2003; Matsumura et al., 2005; Steinert and Beglinger, 2011; Harrold et al., 2012; Mace et al., 2012; Rasoamanana et al., 2012). Nutrients like glucose, free fatty acids and amino acids regulate secretion of secondary messengers from enteroendocrine cells in a dose dependent manner. Glucose sensing by endocrine cells involves active transport via sodium dependent glucose co-transporter 1 (SGLT-1) and facilitated glucose transporter 2 (GLUT2) as well as activation of sweet taste receptors on the surface of endocrine cells analogous to those found on taste buds (Gribble et al., 2003; Gerspach et al., 2011; Mace et al., 2012). Detection of products of protein digestion is mediated by extracellular calcium sensing receptors (CaSR), oligopeptide transporter 1 (PEPT1) and amino acid transporter solute carrier family 38 member 2 (SLC38A2) (Matsumura et al., 2005; Tolhurst et al., 2011; Mace et al., 2012), whereas free fatty acids bind G-protein coupling receptors 40 and 120 (GPR40 and GPR120) (Tanaka et al., 2008; Liou et al., 2010; Xiong et al., 2013). The secondary messengers released by endocrine cells in response to nutrient stimuli include cholecystokinin (CCK), glucagon-like peptide 1 (GLP1), peptide tyrosine-tyrosine (PYY) as well as other peptide hormones. Products of fat and protein digestion are major postprandial stimulators of CCK and PYY release (Tanaka et al., 2008; Liou et al., 2010; Mace et al., 2012). GLP1 is released predominantly in response to luminal sugars (Gerspach et al., 2011; Mace et al., 2012). These hormones act as anorexigenic satiation signals that increase the feeling of fullness thus reducing the size and duration of ongoing meal (French et al., 1993; Gutzwiller et al., 1999; Batterham et al., 2002; Bellinger and Bernardis, 2002; Harrold et al., 2012). An exception is ghrelin, which is the only one among peripherally derived hormones acting as an orexigenic signal to stimulate food intake (Druce et al., 2005; Harrold et al., 2012). Production of ghrelin increases during fast reaching its highest levels immediately before meal and rapidly falls after food is consumed, with secretion pattern following circadian rhythm and habitual meal times (Cummings et al., 2001; Natalucci et al., 2005). Nutrient triggered reduction in circulating ghrelin is thought to be predominantly mediated by postabsorptive events like changes in plasma insulin and intestinal osmolarity, since gastric distension and chemical sensing of nutrients does not affect levels of the peptide (Williams et al., 2003; Overduin et al., 2005; Gagnon and Anini, 2012).

Paracrine activation of receptors expressed on the terminals of vagus nerve by peptide hormones has been suggested as the main mechanism through which information is delivered to the areas within brainstem such as the NTS and *area postrema* that are responsible for control of feeding, although some gastrointestinal hormones like GLP1 travel through the circulation and reach areas of CNS with relaxed blood brain barrier

(Moran et al., 1997; Kanoski et al., 2011; Harrold et al., 2012). Processing of the incoming information delivered via vagus nerve and formation of response partly occurs on the site within the local brainstem circuits as well as is forwarded to the hypothalamus (Norgren, 1978; Huo et al., 2007; Grill and Hayes, 2009; Harrold et al., 2012).

1.1.1.2 Long term regulators of food intake

Although short term regulators like GLP1 and PYY have been shown to contribute to changes in body weight, their primary function is to prevent overconsumption of calories during a sinlge meal (Vrang et al., 2006; Woods and D'Alessio, 2008; Garber et al., 2011). Over longer periods their effects on food intake are balanced with accumulated energy stores. Negative feedback to the hypothalamic feeding centres consistent with stored energy reserves is provided by long term acting „adiposity signals" insulin and leptin secreted proportionally to fat mass (Maffei et al., 1995; Cone, 2005; Benelam, 2009). Central action of insulin and leptin predominantly through the hypothalamic arcuate nucleus (ARC) results in hypophagia in animals and humans (Halaas et al., 1997; Heymsfield et al., 1999; Air et al., 2002; Obici et al., 2002; Hallschmid et al., 2004). Besides acting directly on hypothlamic neurons producing orexigenic and anorexigenic signals leptin and insulin increases sensitivity of the brain towards satiation signals like CCK reducing meal size as long as there are sufficient fat stores (Riedy et al., 1995; Emond et al., 1999). Despite the high circulating levels of insulin and leptin, obesity is characterised by blunted action of both hormones due to hypothalamic resistance and subsequently food intake remains disproportionally high (Lee et al., 2001; Banks et al., 2004; Niswender et al., 2004).

Leptin

Leptin is coded by leptin gene (*LEP* or obese (*OB*) gene) identified in ob/ob mice as the locus bearing mutation responsible for severe obesity observed in homozygous animals (Friedman et al., 1991; Zhang et al., 1994). It is secreted from white adipose tissue and released into the circulation in proportion to body adiposity and adipocyte size (Maffei et al., 1995; Guo et al., 2004). Low levels of leptin have been detected also in other tissue, but physiological significance of the nonadipose tissue production is still to be determined (Masuzaki et al., 1997).

Although leptin release is closely matched to adipose tissue mass over longer period of time, the hormone levels rapidly decrease during fast, exercise and exposure to cold (Trayhurn et al., 1995; Kolaczynski et al., 1996; Weigle et al., 1997; Weimann et al., 1999). The short term fluctuation in plasma leptin occurs before major changes in fat depots and is thought to be an adaptive mechanism preventing excessive loss of energy reserves (Weigle et al., 1997). Inhibition of leptin release due to energy restriction or high energy expenditure is mediated by sympathetic nervous system (Trayhurn et al., 1995; Sivitz et al., 1999). Leptin concentration is negatively correlated also with several circulating factors including growth hormone, peroxisome proliferator activated receptor gamma (PPARγ) agonists, metabolites like free fatty acids and the hormone itself proving the negative feedback loop (De Vos et al., 1996; Zhang et al., 1997; Isozaki et al., 1999; Cammisotto et al., 2003). On the opposite, insulin, glucocorticoids and cytokines like tumour necrosis factor α (TNFα) upregulate expression and secretion of the hormone (Wabitsch et al., 1996; Sarraf et al., 1997). Insulin is one of the most significant determinants of leptin production. Rather than having direct effects it stimulates glucose metabolism in adipocytes which activates transcription factors - sterol regulatory element binding protein 1 (SREBP1), trans-acting transcription factor 1 (Sp1) and others binding leptin gene (Kim et al., 1998; Moreno-Aliaga et al., 2007).

Leptin receptor (LEPR) belongs to glycoprotein 130 family of cytokine receptors (Tartaglia et al., 1995). Inactivating mutation in the *LEPR* gene is a cause of obese phenotype in db/db mice (Chen et al., 1996). Human LEPR has long isoform (LEPRb) and three short isoforms (LEPRa, LEPRc and LEPRd) formed by alternative splicing (Cioffi et al., 1996; Fei et al., 1997; Hileman et al., 2002). Short forms of leptin receptor are involved in its transport across blood brain barrier (Kastin 1999). LEPRb predominantly locates to hypothalamus and is responsible for mediation of leptin action on energy turnover (Chen et al., 1996; Fei et al., 1997). Leptin by binding to its receptor activates Janus activated kinase 2 (JAK2)/signal tranducer and activator of transcription 3 (STAT3) mediated pathway and classical insulin signalling cascade mediated by phosphatidylinositol 3-kinase (PI3K) thus inducing changes in expression of hypothalamic mediators of energy turnover (Niswender et al., 2001; Bates et al., 2003; Niswender et al., 2004). Its ability to activate PI3K confirms that there is a crosstalk between the pathways induced by the two adiposity signals also on the molecular level (Niswender et al., 2001; Niswender et al., 2003).

Insulin

Since its isolation from pancreatic tissue in early twenties of 20th century insulin has been widely studied for its role as a major peripheral regulator of glucose metabolism (Banting et al., 1922). Functions of insulin as an "adiposity signal" also are based on its action on central satiety centers, mainly hypothalamic ARC (Obici et al., 2002; Niswender et al., 2003; Hallschmid et al., 2004; Niswender et al., 2004).

Insulin is expressed and released into circulation by beta cells found in pancreatic islets of Langerhans (Banting et al., 1922). The secretion of the hormone peaks along with postpradial rise of glucose, although small amounts of insulin are secreted constantly (Sanger and Tuppy, 1951). Insulin gene (*INS*) is coding for proinsulin precursor peptide which is post-translationally processed into mature hormone formed by 21 amino acid A chain peptide and 30 amino acid B chain peptide covalently linked with two disulfide bonds (Ullrich et al., 1985; Davidson and Hutton, 1987; Smeekens et al., 1992).

Insulin receptor (IR) is homodimer formed by monomers each consisting of ligand binding alpha subunit and beta subunit comprising tyrosine kinase domain (Kasuga et al., 1982; Ullrich et al., 1985). Insulin binding removes inhibitory effect of alpha subunits on activity of tyrosine kinase enabling autophosphorylation. Autophosphorylation of the IR introduces conformational changes necessary for recruitment of specific adapter proteins coupling the receptor with its downstream effectors (Kasuga et al., 1982; Wilden et al., 1992). Inhibitory effect of insulin on food intake in hypothalamic neurons is induced through the intracellular signalling cascade mediated by insulin receptor substrate (IRS), PI3K and protein kinase B (PKB or Akt; Figure 1.4) (Carvalheira et al., 2003; Niswender et al., 2003). Insulin receptor is expressed in several brain areas including cerebral cortex, hipoccampus, hypothalamus and brainstem (Marks et al., 1990). To reach its target sites in brain like ARC insulin is actively carried across blood brain barrier by receptor mediated transport (Baura et al., 1993; Banks et al., 1997). The transport of insulin across blood-brain barrier is saturable making the process at least partly independent from rapid fluctuations of hormone levels due to postprandial rise of glucose (Banks et al., 1997).

On the contrary to its negative role in the central regulation of energy homeostasis, in periphery insulin enhances nutrient uptake and storage in different tissues, thus leading to increased food intake and weight gain (Carlson and Campbell, 1993; Niswender et al., 2004). Although at the first glance it may seem contradictious, dual action of insulin may represent protective mechanism to soften tissue starving in the absence of insulin by inducing hyperphagia due to lack of central inhibitory signalling by adiposity signals (Niswender et al., 2004).

1.1.2 Centrally regulated signals

Hypothalamus is the key brain structure governing the homeostatic regulation of energy balance. Hypothalamic areas essential for regulation of metabolism are, ARC, dorsomedial hypothalamic nucleus (DMN), paraventricular nucleus of hypothalamus (PVN), lateral hypothalamic area (LHA) and ventromedial nucleus (VMN). Sense of hunger is stimulated in LHA, while VMN, DMN and PVN are involved in induction of satiety and energy expenditure (Olney, 1969; Almli and Golden, 1974; Hill et al., 1981; Cox and Sims, 1988; Bellinger and Bernardis, 2002; Schneeberger et al., 2014).

Figure 1.1 Communication between peripheral and central regulators of energy homeostasis

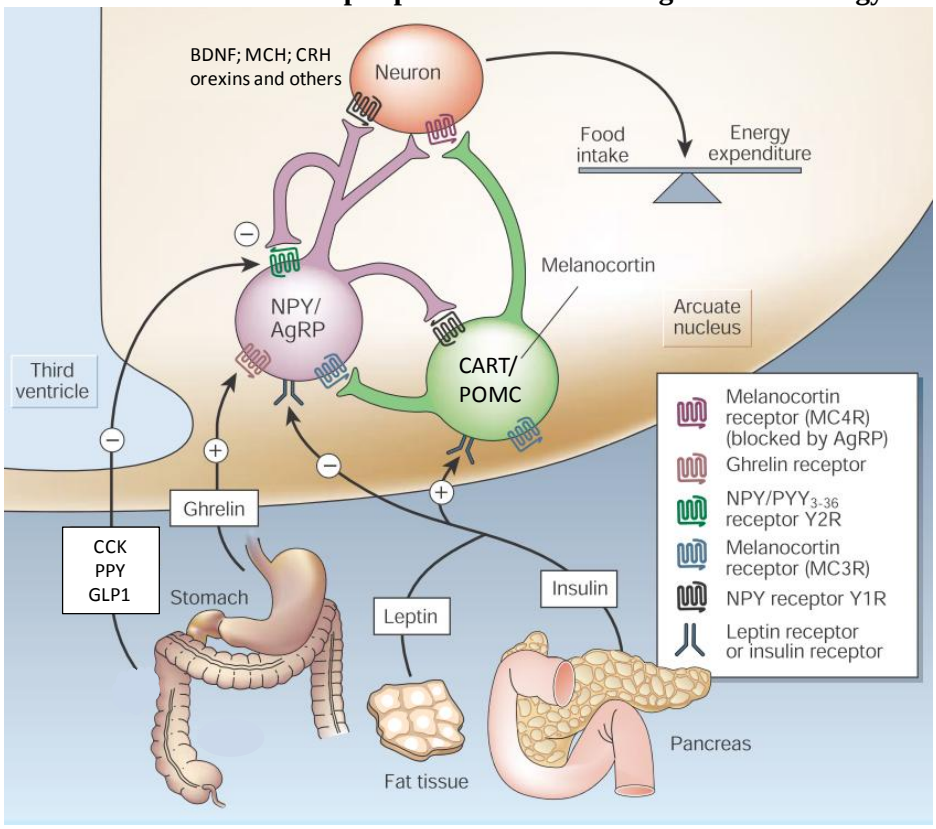

Figure and figure legends adapted from Schwartz and Morton (2002). Peripheral hormones CCK, PPY, GLP1, leptin and insulin inhibit NPY/AGRP producing neurons and stimulate CART/POMC producing neurons in hypothalamic ARC. Since NPY/AGRP neurons stimulate appetite and CART/POMC neurons inhibit it acting via downstream neurons producing neurotransmitters like BDNF, orexins, MCH, CHR and others, the net result is decreased food intake. Ghrelin inhibits CART/POMC neurons and stimulates NPY/AGRP, which subsequently leads to increased food intake.

ARC senses hormonal and nutrient signals from circulation across the weak blood brain barrier of median eminence adjacent to this area as well as receives information from lower regulatory centres (Broadwell and Brightman, 1976; Morita and Miyata, 2013; Schneeberger et al., 2014). The ARC contains two subpopulations of neurons mediating canonical homeostatic pathway relevant to regulation of energy turnover. One expresses appetite stimulators AGRP and neuropeptide Y (NPY), whereas another consists of neurons producing inhibitors of food intake pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) (Hahn et al., 1998; Elias et al., 1999; Elias et al., 2001). Starvation sensitive neurons expressing AGRP and NPY are activated by peripheral stimulators of appetite such as ghrelin and inhibited by satiation and adiposity related signals induced by PYY, GLP1, leptin, insulin, nutrients and related factors (Elias et al., 1999; Batterham et al., 2002; Obici et al., 2002; Cowley et al., 2003; Seo et al.,

2008b) (Figure 1.1). On the contrary, while downregulated during fast by factors responsible for food intake stimulation, activity of neurons expressing POMC and CART is increased in the state of energy abundance after ARC receives signals from satiation and adiposity related messengers, (Cowley et al., 2001; Batterham et al., 2002; Obici et al., 2002; Cowley et al., 2003; Ellacott and Cone, 2004; Seo et al., 2008b) (Figure 1.1). Besides, neurons producing AGRP and NPY have continuous basal inhibitory effect on POMC/CART neurons by releasing neurotransmitter gamma-aminobutyric acid (GABA) (Cowley et al., 2003; Tong et al., 2008). This inhibitory effect is also removed when ARC receives adiposity related signals like leptin; thus further potentiating stimulation of POMC/CART neurons (Cowley et al., 2001). Neurotransmitters released from ARC neurons act within in LHA, VMN, DMN and PVN to induce regulatory pathways further distributed to other brain regions and periphery (Elias et al., 1998; Schneeberger et al., 2014) (Figure 1.1).

1.1.2.1 Neuropeptides released from ARC and downstream pathways

Melanocortin system

Signalling cascades mediated by melanocortin receptors and their ligands are involved in the regulation of diverse physiological processes such as skin and hair pigmentation, adrenal function, functions of cardiovascular system, sexual function, secretive function of sebaceous gland and pheromone secreting glands, inflammation, signalling of pain, peripheral nutrient partitioning and energy homeostasis (Thody et al., 1993; Li et al., 1996; Buggy, 1998; Thiboutot et al., 2000; Wessells et al., 2000; Morgan et al., 2004; Seeley et al., 2004; Hillebrand et al., 2006; Nogueiras et al., 2007; Delaney et al., 2010; Sayk et al., 2010).

Gene coding for POMC is actively transcribed in hypothalamus, pituitary and NTS in the brainstem (Jacobowitz and O'Donohue, 1978; Mains and Eipper, 1979). In periphery its transcription has been observed mainly in skin and hair follicles (Hadley and Haskell-Luevano, 1999). Post-translational processing of the polypeptide coded by the gene yields several active peptides, which are α, β and γ melanocyte stimulating hormones (αMSH, βMSH and γMSH), adrenocorticotropic hormone (ACTH), β and γ lipotropins, β endorphin and corticotropin-like-intermediate lobe peptide (CLIP) (Smith and Funder, 1988; Benjannet et al., 1991; Hook et al., 2009). Melanocortin peptides αMSH, βMSH, γMSH and ACTH bind and activate five melanocortin receptors (MC1R – MC5R) with various affinity (Gantz and Fong, 2003). Melanocortin receptors also have natural antagonists. Agouti protein normally inhibits melanocortin 1 receptor MC1R to determine coat colour in animals (Ollmann et al., 1997), while its human homolog Agouti signalling protein (ASIP) probably regulates pigmentation and melanogenesis (Lu et al., 1994; Voisey et al., 2003). AGRP specifically antagonises αMSH activating effects on MC3R and MC4R (Ollmann et al., 1997; Shutter et al., 1997).

Energy balance is regulated mainly by αMSH and AGRP signalling through melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R). The ARC POMC/CART neurons, when stimulated by peripheral anorectic factors like leptin, release αMSH to activate MC3R and MC4R within PVN and VMN which decreases energy intake and stimulates expenditure (Cowley et al., 2001; Ellacott and Cone, 2004; Cone, 2005; Hillebrand et al., 2006). Accordingly release of AGRP from NPY/AGRP neurons inhibits melanocortin receptors and subsequently regulatory systems are shifted towards energy uptake, economizing and storage (Ollmann et al., 1997; Shutter et al., 1997).

MC4R is expressed exclusively in CNS particularly in hypothalamus and binds αMSH and ACTH with similar affinity, whereas βMSH and γMSH are less potent

activators of the receptor (Mountjoy et al., 1994; Gantz and Fong, 2003). Mice lacking MC4R gain weight due to increased food intake and low locomotor activity (Huszar et al., 1997). Although regulatory pathways downstream of MC4R regulating energy turnover are not yet fully understood, several possible mechanisms have been suggested.

MC4R neurons within hypothalamic PVN coexpress thyrotroin releasing hormone (CRH) and thyrotropin releasing hormone (TRH). Since administration of MC4R agonists enhances production of CRH and TRH, hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-thyroid axis (HPT) axis have been suggested to convey MC4R induced signal to periphery (Kim et al., 2002; Lu et al., 2003; Wiedmer et al., 2011; Xia et al., 2012). Central administration of CRH suppresses energy intake and increases energy expenditure via stimulation of lipolysis in white adipose tissue and heat production in brown adipose tissue (Cerri and Morrison, 2006; Toriya et al., 2010). The HPA axis controlled by CHR is known to regulate response to stress and peripheral metabolism through neuronal pathways projecting to peripheral organs (Muglia et al., 2000; Stanley et al., 2010). Similarly, TRH regulated HPT axis may contribute to MC4R effects on energy expenditure for thyroid hormones enhance resting metabolic rate and thermogenesis (Al-Adsani et al., 1997; Silva, 2001).

Within the VMN one of the proposed downstream targets of MC4R signalling is anorexigenic peptide brain derived neurotrophic factor (BDNF). Depletion of BDNF within VMN induced hyperphagia and obesity (Unger et al., 2007), while intracerebroventricular infusion of BDNF or other agonists targeting BDNF binding tyrosine kinase receptor B (TRKB) led to reduced food intake and weight loss (Toriya et al., 2010). Regulation of BDNF downstream of MC4R is supported by findings that obese and hyperphagic phenotype of *Mc4r* knockout mice displaying decreased expression of BDNF can be rescued by administration of this neurotransmitter (Xu et al., 2003). Moreover stimulation of the MC4R augments BDNF secretion that further indicates that BDNF indeed is one of the mediators of anorectic effects triggered downstream of MC4R (Kim et al., 2002; Nicholson et al., 2007; Toriya et al., 2010).

MC3R expression is broader than that of MC4R and additionally to CNS, MC3R has been found also in peripheral tissues including heart, gastrointestinal tract and immune cells (Gantz et al., 1993; Roselli-Rehfuss et al., 1993). Within hypothalamus highest levels of MC3R mRNA have been detected in ARC and VMN (Roselli-Rehfuss et al., 1993). This receptor displays equal affinity for all four endogenous melanocortin agonists (Gantz et al., 1993; Gantz and Fong, 2003). MC3R plays significant role in regulation of energy storing efficiency and adaptation to conditions of food restriction. Inactivation of mouse MC3R results in increased efficiency in diverting of incoming energy to fat reserves causing adipocyte hypertrophy and accumulation of adipose mass with no significant changes in food intake or metabolic rate (Chen et al., 2000). During fast mice with disrupted MC3R signalling display blunted refeeding, decreased ability to mobilize energy stored in fat depots due to defective lipolysis and show reduced vigilance and locomotor activity during search for food indicating failure in adjustment of feeding behaviour (Begriche et al., 2012; Renquist et al., 2012). It is likely that MC3R also interacts with TRH and CRH induced pathways to convey its effects to periphery (Kim et al., 2002; Renquist et al., 2012). MC3R presence in ARC POMC producing neurons may provide negative feedback regulation on hypothalamic melanocortins (Jegou et al., 2000). Still, role of MC3R in the regulation of food intake is less studied than that of MC4R and mechanisms explaining distinct functions of both receptors are still to be described.

Populations of neurons containing components of melanocortin system outside hypothalamus also have been shown to contribute to maintenance of energy turnover. For example POMC along with MC3R and MC4R are expressed in brainstem, where

melanocortins have negative impact on energy balance similar to that mediated by hypothalamic melanocortin system (Grill et al., 1998; Li et al., 2007a). Activation of MC4R in NTS appears to be necessary for CCK to suppress feeding indicating that brainstem melanocortins facilitate integration of long term and short term homeostatic signals (Fan et al., 2004).

Cocaine and amphetamine regulated transcript (CART)

Douglass and colleagues first described CART as a transcript regulated by acute treatment with psychomotor stimulants cocaine and amphetamine in rat brain within striatum (Douglass et al., 1995; Douglass and Daoud, 1996). Alternative splicing of CART mRNA followed by processing of propeptide yields two active forms of CART, which are CART 55-102 and CART 62-102 in rats and CART 42-89 and CART 49-89 in humans (Douglass et al., 1995; Douglass and Daoud, 1996; Dey et al., 2003). Receptors for CART neuropeptides still are not described, although it has been suggested that they may belong GPCR family (Lin et al., 2011).

Besides striatum the transcript has been found in multiple sites within the rodent and human brain including amygdala, hippocampus, nucleus accumbens, thalamus and pituitary. Yet, the highest level of CART mRNA has been detected in hypothalamic structures ARC, PVN, DMN, lateral and ventromedial hypothalamus (Douglass et al., 1995; Douglass and Daoud, 1996; Koylu et al., 1998; Elias et al., 2001). CART is also expressed in periphery in adrenal glands, pancreas and gastrointestinal tract (Maixnerova et al., 2007). Peptides derived from CART display diverse physiological functions including regulation of reward system, response to stress and energy homeostasis (Kristensen et al., 1998; Lambert et al., 1998).

In respect of maintenance of energy balance CART peptides act as satiety factors. As such they are upregulated in ARC and PVN by leptin and high fat diet (Kristensen et al., 1998; Lambert et al., 1998; Leibowitz and Wortley, 2004). Mice lacking functional CART have been shown to gain weight and adipose mass with more pronounced increase when fat content of meals is high (Asnicar et al., 2001; Wierup et al., 2005).

Hypothalamic ARC neurons containing CART contact neuronal populations in PVN, where increase in CART peptides stimulate production of CRH and oxytocin along with reversing fast induced decrease in TRH (Fekete et al., 2000; Vrang et al., 2000). Oxytocin is an anorexigenic hormone that regulates meal size and delays initiation of first meal. Its effects at least partly can be explained by modulation of brainstem sensitivity to peripheral satiation signals like CCK (Arletti et al., 1990; Blevins et al., 2004). These findings show that CRH and oxytocin may mediate anorectic effects of CART peptides, while interaction with HPT axis may contribute to increased energy expenditure (Fekete et al., 2000; Vrang et al., 2000). Furthermore within PVN CART colocalises with TRH and thus may have direct modulatory impact on HPT axis (Broberger, 1999). Another peptide that may be regulated by CART peptides within PVN is galanin. Like CART peptides orexigenic galanin is increased in response to high fat diet, whereas administration of CART peptides reduces levels of galanin thus possibly acting to prevent overconsumption of energy rich foods (Leibowitz and Wortley, 2004). In DMN and LHA CART is found in neurons producing orexigenic peptide melanin concentrating hormone (MCH) (Broberger, 1999). The MCH has been shown to be particularly effective towards increasing consumption of high calorie diet (Gomori et al., 2003; Karlsson et al., 2012). However, physiological significance of colocalisation of both neurotransmitters thus far has not been explained.

Additionally to its direct impact on energy regulation, CART may modulate NPY signalling. Nerve terminals of ARC neurons producing NPY project to CART/TRH and CART/MCH and intracerebroventriculary injected CART peptides were shown to

attenuate stimulatory effects of NPY on food intake (Lambert et al., 1998; Broberger, 1999).

Neuropeptide Y (NPY)

NPY serves as a strong stimulator of food intake. Central administration of NPY induces hyperphagia, accompanied by enhanced carbohydrate consumption, and weight gain due to the increase in fat mass (Levine and Morley, 1984; Stanley and Leibowitz, 1985; Stanley et al., 1986).

This neurotransmitter shares structural similarity and its receptors with peripherally derived satiation factors PYY and pancreatic polypeptide (PP) (Tatemoto et al., 1982; Takeuchi et al., 1986). Although expression of NPY has been detected throughout the CNS, hypothalamic ARC neurons and DMH are the major sites of its production (Takeuchi et al., 1986). From four functional neuropeptide Y receptor subtypes found in humans neuropeptide Y receptor Y1 (Y1R) and neuropeptide Y receptor Y5 (Y5R) have been suggested to be the two most important in the control of energy balance by NPY. The Y1R is thought to be the major mediator of NPY action on spontaneous and fast induced food intake, while Y5R potentiates effects triggered by Y1R and mediates regulation of thermogenesis and energy expenditure (Hwa et al., 1999; Kanatani et al., 2001; Mashiko et al., 2009; Nguyen et al., 2012). Both subtypes of NPY receptors are expressed in hypothalamus in ARC, PVN and LHA as well as hippocampus, thalamus, amygdala, cerebral cortex and brainstem (Wolak et al., 2003).

Apart from GABA mediated inhibition of POMC/CART neurons, NPY attenuates posttranslational processing of POMC into active neurotransmitters. It decreases levels of one of the key enzymes PCSK2 cleaving the preprohormone (Cowley et al., 2003; Cyr et al., 2013). Reduction in active melanocortin peptides like αMSH decreases activation of signaling cascades downstream from melanocortin receptors, for example prevents stimulation of TRH production, which eventually results in increased food intake and slowing down of energy expenditure (Cyr et al., 2013).

Besides local action, NPY producing neurons from ARC project to other hypothalamic regions relevant to energy homeostasis including to LHA and PVN (Elias et al., 1998; Schneeberger et al., 2014). Long term activation of NPY signaling in LHA and PVN has been linked to positive energy balance due to hyperphagia and decrease in energy expenditure (Tiesjema et al., 2007). Stimulation of food intake was explained by larger size of consumed meals due to NPY signaling in LHA, whereas in PVN NPY appeared to increase meal frequency. Decrease in energy expenditure resulted from NPY mediated downregulation of thermogenesis and locomotor activity via both LAH and PVN (Tiesjema et al., 2007).

To implement its impact on energy turnover NPY suppresses TRH axis within PVN subsequently inducing feeding and inhibiting energy expenditure (Vella et al., 2011). The hormone increases circulating levels of corticosterone, which stimulates food intake, downregulates hypothalamic CRH and provides positive feedback loop increasing expression of NPY in ARC (Albers et al., 1990; Akabayashi et al., 1994; Sainsbury and Zhang, 2012). At the same time, LHA projections of NPY neurons contact orexin containing neurons (Elias et al., 1998; Horvath et al., 1999). Orexin or hypocretin system comprises two peptides orexin A and orexin B with different affinity binding orexin-1 and orexin-2 receptors (Sakurai et al., 1998). Collectively orexins stimulate feeding and regulate arousal and sleep-wake behavior (Sakurai et al., 1998; Edwards et al., 1999; Lee et al., 2005). Injection of NPY into lateral hypothalamus has been shown to activate orexin producing neurons and robustly stimulate food intake with the strongest impact during the active period (Horvath et al., 1999; Campbell et al., 2003). Besides, orexin producing

neurons from lateral hypothalamus contact NPY containing neurons in ARC, indicating reciprocal interaction between both systems (Elias et al., 1998; Horvath et al., 1999).

Neural population in DMN containing NPY also contributes to maintenance of energy balance. Changes in NPY expression within DMH influence food intake, physical activity, metabolic efficiency, thermogenic response to exposure to cold and glucose metabolism (Chao et al., 2011; Zheng et al., 2013). However, neurons in DMH expressing NPY appear to be differently regulated from those in ARC since these neuronal populations do not express leptin receptors and are insensitive to inhibitory effect of leptin (Bi et al., 2003). Instead these neurons may regulate food intake by mediating signaling of centrally produced CCK and other thus far unknown factors (Chen et al., 2008a; Yang et al., 2009; Chao et al., 2011; Zheng et al., 2013). On the other hand regulatory impact of DMN derived NPY on functions of brown adipose tissue is similarly conveyed by sympathetic nervous system (Chao et al., 2011; Zheng et al., 2013).

1.1.3 Hedonic regulation of food intake

In contrast to food intake driven by factors regulating energy balance in concert with metabolic requirements, eating for reward or hedonic eating is driven by desire to receive pleasure from consumption of palatable or rewarding foods. Pleasure associated with eating palatable foods serves as a powerful motivator of food intake even in absence of physiological need to replenish energy resources (Foltin et al., 1988; Rudski et al., 1994; Vucetic and Reyes, 2010; Berthoud, 2011). Reward can be divided into two relatively distinct psychological components "wanting" or incentive, motivational component and "liking" expressing pleasure or hedonic impact of stimulus (Berridge, 2009).

Food, in particular palatable food, stimulates neuronal activity within brain regions responsible for forming reward response including the orbitofrontal cortex, amygdala, *insula*, ventral tegmental area, *nucleus accumbens*, dorsal striatum, and *substantia nigra* (Moore and Bloom, 1978; Schur et al., 2009). Dominant hedonic pathways regulating food consumption according to reward value rather than hunger parallel addiction mechanisms and involve dopamine, opioid and endocannabinoid regulated signalling systems (Gosnell and Levine, 2009; Kirkham, 2009; Vucetic and Reyes, 2010; Berthoud, 2011).

Although the key mediators of homeostatic and reward circuits differ, the two systems normally function in concert. Activity of homeostatic pathways can modify intensity of reward. Hedonic value of food is enhanced by hunger, while satiety related factors render foods less pleasurable (Goldstone et al., 2009; Davis et al., 2010). Some of suggested underlying mechanisms may involve peripheral satiation and satiety factors ghrelin and leptin. Orexigenic ghrelin increases activity of central hedonic circuits (Malik et al., 2008). Leptin on the other hand has been shown to attenuate motivation for palatable food via dopaminergic system (Krugel et al., 2003; Davis et al., 2010). Within central circuits a role in the regulation of intake of palatable foods has been suggested for regulators of energy balance like MCH, orexins and melanocortins (Georgescu et al., 2005; Davis et al., 2011; Choi et al., 2012).

1.1.3.1 Dopamine

Dopamine is produced by neurons within the ventral tegmental area and *substantia nigra*, which project to the nucleus accumbens and dorsal striatum forming the main mesolimbic branch (Moore and Bloom, 1978; Vucetic and Reyes, 2010). Animal studies show that dopamine release within for example *nucleus accumbens* increases in response to sensory cues such as texture and taste linked to anticipating food, particularly sweetened fat rich foods with high hedonic value (Richardson and Gratton, 1996; Liang et al., 2006).

Activation of mesolimbic branch of dopamine signalling enhances wanting to eat for satisfaction, motivates to seek for palatable food and work harder to obtain it (Richardson and Gratton, 1996; Vucetic and Reyes, 2010). In humans treatment with stimulators of dopaminergic signalling induces similar reaction manifesting as craving for food and compulsive eating (Nirenberg and Waters, 2006; Cornelius et al., 2010; Wang et al., 2011a).

Apart from its functions in reward system dopamine also acts within homeostatic hypothalamic circuits, where it is thought to serve as one of meal initiators determining meal frequency and meal size (Meguid et al., 1997; Vucetic and Reyes, 2010).

1.1.3.2 Opioids

Endogenous opioid system consists of enkephalins, dynorphins, and β-endorphin and μ, δ and κ opioid receptors (Knapp et al., 1995; Gosnell and Levine, 2009). The μ opioid receptors expressed within the *nucleus accumbens* and ventral pallidum is predominant mediators of opioid effects on hedonic regulation of food intake (Matthes et al., 1996; Zhang et al., 1998). Administration of opioid receptor agonists in animals demonstrated increased intake of foods, specifically the ones with the most preferable flavour (Gosnell et al., 1990; Woolley et al., 2006). Accordingly, suppression of opioid receptor mediated signalling significantly lessened preference of highly palatable foods versus those with lower reward inducing properties in both animals and humans (Woolley et al., 2006; Gosnell and Levine, 2009; Ziauddeen et al., 2013). Opioids are thought to regulate mainly hedonic perception of food and "liking" aspect of reward rather than triggering intake (Rudski et al., 1994; Yeomans and Gray, 1996; Gosnell and Levine, 2009). These effects are likely mediated by blunting satiety signals induced by oxytocin and melanocortins (Gulati et al., 1991; Olszewski et al., 2001).

1.1.3.3 Endocannabinoids

Both dopamine and opioid regulated pathways interact with endocannabinoid system. Phytocannabinoids or endogenous ligand anandamide activating cannabinoid receptor 1 (CB1), the main mediator of effects on food intake, have been shown to reinforce hunger for sweets and initiate eating even in satiated state in humans and animals (Foltin et al., 1988; Williams and Kirkham, 2002; Kirkham, 2009). The wanting or craving for palatable food component downstream of CB1 is likely mediated via increased dopamine signalling since CB1 antagonists reduce release of dopamine induced by palatable food (Melis et al., 2007). At the same time antagonists specifically binding either CB1 or opioid receptors simultaneously block stimulatory effect of both systems on the intake of palatable food (Kirkham and Williams, 2001). These findings indicate that endocannabinoids are also involved in the regulation of "liking" component of reward, which is supported by studies showing increased enjoyment of palatable food like sucrose after administration of CB1 agonists (Kirkham and Williams, 2001; Mahler et al., 2007).

In addition to interaction with hedonic regulators, endocannabinoids like dopamine also have been involved in homeostatic pathways along with leptin, orexins and MCH (Di Marzo et al., 2001; Huang et al., 2007b).

1.2 Glucose metabolism

Central neuronal circuits essential for maintenance of energy balance also are involved in the regulation of glucose homeostasis. Neurons expressing MCH, orexins, melanocortins, melanocrtin receptors and other components can sense fluctuations in circulating glucose and correspondingly adjust their activity and signal to regulate glucose output and uptake by peripheral tissues (Karnani and Burdakov, 2010; Grayson et al.,

2013). For example, neurons producing orexin are activated by hypoglycemia and can stimulate hepatic glucose production via sympathetic branch of autonomic nervous system (Yi et al., 2009; Karnani and Burdakov, 2010).

However, peripheral mechanisms of glucose homeostasis are much better understood. Hormone insulin produced by pancreatic beta cells is the major, although not the only one, player in the peripheral regulation of glucose homeostasis.

1.2.1 Regulation of insulin production and release

1.2.1.1 Expression of insulin gene and processing of the preprohormone

Beta cell specific transcription of the insulin gene is regulated by complex synergic interaction of several transcription factors, the most important of which are pancreatic and duodenal homeobox 1 (PDX1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) and neuronal differentiation 1 (NEUROD1) interacting with transcription factor E2-alpha or E47 (Petersen et al., 1994; Naya et al., 1995; Ohneda et al., 2000; Zhao et al., 2005; Aramata et al., 2007) (Figure 1.2). Glucose facilitates posttranscriptional modifications predominantly by phosphorylation, nuclear translocation and formation of protein complexes subsequently enhancing DNA binding activity of PDX1, NEUROD1 and MafA (Kataoka et al., 2002; Mosley et al., 2004; Zhao et al., 2005; Andrali et al., 2007). Induction of MafA expression is highly glucose dependent and it is partly subordinated to the activation of PDX1 and possibly NEUROD1 acting on its promoter (Raum et al., 2006). Besides, functional PDX1 is necessary for amplification of insulin production mediated by GLP1 (Li et al., 2005). Glucose also amplifies translation of preproinsulin mRNA via increasing activity of basal translational factors involved in the initiation phase and regulation of factors enhancing stability of mRNA (Wicksteed et al., 2001).

Figure1.2 Main transcription factors regulating insulin gene promoter.

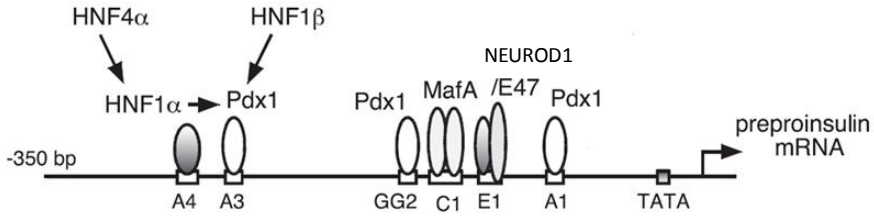

Figure and legend adapted from Aramata et al (2007). Glucose enhances expression of insulin inducing nuclear translocation and activation of key transcription factors PDX1, MafA and NEUROD1 binding A3, C1 and E1 elements of insulin gene promoter. Other transcription factors playing significant role in the regulation of INS gene promoter include hepatocyte nuclear factor 1α (HNF1α), hepatocyte nuclear factor 4α (HNF4α), hepatocyte nuclear factor 1β (HNF1β) directly binding elements on promoter or indirectly by interacting with other transcription factors.

After synthesis and removal of signal sequence insulin prohormone is directed from rough endoplasmic reticulum to trans-Golgi-network and packed in secretory granules (Huang and Arvan, 1995; Eliasson et al., 2008). Here the proprotein convertase subtilisin/kexin type 1 (PCSK1) and 2 (PCSK2) along with carboxypeptidase E accomplish the cleavage of proinsulin (Davidson and Hutton, 1987; Smeekens et al., 1992). Insulin is associated into hexamers incorporating zinc and calcium ions, which assemble forming more complex crystal structures composing dense core of the insulin secretory granule (Emdin et al., 1980; Huang and Arvan, 1995; Dunn, 2005). Zinc and calcium ions for this process are provided by ATP-dependent non-SERCA Ca^{2+} pump and pancreas-specific

zinc transporter 8 (ZnT8) (Mitchell et al., 2001; Lemaire et al., 2009). Assembly of insulin into crystalic structures stabilizes the hormone and protects against excessive proteolytic cleavage, chemical and physical degradation and prevents protein fibrillation, which would inactivate the hormone (Emdin et al., 1980; Ahmad et al., 2003; Dunn, 2005).

1.2.1.2 Regulation of basal insulin secretion

In general four relatively distinct modes or phases of insulin secretion can be recognized, they are - basal insulin secretion, the cephalic phase of insulin secretion and two phases induced by rise in circulating glucose, the first acute secretion phase and more extended and stable second phase insulin secretion (Caumo and Luzi, 2004).

At basal fasting concentrations of glucose insulin is released from beta cells at rate less than 0.01% of total insulin content per minute (Kruszynska et al., 1987; Rorsman and Braun, 2013). Uniform suppression of the hormone secretion is provided by cell to cell communication through the gap junctions and other juxtacrine mechanisms equalizing electrical gradient between the cells with different excitability (Benninger et al., 2011). Basal insulin secretion during fast is necessary to support glucose uptake in peripheral tissues and to control lipolysis and gluconeogenesis, thus, providing normal fasting concentration of circulating glucose (Wilcox, 2005). Subsequently insulin resistance present in majority of obese patients triggers compensatory hyperinsulinemia during intermeal periods (Ratheiser et al., 1990; Tripathy et al., 2000).

At the beginning of a meal before any nutrients can be absorbed the cephalic phase of insulin secretion occurs (Teff, 2000; Teff, 2011). This first burst in insulin secretion is induced as a response to stimulation of oral sweet taste receptors and probably other food cues including olfactory and visual signals (Berthoud et al., 1981; Karhunen et al., 1996; Tonosaki et al., 2007; Just et al., 2008). Signals from taste receptors travel via gustatory nerves and reach the integration sites in brain stem and hypothalamus (Berthoud et al., 1981). The generated response is conveyed by vagus nerve and postganglionic cholinergic nerve terminals innervating pancreatic islets (Secchi et al., 1995; Ahren and Holst, 2001). Preabsorptive increase in insulin has been suggested as an adaptive homeostatic mechanism developed to lessen hyperglycemic effects of the meal (Teff, 2011). Reduced cephalic phase of insulin release is associated with impaired postprandial glucose tolerance (Teff and Engelman, 1996; Ahren and Holst, 2001).

1.2.1.3 Glucose stimulated insulin secretion

Although secretory response of beta cells can be induced by other nutrients, glucose stimulated insulin secretion (GSIS) is the major mechanism governing the release of the hormone (Ashcroft, 1968; Randle and Ashcroft, 1969; Berthoud and Powley, 1990; Brunicardi et al., 1995; Bollheimer et al., 1998; Gromada et al., 1998; Seino, 2012). Approximately in 10 minutes after the beginning of the meal glucose levels in plasma rise to what beta cells respond with increase in insulin release (American Diabetes Association, 2001). This response is mediated by products of glucose metabolism in beta cell through two interconnected mechanisms: triggering pathway and metabolic amplifying pathway (Seino, 2012). The triggering pathway is based on well described mechanism of insulin release involving closure of ATP sensitive potassium (K_{ATP}) channels followed by changes of cytoplasmic Ca^{2+} concentration, whereas metabolic amplifying pathway augments insulin secretion without inducing further changes in intracellular Ca^{2+} (Gembal et al., 1993; Mourad et al., 2010). Metabolic amplifying pathway can be induced only when triggering pathway is activated (Gembal et al., 1993; Mourad et al., 2010; Seino, 2012). In addition, hormones and neurotransmitters, for example, incretins and acetylcholine

intensify glucose induced insulin release, thus, regulating neurohormonal amplifying pathway (Vilsboll et al., 2003; Gautam et al., 2007; Mourad et al., 2012; Seino, 2012).

The triggering pathway

Insulin independent glucose transporters, namely facilitated glucose transporter member 2 (GLUT2) in rodents and glucose transporter member 1 and 3 (GLUT1 and GLUT3) in humans, allow glucose to enter cells, where glycolysis takes place (De Vos et al., 1995). The first step in the intracellular glucose metabolism is phosphorylation catalysed by glucokinase (Alberts et al., 1991a). Glucokinase serves as glucose sensor of the beta cell by defining the rate of beta cell responsiveness to respective glucose concentrations and subsequently the amount of insulin released (Liang et al., 1992). Defects in functions of glucokinase change beta cell ability to adequately evaluate glucose concentration and result in a discordance between levels of released insulin and circulating glucose (Saxena et al., 2007; Sagen et al., 2008; Lorini et al., 2009; Dupuis et al., 2010).

Glucose metabolism has three major stages: glycolysis, Krebs cycle and oxidative phosphorylation (Alberts et al., 1991a). The net result of glucose breakdown is rapid rise in adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio that leads to the closure of inwardly-rectifying K_{ATP} and subsequent depolarisation of plasma membrane (Hopkins et al., 1992). Changes in the membrane potential open L-type voltage gated Ca^{2+} channels providing the major inward flow of Ca^{2+} (Gopel et al., 1999; Wiser et al., 1999; Rorsman and Renstrom, 2003).

Increase in the intracellular Ca^{2+} induces insulin exocytosis. Exocytosis is a three stage process consisting of docking, priming and fusion phases controlled by numerous interacting proteins (Alberts et al., 1991b; Eliasson et al., 2008). At the core of the exocytotic machinery is soluble *N*-ethylmaleimide-sensitive factor activating protein receptor (SNARE) protein complex consisting of plasma membrane associated proteins or t-SNAREs syntoxin 1 and synaptosomal-associated protein 25kDa (SNAP25) and v-SNARE vesicle-associated membrane protein 2 (synaptobrevin 2) (VAMP2) located on the surface of the secretory granule (Alberts et al., 1991b; Wheeler et al., 1996; Lang, 1999). These proteins interact forming helical structure that draws together opposite membranes and catalyses the fusion (Lang, 1999; AlSaleh et al., 2011). Voltage gated L-type Ca^{2+} channels are located close to the SNARE protein complex, although it is unlikely that SNAREs are the primary targets of Ca^{2+} (Lang, 1999; Wiser et al., 1999). Instead, synaptotagmins acting in beta cells have been suggested to be major Ca^{2+} sensors of the exocytotic machinery. They form complex with SNARE proteins which is stabilised by Ca^{2+} thus significantly catalysing fusion of granules with plasma membrane (Mizuta et al., 1997; Gustavsson et al., 2008).

For successful insulin secretion a secretory granule undergoes complete fusion with plasma membrane accompanied by complete emptying of its content (Rorsman and Renstrom, 2003; Ma et al., 2004; MacDonald et al., 2005; Kasai et al., 2010). Secretory granules can also fuse with each other and release their content through the single fusion pore in a process known as compound exocytosis, yet this process is thought to be normally downregulated to avoid exaggerated increase in circulating insulin (Kwan and Gaisano, 2005; Kasai et al., 2010). However, recently it has been demonstrated that about two thirds of exocytosis events in beta cells undergo “kiss and run” exocytosis, when fusion pore is partly and reversibly opened allowing release of only small compounds like ions and ATP (MacDonald et al., 2005). T2D is associated with decreased expression of proteins forming SNARE complex and synaptotagmins that promotes “kiss and run” exocytotic events (Ostenson et al., 2006; Eliasson et al., 2008).

Metabolic amplifying pathway

Increase in ATP and elevated Ca^{2+} are obligatory preconditions for functional metabolic amplifying pathway, which locates it downstream of triggering events (Gembal et al., 1993; Henquin, 2000; Nenquin et al., 2004; Chen et al., 2008b). From physiological aspect the strict hierarchy of the two pathways prevents excess release of insulin, when glucose is low (Henquin, 2000). Other fuels, for example, amino acids also are able to activate amplifying pathway, although this process still needs increased Ca^{2+} and active metabolism (Nenquin et al., 2004). The amplifying pathway enhances sensitivity of exocytotic process to Ca^{2+} doubling productivity of GSIS and, thus, is essential to maintain proper secretory response (Henquin, 2000; Chen et al., 2008b; Mourad et al., 2010). Although it is clear that K_{ATP} channels are not the part of the pathway, exact mechanisms operating in metabolic amplifying pathway are not yet understood (Gembal et al., 1993; Henquin, 2000). Several metabolic products generated in mitochondria such as glutamate and acetyl coenzyme A (acetyl-CoA) have been suggested as possible mediators of this process (Maechler and Wollheim, 1999; Panten et al., 2013). These factors may increase ready for release granule population facilitating the last steps in granule recruitment such as priming (Chen et al., 2008b; Mourad et al., 2010).

Neurohormanal amplifying pathway

Probably the most investigated conductors of neurohormonal amplifying pathway are incretin hormones GLP1 and gastric inhibitory polypeptide (GIP) and neurotransmitter acetylcholine (Vilsboll et al., 2003; Gautam et al., 2007; Mourad et al., 2012). Both GLP1 and GIP have strong amplifying action on GSIS accounting for up to 70% of released hormone (Lauritsen et al., 1982; Nauck et al., 1986). When reaching pancreatic beta cells GLP1 and GIP activate their respective GPCR inducing production of cyclic adenosine monophosphate (cAMP) by adenylate cyclase, a principal secondary mediator of incretin effects on GSIS, and other effectors with smaller contribution (Kashima et al., 2001; Kang et al., 2005; Roger et al., 2011).

cAMP activates pathways downstream of cAMP dependent protein kinase A (PKA) and exchange protein activated by cAMP 2A (Epac2A) enhancing rise in cytoplasmic Ca^{2+} concentration, facilitating mobilization of secretory granules and exocytosis of pre-recruited granules (Renstrom et al., 1997; Hatakeyama et al., 2006; Chepurny et al., 2009; Kaihara et al., 2013). Major mediators of these effects are PKC downstream of PKA and phospholipase C (PLC) located downstream of Epac2A. PLC mediated production of diacylglycerol (DAG) and inositol triphosphate (IP_3) and direct phosphorylation by PKC facilitate opening of calcium channels located within membrane of endoplasmic reticulum mobilizing intracellular Ca^{2+} stores for exocytosis (Kang et al., 2005; Suzuki et al., 2006). In addition, DAG facilitated closure of K_{ATP} channels as well as possibly direct activation of L-type voltage dependent Ca^{2+} channels by PKA reinforce influx of extracellular Ca^{2+} (Light et al., 2002; Suzuki et al., 2006; Chepurny et al., 2009).

Besides promoting release of insulin via mobilisation of calcium, PKA and Epac2A directly interact with proteins involved in exocytosis. PKA phosphorylates proteins like SNAP25 thus increasing release competence of insulin containing granules and prevalence of full fusion events (Hatakeyama et al., 2006; Kaihara et al., 2013). Epac2A on its turn forms complexes with syntabulin, Rim2 and other proteins promoting secretory granule transport towards plasma membrane, mobilization of immediately releasable granules as well as affects later events such as docking and priming (Kashima et al., 2001; Yasuda et al., 2010; Ying et al., 2012).

Cholinergic signalling potentiates GSIS through similar mechanisms like incretins including activation of PLC and PKC, which results in release of intracellular calcium stores as well as sensitization of secretory machinery to calcium (Hughes et al., 1990; Weng et al., 1993; Yada et al., 1995; Zawalich et al., 2000; Yang et al., 2005). Besides, binding of acetylcholine to type 3 muscarinic receptors opens nonselective Na^+ leak channels that stimulates Ca^{2+} influx through L-type voltage gated channels (Yada et al., 1995; Gautam et al., 2007). The latter mechanism has been shown to be active only in the presence of elevated glucose concentrations (Yada et al., 1995).

Biphasic secretion of insulin

Glucose induced insulin secretion from normal pancreatic islets is biphasic (Figure 1.3) (Turner et al., 1971; Caumo and Luzi, 2004; Rorsman and Braun, 2013). It contains the first acute phase of insulin release which lasts less than ten minutes with its peak in about five minutes in human islets in vitro. During the initial burst insulin secretion rate rises from 0.01% to 0.15% of intracellular insulin content released per minute. After the initial burst the hormone secretion rate reaches its plateau at a level close to 0.05%. This stable second phase continues till glucose levels return to normal (Bratanova-Tochkova et al., 2002; Rorsman and Braun, 2013).

Figure 1.3 Granule pools and biphasic insulin secretion.

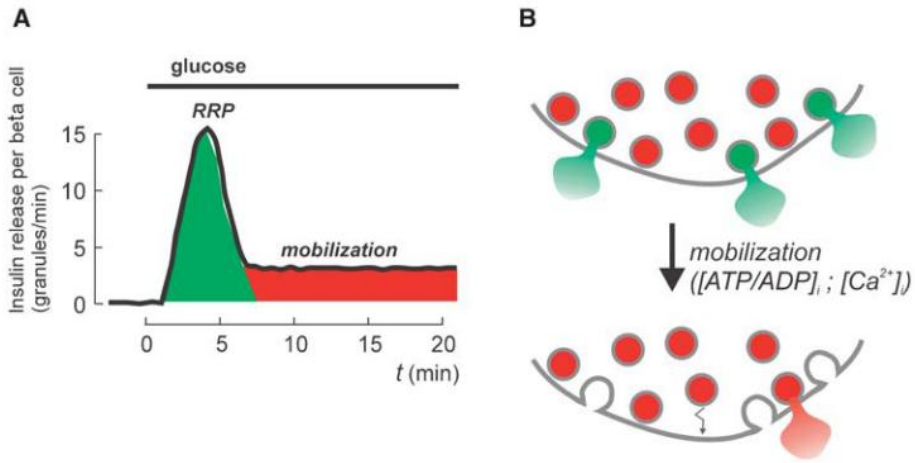

Figure and figure legend from Rorsman and Renstrom (2003). A Schematic representation of biphasic GSIS; B Biphasic release of insulin containing granules from readily releasable pool (green granules) and reserve pool (red granules).

Biphasic mode of GSIS has been explained by difference in exocytotic competence of insulin secretory granules distributed in two distinct pools consisting of up to 13 000 secretory granules per beta cell as shown in rodents. Rapid first phase response is carried out by releasing of readily releasable pool, which consists of predocked granules and makes about 10% of total content of granules (Bratanova-Tochkova et al., 2002; Rorsman and Renstrom, 2003; Straub et al., 2004). During the second sustained phase readily releasable pool needs to be replenished with insulin containing granules newly recruited and docked from the large reserve pool located farther away from the membrane (Figure 1.3) (Ohara-Imaizumi et al., 2002; Straub et al., 2004). This is a slower energy consuming process and requires remodelling of the cytoskeleton (Wilson et al., 2001; Seino et al., 2011). However, more recently it has been shown that docking is not obligatory for granule release and in both phases restoration of readily releasable pool involves newly mobilized

granules termed “restless newcomers” that can be immediately fused and released skipping the docking (Shibasaki et al., 2007).

Acute increase in released insulin concentrations during the first phase of GSIS is important in rapid inhibition of hepatic glucose production and accelerates glucose uptake in peripheral tissues more effectively compared with condition when only the second phase occurs (Luzi and DeFronzo, 1989; Bruttomesso et al., 1999; Kanat et al., 2011). Impaired early insulin secretion is associated with glucose intolerance and T2D (Turner et al., 1971; Bruce et al., 1988; Luzi and DeFronzo, 1989; Bruttomesso et al., 1999; Kanat et al., 2011).

Pulsatile release of insulin

Insulin is released in a pulsatile manner giving rise to oscillations manifesting as rapid pulses of hormone release recurring every 8-15 minutes overlapping larger ultradian fluctuations with period range of 80-120 minutes (Sturis et al., 1991; Bergsten, 2002; Juhl et al., 2002). Rapid oscillations are thought to occur in beta cell in concert with changes in membrane potential and fluctuations in Ca^{2+} linked to intra cellular glucose metabolism (Longo et al., 1991; Chou et al., 1992; Hellman et al., 1994; Bergsten, 2002); while slow ultradian oscillations have been suggested to arise partly from rhythmic amplification of rapid pulses and glucose-insulin reciprocal interaction in periphery (Sturis et al., 1991; Simon and Brandenberger, 2002; Li et al., 2006). Secretory pulses originating from each separate beta cell and islet are synchronized via intercellurar electrical coupling mediated by gap junctions (Pedersen et al., 2005; Head et al., 2012), cholinergic nerves innervating the pancreas along with negative feedback loop due to insulin induced clearance of glucose by liver (Pedersen et al., 2005; Zhang et al., 2008b).

Pulsatile mode of insulin secretion persists under wide range of glucose concentrations including both phases of GSIS and has been suggested to have several advantages over constant release. Oscillations in insulin release may prevent disruption of calcium balance, desensitization of insulin secretary machinery in beta cells and insulin receptor downregulation in target tissues (Goodner et al., 1988; Jones et al., 1992; Peiris et al., 1992; Jahanshahi et al., 2009; Head et al., 2012). On physiological level it significantly improves insulin sensitivity in periphery, particularly in liver (Goodner et al., 1988; Peiris et al., 1992; Matveyenko et al., 2012). In support of these assumptions, defects in pulsatile fashion of insulin secretion are present in conditions of glucose intolerance and *diabetes mellitus* (Peiris et al., 1992; Hollingdal et al., 2000; Head et al., 2012).

1.2.2 Insulin action and glucose metabolism

Insulin is an essential regulator of nutrient usage in peripheral tissues. Major peripheral target tissues of insulin action are liver, skeletal and adipose tissues. To fulfil its mission in the regulation of metabolism in periphery insulin stimulates glucose uptake and storage, synthesis of fatty acids and their deposition in form of lipids and promotes amino acid uptake and utilisation for protein synthesis; at the same time insulin lowers the rate of gluconeogenesis, glycogenolysis and lipolysis (DeFronzo et al., 1981; Foretz et al., 1999; Saltiel and Kahn, 2001; Noguchi et al., 2013) (Figure 1.4). Additionally to its role in nutrient partitioning insulin acts as an antiapoptotic agent and facilitates cell proliferation (Saltiel and Kahn, 2001; Costes et al., 2006). Insulin effects on metabolism are counteracted by glucagon, which is secreted from pancreatic alpha cells in response to low glucose (MacDonald et al., 2007; Habegger et al., 2010). Thus glucagon signalling is important for avoiding hypoglycaemia and restoration of fuel availability. Glucagon increases circulating glucose concentrations via upregulation of gluconeogenesis and

glycogenolysis as well as stimulates lipolysis providing fatty acids for ketone production (Habegger et al., 2010).

The first step in intracellular signalling cascades downstream of activated insulin receptors is binding of specific adaptor proteins. Apart from already described IRS1 and IRS2, adaptor proteins fulfilling the role of intermediaries between insulin receptor and its downstream effectors include four other isoforms of IRS (IRS3-6), Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl), SH2B adaptor protein 1 (SH2B1) and SH2B adaptor protein 2 (SH2B2), Src homology 2 containing transforming proteins (Shc) and Grb2-associated binding protein 1 (Gab1) (Saltiel and Kahn, 2001; Taniguchi et al., 2006a).

Figure 1.4 Key mechanisms of insulin-induced signal transduction.

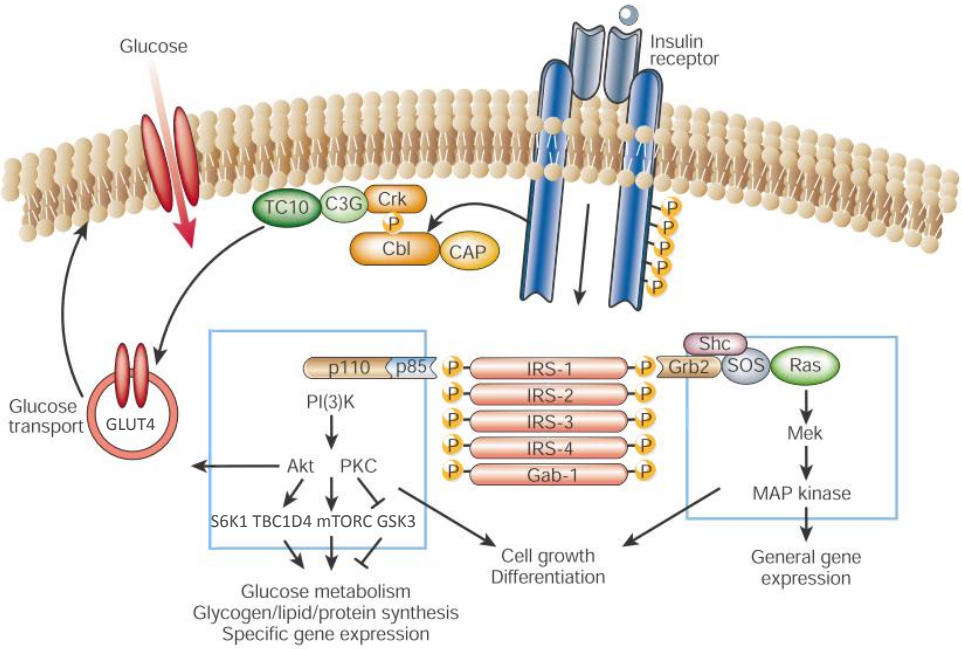

Table adapted from Saltiel and Kahn (2001). Binding of insulin induces autophosphorylation of its receptor. That enables recruitment of adaptor proteins and downstream effectors, which activate signalling cascades implementing effects of insulin within the cells.

Similarly to its signalling in CNS, the central pathway responsible for insulin action in periphery is mediated by IRS1, IRS2 and PI3K. PI3K binds SH2 domains of IRS proteins phosphorylated at tyrosine residues by insulin receptor tyrosine kinase, which brings PI3K consisting of p85 regulatory subunit and p110 catalytic subunit close to its substrate PIP2 at the plasma membrane (Pirola et al., 2004). PI3K converts PIP2 to secondary messenger PIP3; that is needed to facilitate interaction of phosphoinositide-dependent kinase 1 (PDK1) with protein kinase B (PKB or Akt) (Alessi et al., 1997; Wang et al., 1999; Saltiel and Kahn, 2001), ribosomal protein S6 kinase 70-KD 1 (RPS6KB1 or S6K1), members of protein kinase C (PKC) family and other targets. Akt has three isoforms, two of them Akt1 and Akt2 (or PKBα and PKBβ) have been considered as the key enzymes in PI3K induced signalling cascades switching on and off mechanisms involved in the regulation of majority of metabolic effects induced by insulin (Cho et al., 2001; Taniguchi et al., 2006a) (Figure 1.4).

1.2.2.1 Impact of insulin signalling on glucose turnover

Regulation of glucose uptake

Insulin controls glucose uptake by skeletal muscle, major site of glucose disposal, as well as adipose tissue by inducing translocation of insulin dependent glucose transporter GLUT4 to the plasma membrane from specialised intracellular compartments containing GLUT4 storage vesicles (DeFronzo et al., 1981; Saltiel and Kahn, 2001).

Both active PI3K and Akt have been shown to be necessary for successful GLUT4 translocation (Tanti et al., 1996; Wang et al., 1999; Larance et al., 2005). PI3K triggered phosphorylation of Akt by PDK1 enhances its enzymatic activity promoting phosphorylation of guanosine triphosphatase (GTPase) TBC1 domain family, member 4 (TBC1D4 or AS160) initiating its dissociation from GLUT4 vesicles. Removal of TBC1D4 promotes switching of monomeric G proteins known as Rab proteins including Rab8A, Rab13 and Rab14 to active guanosine triphosphate (GTP) bound state. Rab proteins further regulate different steps of vesicle traffic from movements between intracellular compartments to late events like tethering and fusion of vesicles (Larance et al., 2005; Ishikura and Klip, 2008; Ishikura et al., 2008; Sun et al., 2010). Besides TBC1D4 Akt associated GLUT4 translocation may also involve TBC1 domain family member 1 (TBC1D1) and 138 kDa C2 domain-containing phosphoprotein (CDP138) (Ishikura and Klip, 2008; Xie et al., 2011).

Insulin action is also known to be mediated by several different PKC isoforms predominantly activated by PI3K and PDK1 controlled phosphorylation parallel to Akt (Kotani et al., 1998; Chappell et al., 2009). As shown in adipocytes, to stimulate transport of GLUT4 containing vesicles, PKCλ facilitates accumulation of active GTP bound Rab4 protein indirectly stimulating its coupling with motor proteins kinesins that promotes propelling of GLUT4 containing vesicles towards plasma membrane (Kotani et al., 1998; Imamura et al., 2003). Moreover, since Rab4 interacts with t-SNARE proteins this mechanism may aid also granule fusion (Li et al., 2001; Ishikura et al., 2008). Another phosphokinase isoform PKCβII induces recruitment of phospholipase D1 to plasma membrane. That promotes rearrangement of actin net and production of PIP2 necessary for exocytosis augmenting both transport and membrane fusion of GLUT4 storage granules (Huang et al., 2005; Chappell et al., 2009).

Apart from that, cytoskeletal remodelling and targeting of GLUT4 vesicles to plasma membrane in myocytes is also induced via PI3K regulated activation of GTPase Rac1, which interacts with proteins directly restructuring actin network proximal to plasma membrane (Jiang et al., 2002; Ueda et al., 2008; Chiu et al., 2010). On the contrary, the key mechanism of insulin triggered GLUT4 translocation in adipocytes is governed through SH2B2 pathway independently from IRS and PI3K (JeBailey et al., 2004). Phosphorylation of SH2B2 by insulin receptor kinase starts signalling cascade involving protein complex consisting of the Cbl proto-oncogene (CBL), CBL associated protein (CAP) and v-crk avian sarcoma virus CT10 oncogene homolog (CRK), which interacts with guanine exchange factor C3G activating ras homolog family member Q (TC10) (Chiang et al., 2001; Liu et al., 2002; Lodhi et al., 2007). TC10 upregulates targeting of GLUT4 vesicles to the fusion sites by stimulating translocation of secretory granules and exocyst complex (Inoue et al., 2003; Lodhi et al., 2007).

Gluconeogenesis

Inhibition of gluconeogenesis or production of glucose from non-carbohydrate substrates is one of the main mechanisms through which insulin turns down hepatic glucose output (Noguchi et al., 2013). Decrease in glucose production is initiated by Akt

induced phoshorylation of transcription factors forkhead box O1 (FOXO1) and peroxisome proliferative activated receptor-γ coactivator 1α (PGC-1α). Subsequently protein complex formed by FOXO1 between PGC-1α is disrupted, which leads to nuclear exclusion and degradation of FOXO1 (Biggs et al., 1999; Matsuzaki et al., 2003; Puigserver et al., 2003). Since FOXO1 is necessary for transcription of the genes coding for key enzymes involved in gluconeogenesis like phosphoenolpyruvate carboxykinase, fructose 1,6 bisphosphatase and glucose-6-phosphatase, lack of FOXO1 results in decrease of glucose production (Puigserver et al., 2003; Taniguchi et al., 2006b).

Glycolysis

Induction of glycolysis in hepatocytes requires coincident increase in glucose and insulin concentrations (Massa et al., 2011). Similarly to gluconeogenesis, insulin regulates glycolysis principally through stimulation of transcription of genes coding for critical enzymes involved in the metabolism of glucose like GCK and phosphofructokinase-1 (Foretz et al., 1999; Wang et al., 2009; Hagiwara et al., 2012). In contrast to beta cells, hepatic promoter of GCK gene is sensitive to insulin and glucagon signalling (Foretz et al., 1999). At least in the case of GCK, stimulatory effects of insulin on transcription of the gene has been suggested to be induced by PI3K, Akt and mammalian target of rapamicin complex 2 (mTORC2) mediated activation of transcription factors hepatocyte nuclear factor 4α (HNF4α), and hypoxia-inducible factor 1α (HIF1α) and probably SREBP1 (Foretz et al., 1999; Kim et al., 2004; Roth et al., 2004; Hansmannel et al., 2006; Hagiwara et al., 2012).

Glycogenesis

Since breakdown of glucose via glycolysis produces substrates necessary to fuel glycogenesis and lipogenesis, insulin indirectly stimulates these metabolic processes. The hormone has also direct regulatory impact (Saltiel and Kahn, 2001). Glycogen is a polymer in a form of which glucose is stored mainly in liver and skeletal muscle. To promote processing of glucose into glycogen Akt activated via the classical pathway inhibits glycogen synthase kinase 3 (GSK3), two isoforms of which GCK3α and GCK3β are the major negative regulators of glycogen synthase in liver and muscle respectively (Cross et al., 1995; McManus et al., 2005; MacAulay et al., 2007). Inactivation of GSK3 leads to increase in active dephosphorylated form of glycogen synthase that subsequently rises production rate of glycogen. Additionally, insulin may facilitate dephosphorylation of glycogen synthase by stimulating activity of specific protein phosphatases, although this has been suggested to have a minor impact (Suzuki et al., 2001; McManus et al., 2005).

1.2.2.2 Role of the insulin in the regulation of other cellular processes

Lipid metabolism

Insulin increases lipid accumulation within the liver and adipose tissue by slowing down lipolysis and amplifying lipogenesis. Net effect on lipid metabolism is achieved by insulin induced changes in activity and expression of involved proteins.

Decrease in activity of lipolytic enzymes in response to insulin results from activation of phosphodiesterase 3B (PDE3B) by Akt. PDE3B lowers cAMP concentration thus reducing activity of PKA. Since PKA functions as an activator of proteins involved in lipolysis like hormone-sensitive lipase, inactivation of it hinders breakdown of lipids (Kitamura et al., 1999; Berggreen et al., 2009). At the same time to stimulate lipogenesis Akt inactivates AMP-activated protein kinase (AMPK), which is the main inhibitor of the pace-maker in the process of fatty acid synthesis acetyl-CoA carboxylase (ACC). Further

augmentation of lipogenesis is achieved through upregulation of transcription of genes coding for lipogenic enzymes like fatty acid synthase and ACC (Choi et al., 2006; Berggreen et al., 2009). The central pathway controlled by PI3K and Akt leads to mammalian target of rapamicin complex 1 (mTORC1) associated increase in production of SREBP1, which is considered as the principal insulin responsive transcription factor implicated in regulation of lipogenesis (Krakower and Kim, 1985; Li et al., 2010a). Activity of SREBP1 is also regulated by PKC and c-Jun N-terminal protein kinase (JNK) 2 as shown in hepatocytes and in adipocytes (Inoki et al., 2002; Cheng et al., 2010; Duvel et al., 2010). Both kinases are PI3K dependent and induce signalling parallel to that of Akt. PKC may regulate SREBP1 at the transcriptional level, whereas JNK2 is more likely involved in the control of processing and nuclear translocation of SREBP1 (Sajan et al., 2009; Yamamoto et al., 2010; Ito et al., 2013).

Augmentation of protein synthesis

Like in the regulation of other metabolic processes, insulin action on protein synthesis is predominantly mediated by PI3K and Akt signalling. Augmentation of protein production by insulin occurs mainly due to its positive effects on initiation and elongation steps of protein translation (Proud, 2006).

During the initiation step of translation Akt inactivates GSK3β and eIF4E-binding protein 1 (4E-BP1). Inactivation of GSK3β allows eIF2B mediated reloading of eIF2 factor with GTP that accelerates recruitment of methionine loaded transport RNA subsequently amplifying translation (Jaramillo et al., 1991; Haghighat et al., 1995; Welsh et al., 1997; Proud, 2006). When 4E-BP1 is phosphorylated by Akt regulated mTORC1, cap binding eIF4E is released from 4E-BP1 and free to bind its partners helicase eIF4A and anchoring eIF4G forming eIF4F initiation complex (Jaramillo et al., 1991; Haghighat et al., 1995; Gingras et al., 1998; Proud, 2006; Wang et al., 2006b). Besides, formation of the complex is further aided by S6K1 mediated stimulation of interaction between eIF4B and eIF4A downstream of mTORC1. Fully formed eIF4F initiation complex is necessary for association of ribosomes with mRNAs and unwinding of mRNA for translation (Raught et al., 2004; Shahbazian et al., 2006; Dennis et al., 2012).

Insulin induced activation of S6K1 also leads to the upregulation of elongation step of protein translation. This is mediated by inhibitory effect of S6K1 on eEF2 kinase, which normally impairs binding of eEF2 to ribosomes by phosphorylating it (Carlberg et al., 1990; Redpath et al., 1996). Active eEF2 is involved in translocation of ribosomes along mRNA allowing for addition of next amino acid, hence augmenting the rate of protein synthesis (Alberts et al., 1991b; Proud, 2006). Another target of S6K1 is ribosomal protein S6, phopshorylation of which also may influence translational process (Jefferies et al., 1997; Proud, 2006).

Regulation of cell fate

Concurrently with PI3K and Akt mediated signalling cascades, insulin activates pathways induced by mitogen activated protein kinase (MAPK). MAPK signalling has been considered to be the central mechanism of controlling cell fate in response to insulin (Taniguchi et al., 2006a; Alejandro and Johnson, 2008).

Briefly, the first step following the activation of the receptor in insulin triggered MAPK cascade is phosphorylation of Src homology 2 domain containing (SHC) and IRS proteins resulting in recruitment of growth factor receptor bound 2 (GRB2) (Skolnik et al., 1993). The GRB2 associates with son of sevenless (SOS) and induces formation of Ras and v-raf-1 murine leukemia viral oncogene homolog 1 (Raf1) containing complex (Koide et al., 1993; Marais et al., 1995). Kinase Raf1 triggers sequential phosphorylation and

activation of kinase cascade composed of dual specificity kinases MAPK and ERK kinase 1 and 2 (MEK1 and MEK2), extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), S6K1 and p90 ribosomal S6 kinase (RSK), MAPK-interacting kinases (MNK) and mitogen and stress activated kinases (MSK) (Waskiewicz et al., 1997; Deak et al., 1998; Roux and Blenis, 2004). RSK, MSK and MNK control factors regulating numerous cellular processes such as immediate-early gene transcription, translation, cell cycle progression and cell survival. For example, downstream targets of these kinases include transcription factor c-Fos, cyclin dependent kinase p27, death-associated protein kinase (DAPK) and mitochondrial pro-apoptotic protein BCL2 agonist of cell death (BAD) (Shimamura et al., 2000; Fujita et al., 2003; Anjum et al., 2005; Zhu et al., 2008).

Of note, fully functional insulin induced Raf1 signalling via ERK and its direct inhibitory effects on BAD have been shown to be essential for promoting pancreatic beta cell survival and proliferation, while inhibition of ERK or Raf1 leads to beta cell apoptosis (Alejandro and Johnson, 2008). Besides, ERK mediated upregulation of CREB in beta cells may lead to increased expression of mitochondrial protein B-cell CLL/lymphoma 2 (BCL2) and IRS2, thus further improving functional capacity and vitality of beta cells (Jhala et al., 2003; Costes et al., 2006).

1.3 Pathogenesis of Obesity

During the last three decades prevalence of obesity has nearly doubled rising from 4·8% to 9.8% among men and from 7.9% to 13.8% among women. Apart from that, up to 35% of worldwide population can be classified as overweight. In terms of BMI these data translate as 0·4 kg/m² increase for men and 0.5 kg/m² increase for women per decade (Finucane et al., 2011; World Health Organisation., 2011). Proportion of adult residents of Latvia having excess weight ranges from 27% among women to 35% among men, whereas prevalence of obesity is higher in comparison to worldwide average indices with 16.4% of men and 18.6% women being obese (Pudule et al., 2013). Severe obesity is associated with several comorbidities affecting life quality and life expectancy including cardiovascular disease like hypertension, coronary heart disease and stroke, T2D, hyperlipidemia, asthma, renal failure and several types of cancer (Guh et al., 2009; Switzer et al., 2013).

Obesity develops due to disruption of balance in energy turnover occurring, when energy intake persistently exceeds current needs and excessive energy is intensively stored in a form of fats (Prentice and Jebb, 2004; Hill et al., 2012). Responsiveness of intrinsic homeostatic controllers to fluctuations in energy turnover as well as upper threshold, exceeding of which puts in action compensatory mechanisms, is largely predetermined by inheritance. In other words, genetic factors determine capacity of homeostatic systems to maintain energy balance when challenged by changeable environment (Speakman et al., 2008; Speakman et al., 2011). Recent changes in environmental factors like patterns of food consumption and switch to more sedentary lifestyle provide strong challenge to homeostatic systems and are believed to be major contributors to rapid rise in prevalence of obesity (Prentice and Jebb, 2004; Hill et al., 2012).

Measurements of obesity

Classification of overweight and obesity commonly is based on indirect measure of body fatness, body mass index (BMI), obtained dividing weight in kilograms by square of height in meters (kg/m^2). Adults having BMI equal to or above 25 kg/m^2 are classified as overweight, whereas those with BMI at least 30 kg/m^2 are considered as being obese (World Health Organisation., 2000) (Table 1.1). In populations of European origin BMI classifying person as obese corresponds with body percentage over 25% in adult men and

35% in adult women, whereas in severe obesity body fat percentage can reach from 50 to 60% (World Health Organisation., 1995; Deurenberg and Yap, 1999).

Although its accessibility makes BMI a convenient widely used measurement of obesity especially at the population level, this index incorporates both muscle mass and fat mass and does not account for bone structure, gender or age. Thus persons with the same BMI may still differ regarding degree of body fatness with particularly pronounced disparity among those having BMI below 30kg/m^2 (Deurenberg et al., 1998; Frankenfield et al., 2001). Besides, this measure does not allow for recognition between abdominal or central obesity and peripheral obesity or visceral and subcutaneous fat accumulation (Rothman, 2008). Such distinction is clinically significant as there are noticable differences in metabolic profile and predisposition to cardiovascular diseases and T2D depending on type of fat depots. Accumulation of excess fat in abdominal region is recognized the most adverse to health (Bolinder et al., 1983; Jensen et al., 1989; Snijder et al., 2003; Heilbronn et al., 2004; Toss et al., 2011). Abdominal fat depots can be evaluated using measures of waist and hip circumferences and indices derived from these measures such as waist to hip ratio (WHR) (Ashwell et al., 2012; Suchanek et al., 2012; Switzer et al., 2013). For example men with WHR above 1.0 and women with WHR above 0.85 are classified as being abdominally obese. On the other hand, distribution and volume of subcutaneous fat can be assessed by measures of skinfold thickness over triceps, midthigh, subscapular and suprailiac regions (Sievenpiper et al., 2001).

More accurate methods for evaluation of body fat content than those based on simple anthropometric measures distinguishing between fat and fat free mass are bioelectrical impedance analysis, quantitative magnetic resonance, dual energy x ray absorptiometry, isotope dilution or hydrometry, densitometry, magnetic resonance imaging and others (Deurenberg and Yap, 1999; Lee and Gallagher, 2008; Switzer et al., 2013). Multicompartment models dividing body weight into fat, water, protein and mineral, assessed by combining aforementioned methods, are assumed as a relative gold standard for body fat content measuring in vivo (Heymsfield et al., 1990; Wells et al., 1999; Wells and Fewtrell, 2006).

Table 1.1 Classification of adults according to BMI

Classification	BMI (kg/m^2)
Underweight	< 18.50
Normal range	18.50 – 24.99
Overweight	25.00 – 29.99
Obese class I	30.00 – 34.99
Obese class II	35.00 – 39.99
Obese class III	≥ 40.00

Table and table legend are from World Health Organisation Consultation on Obesity (2000).

1.3.1 Changes in eating environment and habits

1.3.1.1 Energy density and macronutrient content of foods

Industrialization of food production has increased availability of food and promoted transition from more traditional diets consisting of predominantly plant derived foods with high percentage of mildly processed fibre or non-starch polysaccharides and lean meat towards consumption of pre-processed energy dense foods, with high amount of calories per specific unit of food, rich in easy absorbable fats and simple carbohydrates (Brand-Miller and Holt, 1998; Cordain et al., 2005).

Diets with high fibre content like vegetables and whole grain typically have low energy density, while requiring longer time for chewing, delaying gastric emptying and displaying slow absorption rate. Longer nutrient exposure within gastrointestinal tract facilitates hormonal response and significantly enhances satiety, thus limiting energy yield from consumed portion of food (Duncan et al., 1983; Burton-Freeman, 2000; Howarth et al., 2001). Inclusion of fibre rich foods in diet has been shown to be beneficial for reduction of total energy intake and weight. Accordingly energy dense diet induces weight gain and body adiposity (Duncan et al., 1983; Poppitt and Prentice, 1996; Bell et al., 1998; Astrup et al., 2000; Howarth et al., 2001; Schulz et al., 2005). The weight gain has been explained by passive overconsumption of energy due to impaired ability to sense caloric content of food and tendency to regulate meal according to weight or volume of consumed food, which prevents adequate decrease in volume of eaten food proportionally to its energetic value (Poppitt and Prentice, 1996; Viskaal-van Dongen et al., 2009).

Dietary fats

Foods rich in fats traditionally fall in the category of energy dense foods. Although dietary fats can induce sensory response through taste, smell, texture and visual cues, in many products sensory properties of fats are masked. These "hidden" fats escape detection, which diminishes satiation and attenuates behavioural compensation for positive energy balance (Drewnowski, 1995; Viskaal-van Dongen et al., 2009).

Moreover, regular consumption of high-fat diet per se desensitises nutrient sensing systems throughout digestive system further impairing satiety response (Duca et al., 2013). Excessive intake of fat rich foods has been suggested to dampen oral gustatory signalling, therefore increasing amount of food needed to induce substantial sense of nutrients (Stewart and Keast, 2011; Maliphol et al., 2013). However, failure to decrease volume of consumed food characteristic to overconsumption of dietary fats has been predominantly attributed to altered feedback from farther parts of gastrointestinal tract. Thus prolonged high-fat feeding leads to aberrations in satiation factor CCK mediated signalling due to downregulation of its receptors and increases rate of gastric emptying. That postpones termination of meal, therefore allowing for intake of larger food portions (Liddle et al., 1986; Covasa and Ritter, 2000; Nefti et al., 2009; Clegg et al., 2011). At the same time to cope with increased supply of dietary fats, gastrointestinal tract undergoes morphological and functional changes that are associated with expansion of absorptive surface of intestines and increase in enzymes involved in processing of fats. This adaptive reaction sufficiently enhances efficiency of energy extraction from food in a form of fats and contributes to weight gain (Reed et al., 1991; Sukhotnik et al., 2004; Duca et al., 2013).

Dietary carbohydrates

If it is clear that dietary fats promote development of obesity and foods with high fibre content are more likely to be protective, then the role of simple sugars like monosaccharides and disaccharides is far more obscure.

Replacement of fat with corresponding amount of total carbohydrates in diet has been correlated with moderate decrease in weight and body adiposity (Astrup et al., 2000; Gibson, 2007). Although carbohydrate rich diets may contain more fibre, the inverse relationship with body weight also tends to remain in studies regarding impact of sugars and complex carbohydrates other than fibre (Astrup et al., 2000; Gibson, 2007; White et al., 2010). Low fat diets with high sugar content have been shown to induce weight loss in obese adults on the background of decreased total calorie intake as well as when consumed *ad libitum* (Saris et al., 2000; Drummond et al., 2004). At the same time other studies have demonstrated comparable effectivity between hypocaloric diets with high and low sugar

contents to reduce body weight (Surwit et al., 1997; White et al., 2010). One of the possible explanations to inverse association of intake of dietary sugars with BMI could be the difference in sugar content among foods with various energy density (Gibney et al., 1995; Gibson, 2000; Ruxton et al., 2010). Gibson in his study showed that sugar content increased along with decrease in energy density of food even after exclusion of contribution from soft drinks. Although this correlation is largely attributable to reciprocal relation between sugar and energy dense fats also known as "sugar fat seesaw", the association still held true after adjustment of fat mediated effects. Thus lower energy density of sugar rich solid foods may attenuate energy overconsumption (Gibney et al., 1995; Gibson, 2000).

Together these observations may suggest that, compared to dietary fats, sugars may have more neutral role in regulation of body weight, casting some doubt towards significance of this class of nutrients in recent increase in obesity prevalence (Gibson, 2000; Ruxton et al., 2010). Yet, other studies provide evidence that contradict this theory showing that decrease in dietary sugars facilitates weight loss in adult populations, while other confirm that subjects displaying increased sugar intake tend to gain weight (DiMeglio and Mattes, 2000; Gibson, 2000; Chen et al., 2009b; de Ruyter et al., 2012). More consistent evidence towards positive correlation between dietary sugar and higher body weight in adults and children comes from studies investigating impact of sugar intake with sugar sweetened beverages (DiMeglio and Mattes, 2000; Chen et al., 2009b; de Ruyter et al., 2012).

Liquid foods induce weaker satiety response than solid foods with equal nutrient and caloric composition (DiMeglio and Mattes, 2000; Mourao et al., 2007; Leidy et al., 2010). Lower satiety inducing capability of nutrients consumed in a liquid form is linked to faster flow through gastrointestinal tract and reduced influence on regulators of satiation and satiety like insulin and ghrelin (Santangelo et al., 1998; Leidy et al., 2010). Similarly impaired satiety response has been observed after consumption of sugared beverages, which may easily lead to energy overconsumption and explain association between weight gain and this class of foods (DiMeglio and Mattes, 2000; Raben et al., 2002; Mourao et al., 2007; Chen et al., 2009b). However, strength of positive association between intake of sugar rich beverages and obesity has been questioned by meta-analysis involving studies in children and adolescents, where effects of sugar containing beverages on changes in BMI were found to be close to neutral (Forshee et al., 2008).

Altogether contradictions in findings from different studies conducted thus so far do not let to judge the significance of dietary sugars in development of obesity with firm confidence. It is most likely that impact of dietary sugars changes depending on form and nutrient composition of foods, energy expenditure and other social habits, assessment of which is methodologically complicated particularly in larger studies (Poppitt et al., 1998; van Baak and Astrup, 2009; Ruxton et al., 2010).

Glycemic index

Apart from contributing to caloric load carbohydrates may affect changes in body weight by their glycemic index (GI). Glycemic index of foods is a measure of how fast and how much blood glucose rise after consumption of specific food relatively to equal load of reference carbohydrate, typically glucose or wheat bread (Jenkins et al., 1981; Food and Agriculture Organization/World Health Organization, 1998). Rapidly digestible, processed and cooked foods with low fibre content, but rich in glucose and carbohydrates, that can be easily converted into glucose, have high GI (based on the bread scale above 70) (Food and Agriculture Organization/World Health Organization, 1998; Atkinson et al., 2008). Examples of foods with high GI include soft drinks, baked potatoes, boiled rice,

cornflakes, instant oat porridge and others (Roberts, 2000; Atkinson et al., 2008). Low GI diets have been shown to induce stronger satiety response and decrease food intake and subsequently display beneficial effects towards maintenance of normal body weight when compared to high GI diet (Slabber et al., 1994; Ludwig et al., 1999; Ludwig, 2000; Krog-Mikkelsen et al., 2011).

Rapid increase in blood glucose after meals with high GI induces strong insulin response, which stimulates glucose uptake by skeletal muscle and liver, while inhibiting hepatic glucose release (Ludwig et al., 1999; Saltiel and Kahn, 2001; Reynolds et al., 2009). At the same time metabolism is switched towards oxidation of dietary carbohydrates, lipolysis is decreased and lipogenesis stimulated, which promotes fat storage. Increased rate of glucose clearance from blood and decrease of fatty acids in circulation promotes hunger. Besides, response of satiation factors like CCK is weaker after high GI meals than low GI meals, that may further facilitate recurrence of hunger and overeating (Ludwig et al., 1999; Reynolds et al., 2009). Accompanied with enhanced accumulation of fat reserves these factors may increase risk of obesity (Ludwig, 2000; Saltiel and Kahn, 2001).

1.3.1.2 Food palatability

Dietary fats and sugars represent highly profitable and readily usable source of energy and therefore foods with high content of these nutrients are recognized as the most palatable or rewarding ones (Drewnowski and Greenwood, 1983; Drewnowski et al., 1992; Drewnowski, 1998). Palatability or hedonic reward induced by food is positively associated with appetite and eating rate (Spiegel et al., 1989; Yeomans et al., 1997). Considering that energy dense foods typically are characterised by increased content of fats and sugars and induced reward is reciprocally associated with satiation, food palatability may significantly encourage excess energy intake (Spiegel et al., 1989; Drewnowski et al., 1992; Yeomans et al., 1997; Drewnowski, 1998).

Although mechanisms regulating energy turnover strictly according to metabolic needs lower reward response in order to limit energy intake, hedonic systems can overrun inhibitory effects on energy intake induced by homeostatic mechanisms and promote positive energy balance (Rudski et al., 1994; Drewnowski, 1998; Kirkham, 2009; Hill et al., 2012). One of the reasons facilitating situation when non-homeostatic factors can overcome homeostatic constraints is that homeostatic systems regulating energy turnover are highly adapted to defend body from energy depletion, but are much less powerful to prevent positive energy balance. However, neuronal pathways involved in upheaval of energy homeostasis by reward signalling are still under investigation (Drewnowski, 1998; Hill et al., 2012).

There is evidence suggesting that overweight and obese persons may differ in their sensitivity to food linked reward that may further aid overcoming of homeostatic signals and predispose these individuals to weight gain (Davis et al., 2004; Benelam, 2009). One of the possibilities is that hypersensitive hedonic system reinforces reward value of food, thereby stimulating overconsumption of palatable food. This suggestion is supported by studies showing that people with excess weight report higher pleasure rates derived from consumption of high fat and high sugar foods than lean (Davis et al., 2004; Salbe et al., 2004; Blundell et al., 2005). On the other hand overeating of palatable food may be a consequence of impaired function of reward system, since more food would be needed to achieve desirable level of pleasure (Stice et al., 2008; Benelam, 2009). Although aberrations in reward response may be pre-existing, overconsumption of hedonic foods per se has been suggested to desensitize hedonic systems by downregulating dopamine signalling (Alsio et al., 2010; Johnson and Kenny, 2010). In such case it may trigger

vicious cycle resulting in further increase in food intake. However, it is possible that both mechanisms play their role in the development of obesity (Davis et al., 2004; Benelam, 2009).

1.3.1.3 Portion size and food variety

Apart from palatability non-homeostatic food related factors affecting eating pattern include served portion size and variety of served foods. Larger portion size of, for example, pre-packaged snacks or restaurant foods stimulated people to eat more and this relation was shown to remain positive over study period for up to two months (Diliberti et al., 2004; Rolls et al., 2004; Jeffery et al., 2007). Moreover, people have difficulties to evaluate difference between presented larger portion and usually consumed amount of food (Diliberti et al., 2004). Increased caloric intake typically is not compensated by subsequent reduction in energy consumption indicating that homeostatic regulators indeed are not powerful enough to oppose external food linked stimuli (Schwartz et al., 2003; Benelam, 2009; Mariman, 2012).

People eat more also when presented with greater choice of foods during a meal. Stimulatory effects of variety in offered foods largely can be explained by attenuation of sensory-specific satiety (Benelam, 2009; Brondel et al., 2009). Sensory-specific satiety is satiation related mechanism defined as temporary reduction in pleasantness of taste of food being consumed in contrast to other unconsumed foods. This phenomenon does not require food entering gastrointestinal tract and is independent of digestion or caloric intake (Rolls and Rolls, 1997), although capability to avoid it may be more pronounced for palatable foods (Brondel et al., 2009). Other probable mechanisms mediating increased food intake seen in presence of greater variability of food items may be, for example, encouraged interest in eating and avoidance of habituation (Myers Ernst and Epstein, 2002; Raynor et al., 2006).

1.3.1.4 Television viewing

Studies that aimed to evaluate impact of television viewing on food intake have demonstrated that both children and adults eat more, when watching television during meal (Vioque et al., 2000; Bellissimo et al., 2007; Temple et al., 2007; Rey-Lopez et al., 2012). This occurs due to decreased sensitivity to satiety signals induced by previous meal and delayed formation of mealtime satiation (Bellissimo et al., 2007). Impaired response to homeostatic control of energy intake may be related to novel external signals presented by television, which decrease awareness of food being eaten (Temple et al., 2007; Higgs and Woodward, 2009). When paying less attention to food being eaten, quantity of consumed food is often underestimated. Besides it disrupts memory formation about satiating quality of previous meal, thus, increasing later food intake (Robinson et al., 2013). Additionally, television viewing is associated with higher preference towards energy dense, palatable foods and more sedentary lifestyle, which may also contribute to weight gain (Ranjit et al., 2010; Thorp et al., 2013; Wennberg et al., 2013).

1.3.1.5 Social situation

Less attention will be paid to eating during meals eaten together with companions (Hetherington et al., 2006; Robinson et al., 2013). Indeed people usually consume larger amount of food per meal in presence of others than when being alone (Hetherington et al., 2006). Apart from reduced awareness of eating due to disturbance by social interaction, meal size may be also influenced by prolonged meal time characteristic to such social situations or, for example, mimicking persons with larger food intake (Feunekes et al., 1995; Hermans et al., 2012). Still, correlation between size of consumed meal and eating in

company is not so straightforward, since it depends on type of companions, whether they are, for example, members of family or strangers (Kristensen et al., 2002). Besides, people eating away from home also are more likely to increase their energy intake and high frequency of such occasions may lead to regular overconsumption and weight gain (Ayala et al., 2008; de Castro et al., 2012).

1.3.1.6 Sleep deprivation

Insufficient sleep has been frequently associated with weight gain and increased risk of developing obesity in children and adults (Singh et al., 2005; Cappuccio et al., 2008; Markwald et al., 2013). Data from meta-analysis including more than 600 000 adults with mixed ethnic background showed that one hour reduction of daily sleep correlated with 0.35 kg/m^2 increase in BMI (Cappuccio et al., 2008). One of the causes of weight gain due to sleep deprivation has been suggested increased hunger due to drop in levels of leptin and PYY along with increase in major peripheral stimulator of appetite ghrelin (Spiegel et al., 2004; Magee, 2009). Yet, in another study overeating was observed despite no aberrations in postprandial release of these hormones, indicating that other mediators may also be involved (Markwald et al., 2013). Findings from animal studies point towards orexins regulating food intake and wakefulness as plausible candidates for this role (Martins et al.; Markwald et al., 2013). Besides intervention with satiety mechanisms, shorter sleep time prolongs active period during which food consumption may occur and increases snacking at night that adds to total energy intake (Markwald et al., 2013; Spaeth et al., 2013). Moreover, sleep deprived persons more often display enhanced preference toward energy dense and palatable foods (Spiegel et al., 2004; Markwald et al., 2013). Detrimental changes in patterns of food intake associated with restricted sleep can be accompanied by decrease in energy expenditure. After insufficient sleep both children and adults feel more weary and tend to lower physical activity preferring sedentary pastimes like television viewing that further augments accumulation of energy surplus (Ortega et al., 2010; Benedict et al., 2011).

Apart from direct effects on energy intake and expenditure, altered sleep/wake pattern can lead to disruption of circadian rhythms. Circadian rhythms are maintained by intrinsic mechanisms that display oscillation period of 24 hours and serve to adjust functions of tissues to temporal changes in its environment like diurnal fluctuations in levels of circulating nutrients and hormones (Bray and Young, 2007). Long term disturbance of sleep/wake rhythm like characteristic to shift workers has been associated with increased risk of obesity as well as T2D (Scheer et al., 2009). Suggested mechanisms leading to adverse metabolic outcome include altered regulation of appetite due impaired suppression of leptin release at the end of the day and impaired insulin signalling (Mullington et al., 2003; Scheer et al., 2009).

1.3.2 Changes in energy expenditure

Energy is expended through resting metabolic rate, which describes energy needed to maintain vital functions of body at rest, thermogenesis including the thermic effects of food and thermoregulation, and energy used for physical activities. Resting metabolic rate accounts for 50-70% of expended energy and is highly correlated with body mass, particularly fat free mass. Energy use through physical activity is the most variable component and the only one under voluntary control (Johnstone et al., 2005; Blundell et al., 2012; Hill et al., 2012).

Mechanisation of daily life undoubtedly has decreased necessity to be physically active and there is evidence indicating that physical activity levels in modern societies

indeed are lower than in those living so called traditional lifestyle (Bassett et al., 2004; Hill et al., 2012; Pontzer et al., 2012; Rind et al., 2013). However, it has been doubted, whether the actual decline in total energy expenditure through physical exercises has been significant enough to be the main trigger of rapid increase in recent obesity prevalence, since total energy expenditure seems to have remained relatively unchanged (Dugas et al., 2011; Pontzer et al., 2012). Instead changes in levels of daily physical activity have been assumed to play role in modulation of impact of energy intake on weight stability. In theory, body weight will remain stable if energy intake will be lowered to match decreased needs of sedentary lifestyle. Yet, in practice it is hard to achieve, because homeostatic mechanisms display stronger resistance to weight loss than to excess energy intake. Being physically active allows one to consume more food without risking energy overconsumption as well as improves metabolic profile. Increasing rate of physical activity helps to optimise balance between incoming and expended energy, while sedentary lifestyle intensifies positive energy balance leading to obesity (Schwartz et al., 2003; MacLean et al., 2009; Hill et al., 2012; Mariman, 2012).

This hypothesis has been supported by studies showing that physically active lifestyle associates with protective effects against excessive weight gain across different age groups (Di Pietro et al., 2004; Byrd-Williams et al., 2010; Mitchell et al., 2013). Moreover, increasing physical activity has been reported to proportionally attenuate genetic predisposition to obesity. Paul T. Williams in his studies showed that moderate physical activity expressed as walking more than 4.5km per day or running more than 3km per day reduced parental contribution to BMI of their offspring by 36% compared to those walking less than 1.5km per day, whereas running more than 9km per day further reduced correlation with parental adiposity for around 50% (Williams, 2011; Williams, 2012). These findings are supported by study showing that higher working or leisure time physical activity reduced genetic predisposition to obesity for up to 40%, as estimated from joint effects of 12 common susceptibility SNPs (Li et al., 2010b).

Apart from protective effects against weight gain, regular physical activity has beneficial effects on regulation of blood glucose and insulin sensitivity as well as lowers risk of developing cardiovascular diseases (Colberg et al., 2010; Shiroma and Lee, 2010).

In addition to energy necessary for completing physical exercises, changes in environmental temperature may have impact on energy turnover. Lowering indoor temperature below comfort zone, like for example from about 24˚C to 19˚C, has been shown to result in subtle yet significant increase in energy expenditure (Celi et al., 2010). Ambient temperatures above the comfort zone on the other hand may have more pronounced effects on energy intake through lowering appetite and consumption of energy dense foods. Thus possibility to spend more time in comfort temperatures provided climate control systems may promote weight gain and development of obesity (McAllister et al., 2009; Moellering and Smith, 2012).

1.3.3 Intrauterine environment and predisposition to obesity

Recently more attention has been paid to the impact of environmental signals on programming of homeostatic systems and organ functions during fetal development. Both intrauterine nutrient restriction and overnutrition can lead to development of obesity later in life (Ravelli et al., 1999; Kensara et al., 2005; Ibanez et al., 2008; McAllister et al., 2009; Schack-Nielsen et al., 2010; Yu et al., 2011).

Organism is able to modify its development in response to environmental stimulus to improve its chances to meet requirements of its anticipated environment. Yet, mismatch between actual environmental conditions and the conditions to which regulatory systems

have been adapted puts an organism at disadvantage (Gluckman and Hanson, 2004; Kuzawa, 2005; McAllister et al., 2009). Thus, individuals exposed to intrauterine nutrient restriction due to maternal undernutrition, are prone to develop obesity, when confronting energy rich environment later in life, because their inner regulatory systems are rendered to gain maximal profit from available energy (Ravelli et al., 1999; Kensara et al., 2005; Ibanez et al., 2008; Begum et al., 2012). Hypernutrition during fetal development predominantly linked to maternal diabetes or obesity, on the other hand, rather induces functional changes in central and peripheral homeostatic pathways that form foundation for further progress of pathogenic effects of obesogenic factors confronted later in life (Chen et al., 2009a; Vucetic et al., 2010; Borengasser et al., 2013).

Information on nutrient availability is thought to be integrated into developmental programming predominantly through epigenetic regulation of gene expression (Vucetic et al., 2010; Borengasser et al., 2013). For example, maternal obesity has been associated with increased expression of genes involved in adipogenic and lipogenic pathways in rat white adipose tissues (Borengasser et al., 2013). Among central homeostatic and hedonic regulators of food intake exposure to maternal obesity in animal models was demonstrated to induce changes in genes involved in NPY, dopamine and opioid mediated signalling, which could explain enhanced food intake and increased preference of energy dense foods seen in offspring (Chen et al., 2009a; Vucetic et al., 2010). In conditions where palatable, energy dense foods are freely available such behavioural changes would strongly predispose to obesity.

1.4 Pathogenesis of T2D

Today 382 million people or 8,3% from global population have diabetes and 316 million or over 6% have impaired glucose tolerance with the greatest number of patients being between 40 and 59 years old (International Diabetes Federation, 2013). On average 7000 new diabetes cases are registered every year in Latvia. Prevalence of diabetes among adult residents of Latvia exceeds 6%, while 11% have glucose intolerance (International Diabetes Federation, 2013; Slimību profilakses un kontroles centrs, 2013).

The term "*diabetes mellitus*" describes heterogeneous group of metabolic diseases characterised by presence of hyperglycemia caused by impaired insulin secretion, insulin resistance or combination of both (Kaul et al., 2012; Goldenberg et al., 2013). Major symptoms of severe hyperglycemia include polydipsia, polyuria, weight loss and polyphagia (American Diabetes Association, 2008; Kaul et al., 2012). Acute life threatening conditions caused by pronounced hyperglycemia are ketoacidosis and hyperosmolar nonketotic syndrome, whereas chronically elevated blood glucose leads to development of microvascular diseases like retinopathy, nephropathy, ulceration and, in extreme cases, amputation of lower part of extremities, cerebrovascular and cardiovascular diseases as well as peripheral neurophaties (American Diabetes Association, 2008; Cade, 2008). The most common forms of diabetes are T2D (formerly known as non-insulin-dependent *diabetes mellitus*) and an autoimmune disorder, type 1 diabetes, (T1D or insulin-dependent *diabetes mellitus*) accounting correspondingly for 85 to 90% and 5 to 10% of diabetic cases (American Diabetes Association, 2008; Maraschin, 2012; International Diabetes Federation, 2013). Other forms of diabetes include gestational diabetes referring to glucose intolerance first recognized during pregnancy, maturity-onset diabetes of the young (MODY) and rare severe conditions including diabetes as one of the clinical features like Rabson-Mendenhall syndrome (Odawara et al., 1989; Gloyn, 2003; Ellard and Colclough, 2006; Yamada et al., 2006; Winckler et al., 2007; American Diabetes Association, 2008). Diabetes may also develop as a consequence of

endocrinopathies, medication, hormones, chemicals, infections and other external factors (American Diabetes Association, 2008; Maraschin, 2012).

The T2D is characterised by coexistence of insulin resistance and impaired insulin secretion due to progressive failure of pancreatic beta cells caused by complex interaction of genetic and environmental factors. Ketoacidosis is unusual and, in contrast to T1D, the disease rarely reaches the stage of complete insulin dependency to treat hyperglycemia (American Diabetes Association, 2008; Maraschin, 2012).

Diagnostic criteria for diabetes mellitus

Disorders of glycaemia are diagnosed following criteria based on thresholds of plasma glucose set according increase in risk of developing microvascular complications, in particular diabetic retinopathy (Goldenberg et al., 2013). Fasting plasma glucose value from 6.1 to 6.9 mmol/l along with oral glucose tolerance test (OGTT) plasma glucose below 7.8 mmol/l is classified as impaired fasting glycaemia, whereas fasting plasma glucose up to 7.0 mmol/l (126 mg/dl) and OGTT plasma glucose ranging from 7.8 to 11.0 mmol/L indicates towards impaired glucose tolerance (Schneider et al., 2003; World Health Organisation., 2006; Goldenberg et al., 2013). Glycaemic levels between norm and diabetes or glycated haemoglobin of 6.0% to 6.4% sometimes are referred to as "prediabetes" as each significantly predicts development of diabetes and its complications. Patient is diagnosed with diabetes if fasting plasma glucose is above or equal to 7.0 mmol/l, OGTT plasma glucose is above or equal to 11.0 mmol/l and/or glycated haemoglobin is above or equal to 6.5% (Schneider et al., 2003; World Health Organisation., 2006; Goldenberg et al., 2013) (Table 1.2).

Table 1.2 Diagnostic criteria of diabetes according to measurements of glucose

Diagnosis	Fasting plasma glucose	2h 75g OGTT glucose
Impaired fasting glycaemia	6.1 - 6.9 mmol/l (110–125 mg/dl)	< 7.8 mmol/l (140 mg/dl)
Impaired glucose tolerance	< 7.0 mmol/l (126 mg/dl)	7.8 - 11.0 mmol/L
Diabetes	≥ 7.0mmol/l (126mg/dl)	≥ 11.0 mmol/l (200mg/dl)

Table is adapted from Schneider et al (2003).

OGTT is performed measuring blood glucose concentration after an overnight fast and then two hours after a 75g oral glucose load (World Health Organization., 1985). Basal measurements of plasma glucose and insulin after an overnight fast may be used to derive surrogate indexes characterising insulin sensitivity and beta cell function. Most widely used indexes in epidemiological studies are homeostasis model assessment of insulin resistance (HOMA-IR= (fasting plasma insulin (mU/l)* fasting plasma glucose (mmol/l))/22.5) and beta cell function (HOMA-B= (20* fasting plasma insulin (mU/l))/(fasting plasma glucose (mmol/l) * 3.5)). Although easy to apply, there are several drawbacks in their usage like high variation depending on type of insulin assay used and limited sensitivity in extreme cases (Matthews et al., 1985; Wallace et al., 2004). Hyperinsulinemic euglycemic glucose clamp and hyperglycemic clamp techniques are considered as more precise methods for assessment of insulin sensitivity and secretion. Both approaches are based on direct measurements of glucose and insulin dynamics *in vivo* reflecting glucose uptake by tissue and secretion of insulin to compensate for hyperglycemia. Moreover, hyperinsulinemic euglycemic glucose clamp allows distinguishing between muscle and hepatic insulin resistance. Although more informative and precise than simple indexes, these methods are time consuming, expansive and require skilled personnel (DeFronzo et al., 1979; Elahi, 1996).

1.4.1 Obesity and development of insulin resistance

Obesity indisputably is one of the most significant risk factors for development of insulin resistance and T2D. Excessive caloric intake can overload the storage capacity of adipose tissue leading to adipocyte hypertrophy. Hypertrophic adipocytes with large intracellular lipid content display enhanced lipolysis and changes in profile of secreted hormones with perceptible shift towards proinflammatory factors, simultaneously decreasing release of insulin sensitizers like adiponectin (Jacobsson and Smith, 1972; Weisberg et al., 2003; Heilbronn et al., 2004; Bruun et al., 2005; Skurk et al., 2007; O'Connell et al., 2010). Development of adipose tissue inflammation further worsens dysfunction of adipocytes. Consequently, rise in circulating free fatty acids augments lipid accumulation within non-adipose tissue like liver and skeletal muscle (Perseghin et al., 1999; Ryysy et al., 2000; Heilbronn et al., 2004). These metabolic defects become further aggravated in presence of impaired subcutaneous adipogenesis along with increased visceral fat deposition within the abdominal region. Due to higher metabolic and lipolytic activity and lower insulin sensitivity in comparison to subcutaneous adipose tissue, visceral adipose tissue are particularly important in metabolic disease (Bolinder et al., 1983; Jensen et al., 1989; Cnop et al., 2002; Heilbronn et al., 2004).

Accordingly, causative link between increase in adiposity and aberrations in insulin signalling and glucose metabolism can be explained by several basic peripheral mechanisms including fat deposition in non-adipose tissues or ectopic fat accumulation, inflammation, mitochondrial deficiency and endoplasmic reticulum (ER) stress associated unfolded protein response (UPR). All these defects have been shown to be present in patients with obesity and/or T2D (Hotamisligil et al., 1995; Yki-Jarvinen, 2002; Petersen et al., 2004; Bhattacharya et al., 2007; Boushel et al., 2007; Qatanani and Lazar, 2007). Capacity of these mechanisms to induce insulin resistance and cell capability to confront metabolic challenge is modulated by external factors or a priori efficiency of intracellular metabolic pathways defined by inheritance (Samuel and Shulman, 2012).

General metabolic defects induced by peripheral insulin resistance are decline in glucose uptake particularly by skeletal muscle, rise in hepatic glucose output and increase in lipolysis. These metabolic aberrations result in increased circulating glucose and free fatty acids, thus, triggering feedback loop that further intensifies insulin resistance (Qatanani and Lazar, 2007).

1.4.1.1 Accumulation of fats in non-adipose tissues

High intracellular concentration of lipids leads to the increased generation of intermediate products of lipid synthesis from which DAG and ceramides have been proposed to be the most significant secondary messengers responsible for aberrations in insulin signalling (Erion and Shulman, 2010; Chavez and Summers, 2012).

Accumulation of intracellular plasma membrane associated DAG enhances activity of several novel isoforms of protein kinase C. PKCθ, PKCδ and PKCε and conventional isoform PKCβII interacting with protein components of insulin receptor signalling cascades (Itani et al., 2002; Dey et al., 2006; Cantley et al., 2013; Galbo et al., 2013). Dominant isoforms responsible for deleterious effects of DAG in myocytes are PKCθ, PKCδ and PKCβII, which phosphorylate IRS1 at serine residues, thus, preventing its activating phosphorylation at tyrosine residues induced by insulin receptor linked kinase (Itani et al., 2002). In adipose tissue phosphorylation of IRS in response to lipid signals is performed by PKCθ and PKCδ, whereas in the liver PKCε is the major DAG activated kinase, which interrupts insulin signal transduction by preventing stimulation of IRS2 (Frevert and Kahn, 1996; Dey et al., 2006; Samuel et al., 2007).

In contrast to DAG responsive kinases disrupting the chain of phosphorylation events downstream of insulin receptor at early steps mediated by the IRS, ceramides induce atypical kinase PKCζ and protein phosphatase 2A (PPA2) directly inactivating Akt (Galbo et al., 2011; Chavez and Summers, 2012). Modification of Akt by PKCζ reduces translocation of the enzyme to the plasma membrane its major site of action, whereas PPA2 induced dephosphorylation attenuates enzymatic activity of Akt (Chavez et al., 2003; Stratford et al., 2004; Chavez and Summers, 2012). Coexistence of both mechanisms has been shown in myocytes and adipocytes and at least PP2A mediated pathway is active in hepatocytes (Powell et al., 2004; Blouin et al., 2010; Stretton et al., 2010; Galbo et al., 2011). Apart from PKCζ, in myocytes another atypical kinase PKCλ has been shown to be involved in Akt inactivation, if not serving as the major negative regulator of this kinase (Stretton et al., 2010). However, despite the evidence supporting involvement of ceramides in impairment of insulin signalling, there is still some uncertainty towards the role of these lipid intermediates play in causation of insulin resistance (Chavez et al., 2003; Holland et al., 2007; Chavez and Summers, 2012). For example, some studies show that at least in hepatocytes deleterious effects of saturated and unsaturated fats are mediated predominantly by DAG, whereas changes in ceramide mediated pathways are irrelevant for transduction of insulin signal (Itani et al., 2002; Galbo et al., 2013).

1.4.1.2 Inflammation

Obesity is characterised by low grade persistent inflammation associated with increased release of such proinflammatory factors as tumor necrosis factor α (TNFα) from adipose tissue (Hotamisligil et al., 1995; Weisberg et al., 2003). Along with TNFα, adipocytes oversaturated with lipids produce high levels of chemoattractants, for example, monocyte chemotactic protein-1 (MCP-1), which stimulates invasion of macrophages in adipose tissue (Bruun et al., 2005; Christiansen et al., 2005). Macrophage content in adipose tissue from obese patients has been estimated to be close to 40% compared to 10% in lean subjects. Since immune cells are the major producers of proinflammatory cytokines, enhanced recruitment of macrophages significantly intensifies inflammatory process (Weisberg et al., 2003).

Within the cells TNFα triggers activation of several kinases including inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta (IKKβ), mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) and protein tyrosine phosphatase 1B (PTP1B) (Tang et al., 2006; Bouzakri and Zierath, 2007; Nieto-Vazquez et al., 2007; Austin et al., 2008). MAP4K4 promotes hyperphoshorylation of JNK and ERK1/2, while IKKβ activation has been suggested to be mediated by mTORC in PI3K independent manner (Zhang et al., 2008a). Collectively IKKβ, JNK and ERK1/2 inhibit IRS1 by phosphorylating sites of negative regulation containing serine residues and impair functions of Akt and its downstream target TBC1B4 (Tang et al., 2006; Bouzakri and Zierath, 2007; Austin et al., 2008), whereas PTP1B located downstream of JNK neutralizes stimulatory effect of insulin by dephosphorylating IRS (Nieto-Vazquez et al., 2007). Together induction of these detrimental mechanisms downregulates GLUT4 translocation, impairs expression of GLUT4 and adipogenic factors like PPARγ, as well as may induce cell death via nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-kB) dependent pathways (Kamata et al., 2005; Tang et al., 2006; Austin et al., 2008). Besides, TNFα is known to accelerate mitochondrial production of reactive oxygen species (ROS) and hence stimulate pathways regulated by ROS induced kinases (Kamata et al., 2005; Nishikawa et al., 2007). One of such kinases likely activated by TNFα via ROS signalling is apoptosis signal-regulating kinase 1 (ASK1). ASK1 induces mitogen-activated protein kinase kinase 4 (MAP2K4) and other mitogen activated kinases serving as switches for

JNK and p38 mitogen-activated protein kinase (p38 MAPK), which further mediate negative phosphorylation of IRS1 through ErbB and PI3K pathway (Ichijo et al., 1997; Imoto et al., 2006; Bluher et al., 2009; Hemi et al., 2011).

Apart from activating enzyme cascades TNFα upregulates expression of suppressor of cytokine signalling 3 (SOCS3) (Emanuelli et al., 2001). SOCS3 interacts with IRS1 and IRS2 and directs them for degradation and competes with IRS1 for binding sites at insulin receptor, thus lowering activation rate of IRS proteins (Emanuelli et al., 2001; Rui et al., 2002).

1.4.1.3 Mitochondrial dysfunction

Impaired oxidative capacity and increase in production of ROS, a byproduct of oxidative metabolism, has been suggested as primarily mechanisms linking mitochondria with defects in insulin signal transduction (Kahn, 2003; Kim et al., 2008; Phielix et al., 2008; van den Broek et al., 2010). Decrease in fatty acid breakdown due to low oxidative capacity facilitates intracellular fat accumulation in non-adipose tissues and amplifies adipocyte hypertrophy (Vankoningsloo et al., 2005; van den Broek et al., 2010; Grattagliano et al., 2012). Concurrently, as potent oxidants ROS directly damage cellular proteins, lipid structures and DNA and enhance enzymatic activity of stress regulated kinases like IKKβ, JNK and p38MAPK interfering with signal transduction downstream of insulin receptor (Ichijo et al., 1997; Nishikawa et al., 2000; Itani et al., 2002; Kamata et al., 2005; Imoto et al., 2006; Kim et al., 2008). Thus, defects in mitochondrial function may lead to insulin resistance *per se* as well as enhance deleterious effects of fatty acids and proinflammatory cytokines (Ichijo et al., 1997; Itani et al., 2002; Bloch-Damti et al., 2006). However, whether the mitochondrial failure is a cause or only consequence of insulin resistance is still under question, since abnormalities present in T2D also impair mitochondrial function (Boushel et al., 2007; Turner et al., 2007).

1.4.1.4 Endoplasmic reticulum stress

ER is a protein factory of the cell, where synthesis and folding of newly synthesised proteins take place. If capacity of protein folding step mismatches existing demands, accumulation of misfolded proteins occurs inducing ER stress. That activates UPR, which normally facilitates adaption of ER machinery to enhanced demand for protein folding (Hotamisligil, 2010; Wang and Kaufman, 2012). The inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1), double stranded RNA activated protein kinase (PKR) like endoplasmic reticulum kinase (PERK) and the activating transcription factor-6 (ATF6) govern the three canonical pathways shaping UPR. These UPR sensors are activated by misfolded protein initiated dissociation of inhibitory complexes formed with chaperone/glucose regulated protein 78 (BiP/GRP78) (Bertolotti et al., 2000; Shen et al., 2002; Hotamisligil, 2010). Activation of IRE1 induces translation of transcription factor X-box binding protein 1 (XBP1) (Lee et al., 2003). XBP1 and ATF6 initiate transcription of genes coding for chaperones, proteins involved in ER biogenesis and responsible for removal of misfolded proteins, as well as factors regulating ER redox control, inflammation and apoptosis (Yoshida et al., 2001; Lee et al., 2003; Rutkowski et al., 2006; Wang and Kaufman, 2012). PERK upregulates gene transcription via activation of activating transcription factor-4 (ATF4) as well as its partner in UPR ATF6. Apart from that, PERK acts to slow down protein synthesis via inhibition of eIFα, hence decreasing supply of newly synthesised proteins for folding (Harding et al., 1999; Rutkowski et al., 2006; Wang and Kaufman, 2012).

Although ER stress particularly affects highly loaded endocrine cells like insulin producing beta cells, chronic ER insufficiency accompanied by dysregulation of proteins

involved in UPR corrupts intracellular processes also in adipocytes and hepatocytes (Lipson et al., 2006; Hotamisligil, 2010; Pfaffenbach et al., 2010; Han et al., 2013). Several proteins together with their role in UPR also participate in the regulation of lipid metabolism. For example, changes in expression of GRP78 have been linked to enhanced hepatic lipid accumulation (Lee et al., 2003; Kammoun et al., 2009; Hotamisligil, 2010). ER stress associated hyperactivation of IRE1 induces JNK mediated disruption of insulin signalling cascades in adipose tissue and liver , whereas PERK hyperactivity stimulates FOXO1 opposing its inhibition by Akt and hence insulin signalling (Ozcan et al., 2004; Gregor et al., 2009; Pfaffenbach et al., 2010; Zhang et al., 2013). Both kinases may also exacerbate insulin resistance indirectly through NF-kB and JNK regulated inflammation (Urano et al., 2000; Deng et al., 2004). Moreover, PERK signals via eIF2α phosphorylation to enhance production of CCAAT/enhancer-binding protein homologous protein (CHOP) regulating PPARγ and to subsequently inhibit adipogenesis thus contributing to metabolic aberrations in adipose tissues (Han et al., 2013).

If capacity of UPR is insufficient for reversion of ER impairment, chronic activation of the pathway can lead to cell death mainly through the PERK, eIF2α and ATF4 regulated induction of proapoptotic genes (Pfaffenbach et al., 2010; Saito et al., 2010; Cao et al., 2012). In addition, overburden of UPR is linked to increased production of ROS, which further aggravates defects in insulin sensitivity and contributes to decreased cell vitality (Malhotra et al., 2008).

1.4.2 Loss of beta cell function in T2D

Pancreatic beta cells are highly adaptive and capable to match insulin secretion in wide amplitude of physiological requirements by boosting production of insulin and increase in beta cell mass via hypertrophy and proliferation (Steil et al., 2001; Rhodes, 2005; Ackermann and Gannon, 2007; Spellman, 2007). However, with progression of insulin resistance the threshold can be reached where functional capacity of beta cells is challenged. This is accompanied by defects in insulin production and secretion like loss of early response and pulsatile pattern of hormone release (Turner et al., 1971; Bruce et al., 1988; Peiris et al., 1992; Kahn, 2003). Continuous overload of beta cells results in high apoptosis rate eventually exceeding proliferation rate of beta cells, leading to loss of beta cell mass. Subsequently, increasing demand for insulin cannot be compensated inducing overt hyperglycemia (Butler et al., 2003; Kahn, 2003; Del Prato et al., 2004; Rhodes, 2005).

Still, not all individuals with insulin resistance develop glucose intolerance, whereas impaired beta cell function has been shown to be present before hyperglycemia occurs (Ehrmann et al., 1995; Elbein et al., 2000; Kahn, 2003). Although it supports the role of beta cell impairment in the initiation of T2D, despite intensive research, genetic and environmental background determining compensatory capacity of beta cells and mechanisms triggering beta cell dysfunction in prediabetic stage are still poorly understood (Kahn, 2003). At the same time functional overload and metabolic abnormalities present in obesity and T2D like glucotoxicity, lipotoxicity and inflammation significantly contribute to further cellular damage inducing vicious cycle accelerating beta cell failure and apoptosis (Kahn, 2003; Del Prato et al., 2004; Rhodes, 2005). Mechanisms underlying this relatively secondary causative relationship are better characterised, although some uncertainties remain.

1.4.2.1 Glucotoxicity

Within the physiological range, glucose is indispensable for maintenance of beta cell functions, vitality and proliferation, whereas prolonged exposure to high concentrations is toxic and leads to beta cell exhaustion (Porat et al., 2011; Bensellam et al., 2012). Deleterious effects of exposure to exaggerated glucose levels are caused by increased metabolic load on ER leading to ER stress and mitochondrial dysfunction accompanied with rise in production of ROS, as well as hyperactivation of such processes as glycation and hexosamine pathaway. Similar glucotoxicity associated mechanisms underlie development of complications of T2D in particular damage of vascular endothelial cells (Nishikawa et al., 2000).

Pancreatic beta cells are particularly vulnerable towards ER stress as highly active secretory factory. Insulin is major protein product comprising 40-50% of total amount of synthesized proteins in beta cell (Schuit et al., 1988). Long term high rate production of insulin prohormone and amyloid polypeptide coproduced with insulin is the main source of excess misfolded proteins causing ER stress and damage of ER membranes (Mulder et al., 1996; Huang et al., 2007a; Oslowski and Urano, 2011). In rare cases increase in proinsulin misfolding may also occur due to mutations in insulin gene regions coding for the fragments relevant for correct folding (Stoy et al., 2007). Recently it has been shown that ER stress and UPR mediated beta cell death can be also caused by hypoxia associated with rise in metabolic rate to cope with increasing glucose supply (Zheng et al., 2012).

Acute activation of UPR components IRE1, ATF6 and PERK by short term exposure to elevated glucose is obligatory for maintenance of insulin production and beta cell survival (Lipson et al., 2006; Gao et al., 2012; Engin et al., 2013), whereas exaggerated ER stress triggers deleterious mechanisms in general resembling those in other cell types eventually resulting in cell apoptosis like PERK and CHOP mediated pathway (Oyadomari et al., 2002; Hotamisligil, 2010). Specifically for beta cells, prolonged activity of ATF6 represses transcription of insulin gene due to impaired expression and function of transcription factors activating insulin gene promoter like PDX1, NEUROD1 and MafA, while hyperactivation of IRE1 precipitates degradation of proinsulin mRNA (Lipson et al., 2006; Seo et al., 2008a).

Along with ER stress, high intracellular glucose concentration promotes glycation and production of hexosamines. Glycation is spontaneous, non-enzymatic attachment of sugar molecules to proteins, lipids and nucleic acids, whereas hexosamines serve as substrates for enzyme mediated O-linked N-acetylglucosamine linkage to proteins or O-GlcNacylation (Alberts et al., 1991b). Uncontrolled activity of these mechanisms enforces modifications of cellular components that directly corrupts cellular functions like GSIS and decreases viability of beta cells. Stimulation of hexosamine pathway reduces PDX1 activity and lowers expression of its target genes coding for insulin, GLUT2 and GCK (Liu et al., 2000; Kaneto et al., 2001). Meanwhile, activation of specific receptors of advanced glycation endproducts (AGEs) leads to decline in GSIS associated with decreased production of ATP, which disrupts the essential link between glucose concentration and insulin secretory response. AGE signalling also induces cell death, for example, via decrease in antiapoptotic proteins like BCL2 and activation of apoptotic pathways that are induced by mitochondrial damage and mediated by caspases (Zhao et al., 2009; Zhu et al., 2010).

Both glycation and hexosamine pathway can stimulate mitochondrial ROS production (Kaneto et al., 2001; Lim et al., 2008). Mitochondrial oxidative phophorylation is the major glucose responsive source of ROS (Leloup et al., 2009), although alternative pathways utilising glucose and ER stress also have their contribution to ROS production (Kaneto et al., 2001; Back et al., 2009). Under physiological concentrations of glucose,

balanced ROS production coupled with glucose metabolism is necessary for stimulation of GSIS (Leloup et al., 2009). During prolonged hyperglycemia as in T2D, protective antioxidant systems fail to neutralise accumulating ROS resulting in oxidative stress (Tanaka et al., 2002). Low expression levels of antioxidant enzymes such as catalase, glutathione peroxidase 1 (GPX1), and superoxide dismutase 1 (SOD1) renders beta cells particularly sensitive to oxidative stress (Lenzen et al., 1996). Increase in ROS, especially hydrogen peroxide levels, impairs GSIS through interfering with glucose metabolism and decline in mitochondrial ATP production due to decreased activity of enzymes regulating glycolysis and Krebs cycle (Tretter and Adam-Vizi, 2000; Kajikawa et al., 2002). Exacerbation of oxidative stress stimulates JNK. Stress responsive elements downstream of JNK like c-Jun decrease expression and DNA binding ability PDX1 and MafA lowering promoter activity of insulin gene (Kaneto et al., 2002; Matsuoka et al., 2010). Furthermore hydrogen peroxide disrupts mitochondrial membrane potential and causes mitochondrial damage. Such functional and morphological abnormalities of mitochondria are present in beta cells derived from patients with T2D (Anello et al., 2005).

1.4.2.2 Glucolipotoxicity

Free fatty acids can exert beneficial as well as deleterious effects on beta cells. It is concentration, period of exposure and type that settles the mode of action of fatty acids (Stein et al., 1997; Maedler et al., 2003; Kim and Yoon, 2011). Moderate, temporary increase of fatty acid concentration facilitates insulin exocytosis through the pathways such as mobilisation of Ca^{2+}, ROS production and probably regulation of exocytotic machinery by intermediates of lipid metabolism including long chain acyl coenzyme A (acyl-CoA) and direct activation of fatty acid receptor GPR40 (Komatsu et al., 1999; Fujiwara et al., 2005; Schnell et al., 2007; Saadeh et al., 2012). Long chain saturated fatty acids like palmitic acid have been demonstrated to be major mediators of lipid action on functions of beta cells (Stein et al., 1997; Gravena et al., 2002; Maedler et al., 2003). However, unsaturated fatty acids still contribute to maintenance of insulin secretion. Oleic and palmitoleic acids were demonstrated to counteract deleterious impact of overexposure to saturated fatty acids such as palmitic acid and improve insulin secretion and beta cell proliferation (Maedler et al., 2003).

Glucolipotoxicity hypothesis states that fatty acids cause significant beta cell damage only when glucose concentration is increased and vice versa (Prentki et al., 2002). Beta cells are able to cope with increased glucose or fatty acid concentration by adjusting metabolism towards oxidation of the most abundant fuel, yet co-abundance of both can eventually overwhelm metabolic processes and lead to impaired production of insulin and cell damage. Hyperglycemia is associated with elevated glucose-derived malonyl-CoA, which regulates lipid partitioning or ratio between mitochondrial fatty acid β-oxidation and esterification (Prentki et al., 2002; Roduit et al., 2004). Malonyl-CoA inhibits β-oxidation leading to accumulation of long chain acyl-CoA and lipid related products like DAG and ceramides (Prentki et al., 2002; Roduit et al., 2004; El-Assaad et al., 2010; Somesh et al., 2013).

Increase in intracellular ceramide content leads to activation of kinases JNK and ERK1/2 that prevents PDX1 and MafA mediated activation of insulin gene promoter (Poitout et al., 2006; Fontes et al., 2009; Guo et al., 2010). Apart from loss of PDX1 and MafA induced signal, transcription levels of insulin gene are decreased by overexpression of another mediator of glucolipotoxicity, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α). This central regulator of mitochondrial biogenesis is associated with downregulation of NEUROD1 (Kim et al., 2009).

At the same time, alternations in sphingolipid metabolism, including ceramide and sphingomyelin turnover, impair protein trafficking and augment ER stress (Boslem et al., 2010; Boslem et al., 2011). Activation of UPR decreases insulin gene transcription and destabilises insulin mRNA (Pirot et al., 2007; Allagnat et al., 2010). Since ceramides induce mitochondrial dysfunction and subsequent drop in ATP production, these lipid metabolites can also attenuate insulin release. Secretion of the hormone is even further reduced by fatty acid mediated upregulation of uncoupling protein 2 (UCP2) (Maedler et al., 2003; Kim and Yoon, 2011; Yano et al., 2011). Mitochondrial failure induced by ceramides and high metabolite flux along with activation of UCP2 results in oxidative stress (Joseph et al., 2004). ER stress synergises with oxidative stress and mitochondrial apoptotic pathways to induce beta cell apoptosis, for example, via JNK mediated mechanisms, that eventually results in loss of beta cells observed during progress of the disease (Maedler et al., 2003; Allagnat et al., 2010; Boslem et al., 2010; Kim and Yoon, 2011; Somesh et al., 2013).

Overproduction of DAG is associated with disruption of Ca^{2+} currents essential for exocytosis and activation of PKC. One of PKC isoforms PKCε has been shown to attenuate amplifying pathway of GSIS (Cantley et al., 2009; Somesh et al., 2013). Ion flux necessary for exocytosis has been suggested to be abolished also by prolonged GPR40 induced opening of K_{ATP} accompanied by closure of inwardly rectifying volume regulated anion channels (Zhao et al., 2008; Best et al., 2011). However, described mechanisms provide only an insight in pathogenic pathways induced by synergy of glycotoxicity and lipotoxicity and more are still to be described.

1.5 Genetic background of common diseases

Currently two contrasting hypothesis exist explaining evolution of genetic background predisposing for obesity. The „thrifty gene" hypothesis originally proposed by J.Neel states that individuals genetically programmed to rapidly accumulate energy stores during periods of food abundance had greater chances to survive and reproduce during periods of famine in developing agricultural societies. Accordingly, positive selection for genotypes underlying such beneficial traits occurred (Neel, 1962; Prentice et al., 2008). Instead, "drifty gene" or "predation release" hypothesis is based on assumption that accumulation of variants predisposing for obesity has occurred due to genetic drift in the absence of selective pressure (Speakman, 2007; Speakman, 2008). In line with this hypothesis, it has been suggested that in early stages of human evolution lower threshold of BMI was defined by selective pressure linked to risk of starvation and capability to cope with disease, whereas major selective factor against increased body fatness was capability to escape the risk of predation. Development of social behaviour, use of fire and weapons significantly improved protection against predators, hence removing the selective pressure regulating upper threshold and allowing spreading of genetic variants linked to increased adiposity (Speakman, 2007; Speakman, 2008). Furthermore, genetic variants potentially predisposing to adiposity could have remained neutral in harsh environmental conditions human ancestors had to cope with, which could facilitate their random dispersion and escape from selection (Speakman, 2008).

Similarly, according to „thrifty genotype" hypothesis higher insulin resistance may have been advantageous during periods of famine, because low insulin response increases blood glucose by slowing down its uptake and facilitating gluconeogenesis. This helps to secure enough fuel for carbohydrate demanding brain sparing skeletal muscle mass, thus providing reproductive and survival advantage (Neel, 1962; Reaven, 1998). Alternatively, these mechanisms could have helped to cope specifically with low carbohydrate content in

diet of hunter-gatherer societies before agricultural development (Brand-Miller et al., 2012). Although this hypothesis is supported by findings showing high frequency of genetic variants linked to energetic efficiency in populations, which underwent strong natural selection for such traits, like Polynesians, it contradicts results from genetic studies performed in populations of European origin, where majority of revealed T2D susceptibility variants have been linked to impaired beta cell functions (Myles et al., 2007; Morris et al., 2012). On the other hand, high prevalence of risk variants linked to beta cell dysfunction may be explained by genetic drift allowed by relative absence of selective pressure in conditions of low demand for insulin (Southam et al., 2009; Pijl, 2011). While functionally neutral on the background of diet with low glycemic index, deleterious impact of these variants becomes apparent with increase in dietary carbohydrate content and its absorbency presenting much higher functional burden on beta cells (Pijl, 2011; Brand-Miller et al., 2012).

Although it is likely that both positive selection and genetic drift have shaped genetic background of common diseases, further increase in understanding about nature of variants forming genetic architecture of these traits may help to clarify which mechanism had dominated. So far four hypotheses have been suggested to explain the structure of genetic component underlying common complex traits.

"Common disease common variant" hypothesis states that disease risk can be explained by limited number of loci with relatively high frequencies and moderate effects. However, this statement has been recently challenged by GWAs showing that this class of variants contributes only small proportion of observed genetic risk (Gibson, 2009; Schork et al., 2009; Gibson, 2012; Morris et al., 2012). Subsequently, on the bases of incoming information from large scale studies, "common disease common variant" hypothesis has been substituted with "infinitesimal model" assuming existence of numerous susceptibility alleles with small effects covering wide frequency range (Gibson, 2012; Morris et al., 2012). On the other hand, according to the "common disease rare variant" hypothesis, pool of genetic susceptibility variants is enriched in rare alleles with frequencies under 1%, but strong functional impact. This model is largely based on assumption that spread of disease causing alleles ought to be limited by selective pressure. Significance of these variants in predisposition to disease hopefully will be resolved by next generation sequencing (Pritchard, 2001; Gibson, 2009; Schork et al., 2009).

Alternatively to described narrow sense heritability models, broad sense heritability models state that additive genetic effects alone are not sufficient to explain observed heritability of common traits and hence disease susceptibility is significantly modified by genotype-genotype, genotype-environment interactions and epigenetic effects (Visscher et al., 2008; Gibson, 2012). To assess patterns of mutual interactions between different categories of genetic variation and environment probably is the major issue to fully understand development of obesity and T2D as well as other complex diseases, as disease susceptibility is unlikely based on impact of solely one class of factors (Gibson, 2012; Loos, 2012).

1.5.1 Genetic background of obesity

1.5.1.1 Obesity associated syndromes

Obesity is one of the clinical characteristics of about 30 syndromes with complex phenotype also including developmental abnormalities, physical dysmorphology and more or less pronounced mental retardation (Chung and Leibel, 2005; Garver et al., 2013). These are predominantly cases of severe obesity related to inadequate response of central satiety centres (Shapira et al., 2005; Garver et al., 2013). Genetic basis of obesity associated

syndromes is complex, for it usually involves large scale structural and epigenetic changes often interfering with more than one candidate gene. Examples of obesity associated syndromes are Bardet-Biedl syndrome, Alstrom syndrome, Albright hereditary osteodystrophy and others, although probably one of the best recognized of these syndromes is Prader-Willi syndrome (PWS) (Chung and Leibel, 2005; Garver et al., 2013).

Apart from other symptoms, PWS is characterised by poor feeding in early infancy opposed with hyperphagia and massive weight gain later in early childhood (Cassidy et al., 2012). Exaggerated food intake and craving for food in patients with this syndrome is thought to be explained by impaired hypothalamic satiety control (Shapira et al., 2005; Cassidy et al., 2012). Weight gain is reinforced by increased physical inactivity and lower resting energy expenditure (Butler et al., 2007). The cause of PWS is the loss of expression of paternally expressed genes on 15q11.2-q13 and cluster of noncoding small nucleolar RNAs genes (snoRNA) (Cassidy et al., 2012). Hyperphagia and obesity have been shown to be present in patients lacking expression of cluster snoRNA C/D box 116-1 (SNORD116) due to small deletions (de Smith et al., 2009; Duker et al., 2010).

1.5.1.2 Monogenic obesity

The first successful steps towards deciphering single genes implicated in obesity, were made in studies of rodent models displaying morbid obesity, namely *ob/ob* and *db/db* mouse strains bearing mutations in genes coding for leptin and its receptor (Zhang et al., 1994; Chen et al., 1996). Shortly afterwards loss of function mutations in homologous loci were confirmed as a cause of monogenic forms of obesity in humans. Carriers of wide spectrum of deleterious mutations including frame-shift, missense, nonsense and splice site changes in one of these loci become obese early in childhood due to hyperphagia and lack of leptin induced satiety signal (Montague et al., 1997; Clement et al., 1998; Strobel et al., 1998; Farooqi et al., 2007b).

These findings served as the take-off point for further description of causative mutations in seven additional genes by linkage studies in cases with extreme phenotype. All of these genes code for proteins involved in the central regulatory pathway of the hypothalamic control of energy balance governed by leptin and melanocortins. Mutations leading to early onset obesity linked to impaired satiety response have been found in *POMC, MC4R, BDNF, PCSK1,* neurotrophic tyrosine kinase receptor type 2 (*NTRK2*), singleminded homolog 1 (*SIM1*) and melanocortin 2 receptor accessory protein 2 (*MRAP2*) genes (Hinney et al., 1998; Holder et al., 2000; Farooqi et al., 2003; Yeo et al., 2004; Gray et al., 2006; Farooqi et al., 2007a; Asai et al., 2013).

Yet, patients being obese because of a single gene defects with high penetrance make only a small proportion of total obesity cases. Thus, mutations in *MC4R* gene, the most frequent causes of monogenic obesity, are found in about 6% of obese patients, whereas only one patient has been described with missense mutation in *NTKR2* resulting in obesity (Farooqi et al., 2003; Yeo et al., 2004).

1.5.1.3 Common type obesity

Information provided by investigation of genetic risk factors in rare cases with extreme phenotypes has been integrated into candidate gene studies aimed to describe genetic background of common obesity. Scanning of loci containing genes linked to monogenic forms of obesity allowed mapping of more frequent susceptibility variants with less severe impact on phenotype, for example, in genes coding for leptin, leptin receptor, POMC, PCSK1 and BDNF (Chagnon et al., 2000; Geller et al., 2004; Baker et al., 2005; Benzinou et al., 2008b; Skledar et al., 2012). The list of plausible risk genes for common type obesity has been further extended by genetic analysis of candidate genes selected for

their role in physiological pathways regulating energy balance. Among such genes containing common variants associated with BMI are those coding for peripheral homeostatic regulators like PYY and PPARγ involved in adipocyte functions represented by well replicated susceptibility variant rs1801282 (Pro12Ala) (Deeb et al., 1998; Ahituv et al., 2006). Other examples include endocannabinoid receptor 1 gene (*CNR1*), dopamine receptor 2 (*DDR2*) and dopamine receptor 4 (*DDR4*) genes that are known to play role in food reward (Benzinou et al., 2008a; Roth et al., 2013). However, reliance on pre-existing hypothesis limits capacity of candidate gene studies to discover novel regulatory pathways.

Such problems do not concern family based linkage studies. Yet, highly useful for description of novel mutations with strong impact on the trait, this approach has proved to be less successful for deciphering common variants with moderate effects. Although this approach has revealed over 250 potential candidate loci linked to obesity or obesity related quantitative traits such as BMI and fat mass, only few potential risk genes, including glutamic acid decarboxylase enzyme (*GAD2*), ectonucleotide pyrophosphatase/ phosphodiesterase 1 (*ENPP1*) and amino acid transporter solute carrier family 6 member 14 (*SLC6A14*) have been picked out in positional cloning studies based on linkage data (Boutin et al., 2003; Suviolahti et al., 2003; Meyre et al., 2005; Rankinen et al., 2006; Walley et al., 2009). Moreover, replication of majority of indicated loci has been rather inconsistent with 15 finds confirmed in more than three studies and no significant hits in meta-analysis combining data from 37 linkage studies (Rankinen et al., 2006; Saunders et al., 2007).

So far GWA studies have proved to be the most advantageous approach used for discovery of novel variants contributing to risk of developing common diseases. GWA studies are based on screening of large number of SNP markers with frequencies above 0.05 covering the entire genome (Walley et al., 2009). It is hypothesis free approach that allows capture of common variation with moderate effects in general representing narrower susceptibility regions compared to those provided by linkage analysis (Walley et al., 2009; Roberts et al., 2010). Since first studies conducted in 2007, GWA approach has yielded over 30 readily replicated risk loci for common obesity (Walley et al., 2009; Loos, 2012) (Figure 1.5).7

The initial GWA studies, including about 5000 samples in a discovery cohort, revealed the most robustly replicated susceptibility locus for common type obesity comprising fat mass and obesity-associated (*FTO*) gene (Frayling et al., 2007; Scuteri et al., 2007). Identification of *FTO* was followed by discovery of common variants near well known risk gene for monogenic obesity *MC4R* showing association with BMI in study population comprising more than 16 000 samples (Loos et al., 2008). Effects of these common variants are small, when compared to those implicated in monogenic forms of obesity. Thus, approximate difference in BMI between male and female carriers of rare missense or frame shift variants in *MC4R* and non-carriers has been estimated to be 4kg/m^2 and 9.5 kg/m^2 respectively (Dempfle et al., 2004). On the other hand, average effect per risk allele of common variant rs17782313 representing the same locus was 0.22 kg/m^2 (Loos et al., 2008), whereas for variants in *FTO* 0.4 kg/m^2 per risk allele (Frayling et al., 2007). Following studies using sample size reaching 34 000 highlighted ten novel associations signals including one near transmembrane 18 (*TMEM18*) gene, displaying stronger effect on the changes in BMI than estimated for variants near *MC4R* locus, yet previously overlooked due to its lower frequency (Thorleifsson et al., 2009; Willer et al., 2009).

Results from these GWA studies were replicated in meta-analysis performed within the framework of GIANT consortium comprising 249 796 subjects. Apart from that, the large sample size provided enough power to confirm association with BMI for loci

overlapping transcription factor AP-2 beta (activating enhancer binding protein 2 beta) (*TFAP2B*) and neurexin 3 (*NRXN3*) genes initially associated with waist circumference as well as to detect 18 novel association signals including glutaminyl-peptide cyclotransferase-like/gastric inhibitory polypeptide receptor (*QPCTL/GIPR*), solute carrier family 39 (zinc transporter), member 8 (*SLC39A8*), cell adhesion molecule 2 (*CADM2*) (Lindgren et al., 2009; Speliotes et al., 2010). Furthermore, variants near olfactomedin 4 (*OLFM4*) and homeobox B5 (*HOXB5*) genes showing tendency towards association with BMI in adults according to GIANT consortium data, reached genome wide significance in subsequent meta-analysis of childhood obesity (Bradfield et al., 2012).

Figure 1.5 Common obesity susceptibility loci discovered in GWA studies for obesity and related quantitative traits.

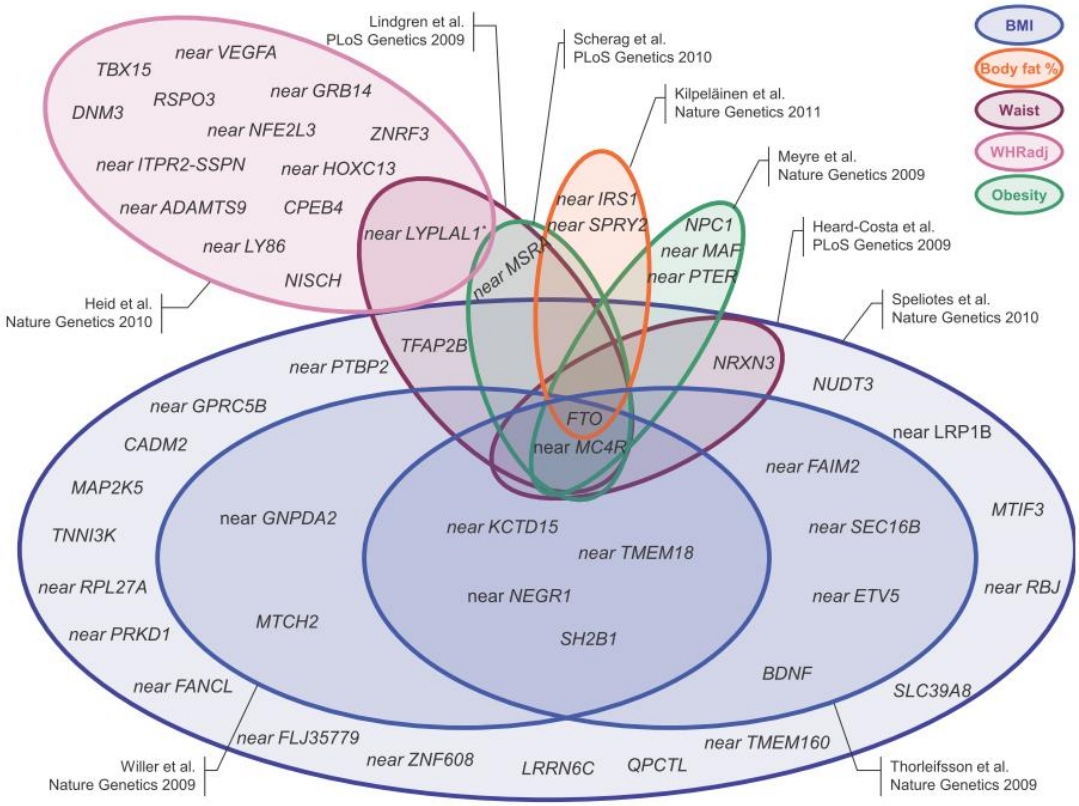

Table and table legends adapted from Loos (2012). Obesity susceptibility loci discovered in GWA studies for BMI (blue), genome-wide meta-analysis for body fat percentage (orange), GWA studies for waist circumference and WHR (pink) GWA studies for extreme and early-onset of obesity (green).

Several other studies focused on analysis of specific adiposity related traits added 16 loci like lysophospholipase-like 1 (*LYPLAL1*) and growth factor receptor-bound protein 14 (*GRB14)* associated with metabolically detrimental abdominal obesity and two more, namely *IRS1* and sprouty homolog 2 (*SPRY2*) genes, showing significant correlation with body fat percentage (Lindgren et al., 2009; Heid et al., 2010; Kilpelainen et al., 2011).

Majority of these findings from GWA studies in European populations have been confirmed in Asian, African and American Indian populations, with the most widely replicated signals represented by loci comprising *FTO*, *TMEM18*, *BDNF*, *MC4R*, SEC16 homolog B (*S.cerevisiae*) (*SEC16B*) and *QPCTL/GIPR* genes (Dorajoo et al., 2011; Takeuchi et al., 2011; Okada et al., 2012; Wen et al., 2012; Monda et al., 2013). Besides, studies in non-European populations have yielded several new risk loci for obesity, which initially have remained below genome wide significance thresholds, though afterwards have been replicated also in Europeans. Such examples are risk variants near mitogen-

activated protein kinase kinase 3 (*MAP2K3*) and ataxin binding protein 1 (*A2BP1*) identified in American Indians (Ma et al., 2010; Bian et al., 2013), two susceptibility loci containing genes microRNA 148a/ nuclear factor, erythroid 2-like 3 (*MIR148A/NFE2L3*) and polypeptide N-acetylgalactosaminyltransferase 10 (*GALNT10*) detected in Africans and several other risk loci found in Asians (Okada et al., 2012; Wen et al., 2012; Monda et al., 2013). Together observed concordance suggests that genetic component of obesity is largely shared across diverse populations and functional alleles underlying observed associations may be of ancient origin (Ma et al., 2010; Okada et al., 2012; Wen et al., 2012; Bian et al., 2013; Monda et al., 2013).

Apart from extensive studies of single nucleotide changes, interest has grown towards significance of copy number variants (CNV) or deletion/insertion variants affecting DNA segments greater than kilobase in length (Feuk et al., 2006). Lately several CNV have been linked to common type obesity in children and adults like insertion/deletion variant at 10q26.3 or extreme early onset obesity like 220kb deletion at 16p11.2 (Glessner et al., 2010; Yang et al., 2012; Walters et al., 2013; Wheeler et al., 2013). Besides, two of top SNPs from GWA studies in neuronal growth regulator 1 (*NEGR1*) and G protein-coupled receptor, class C, group 5, member B (*GPRC5B*) loci were found to be actually in high linkage disequilibrium with common potentially functional CNVs (Willer et al., 2009; Speliotes et al., 2010; Wheeler et al., 2013).

In general, data from GWA studies support findings from research of extreme obesity cases proposing aberrations in centrally regulated processes as the dominant cause also for common forms of obesity. However, other peak variants associated with risk of obesity or obesity measures locate in or near genes involved in the maintenance of peripheral metabolism, adipocyte functions, regulation of gut development and gut microbiome and immune processes (Ma et al., 2010; Speliotes et al., 2010; Kilpelainen et al., 2011; Bradfield et al., 2012; Bian et al., 2013).

Although it is beyond doubt that during the last two decades genetic research has enormously expanded our knowledge about biology of obesity, much more remains to be discovered. Common variants representing 32 loci from GWA studies explain from only 2% to 4% of variation in BMI from totally suggested 40-70% (Speliotes et al., 2010). Factors explaining "missing heritability" are still a major issue under discussion. One of the answers might be that numerous susceptibility variants have effects and/or frequencies below the detectible threshold of the currently used methods. For example, rare variants with small to medium effects are particularly prone to escape being detected (Roberts et al., 2010; Wray et al., 2011). Moreover, at least part of the heritability may be lost from estimations because of dilution of strong effects of rare variants in incomplete LD with common genotyped SNPs (Dickson et al., 2010; Wray et al., 2011; Gibson, 2012).

Another possibility is that heritability levels derived from initial family studies are overestimated (Heijmans et al., 2008; Zuk et al., 2012). Heritability, especially in complex traits, can be artificially inflated by gene to gene interactions (Zuk et al., 2012). Spurious heritability may also be introduced by epigenetic modifications transferred from parents or directed by prenatal environmental conditions inducing changes in gene expression without affecting DNA sequence (Heijmans et al., 2008; Vucetic et al., 2010; Feil and Fraga, 2012). Furthermore, epigenetic changes may serve as a causal link between external factors like diet and aberrations in systems regulating food intake also during adulthood and therefore interfere with genetic association signals (Vucetic et al., 2010; Vucetic et al., 2012).

1.5.2 Genetic background of T2D

1.5.2.1 Complex conditions with diabetes

Decoding process of the genetic base and pathogenic mechanisms of *diabetes mellitus* resembles the scenario followed to discover genetic causes of obesity, where information is gathered and integrated from studies of rare, severe cases along with those focusing on more common forms of the disease.

There are several rare disorders characterised by diabetes associated with specific pathogenic pathways. For example, severe beta cell dysfunction in patients with Wolfram syndrome 1 is thought to be caused by exaggerated ER stress induced by deficiency of ER transmembrane protein wolframin due to mutations within the wolfram syndrome 1 (wolframin) (*WFS1*) gene (Yamada et al., 2006; Marshall et al., 2013), whereas Wolcott-Rallison syndrome is caused by mutations in immediate early response 3 interacting protein 1 (*EIF2AK3*) gene coding for ER stress mediator PERK (Delepine et al., 2000). In Wolfram syndrome 2 and maternally inherited diabetes and deafness (MIDD) impaired insulin secretion results from decrease in ATP production and mitochondrial degeneration caused by mutations in CDGSH iron sulfur domain 2 (*CISD2*) and mitochondrial genes like mitochondrially encoded tRNA leucine 1 (*MTTL1*) respectively (Maassen et al., 2004; Amr et al., 2007; Chen et al., 2009c). In developmental delay, epilepsy and neonatal diabetes (DEND), on the other hand, insulin release is blunted due to gain of function mutations in potassium inwardly-rectifying channel, subfamily J, member 11 (*KCNJ11*) gene coding for subunit Kir6.2 of K_{ATP} channel preventing closure of channels in response to ATP (Gloyn et al., 2006).

Patients with Donahue syndrom, Rabson-Mendenhall syndrome and hyperinsulinemic hypoglycemia familial 5 having insulin resistance usually are homozygous or compound heterozygous for loss of function mutations within *INSR* gene or its regulatory region. Severity of phenotype among these cases may vary depending on pathogenicity of mutations and potency of beta cells to compensate for insulin resistance (Odawara et al., 1989; Hojlund et al., 2004; Jiang et al., 2011). Other monogenic causes of insulin resistance are mutations in gene coding for AKT2 and risk genes for congenital generalized lipodistrophy, 1-acylglycerol-3-phosphate O-acyltransferase 2 (*AGPAT2*) and Berardinelli-Seip congenital lipodystrophy 2 (seipin) (*BSCL2*), where insulin resistance occurs secondary due to loss of adipose tissue (Simha and Garg, 2003; George et al., 2004).

1.5.2.2 Monogenic forms of diabetes

Genetic studies of cases with neonatal *diabetes mellitus* (NDM) and MODY apart from confirming known factors have revealed several novel candidate genes linked with regulation of beta cell functions.

One of such candidate genes is found in maternally imprinted region on chromosome 6q24. Diabetic phenotype is most likely induced by overexpression of pleiomorphic adenoma gene-like 1 (*PLAGL1*) gene involved in regulation of cell cycle induced by sporadic or inherited genetic and epigenetic alternations (Docherty et al., 2010). Other mutations causing NDM have been described in *KCNJ11*, ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (*ABBC8*) encoding SUR1 subunit of K_{ATP} channel, *INS* gene itself and binding sites of transcription factors like Krüppel-like factor 11 (*KLF11*) in promoter region of *INS* (Vaxillaire et al., 2012). In rare cases NDM is caused by homozygous or compound heterozygous mutations in glucokinase (hexokinase 4) (*GCK*) gene leading to complete loss of enzymatic activity of encoded glucose sensor (Gloyn, 2003).

To date 13 MODY linked genes have been identified in families with autosomal dominant diabetes with some coincidence with risk loci for NDM. These genes are previously mentioned *KCNJ11*, *ABBC8, GCK* and *INS,* genes coding for transcription factors regulating insulin expression, beta cell differentiation and growth *HNF1A, HNF4A, HNF1B, PDX1, NEUROD1, KLF11,* paired box 4 (*PAX4*) and genes coding for enzymes carboxyl ester (*CEL*) and B lymphoid tyrosine kinase (*BLK*). GCK-MODY or MODY2 in contrast to more severe forms of diabetes is caused by heterozygous mutations in *GCK* gene (Stoffers et al., 1997; Kristinsson et al., 2001; Furuta et al., 2002; Gloyn, 2003; Neve et al., 2005; Ellard and Colclough, 2006; Plengvidhya et al., 2007; Molven et al., 2008; Borowiec et al., 2009; Bonnefond et al., 2012; Bowman et al., 2012).

1.5.2.3 Genetic risk factors for T2D

In common with genetic studies of obesity, linkage approach has been indispensable for deciphering genetics of monogenic forms of diabetes, but thus far has brought little contribution in research of T2D. Although more than 50 genome wide linkage studies of T2D have been conducted, replication of majority of the findings is inconsistent, with only few potent risk variants located within the linkage regions. Susceptibility genes for T2D containing such variants revealed by linkage studies include calpain-10 (*CAPN10*), adiponectin, C1Q and collagen domain containing (*ADIPOQ*), ectonucleotide pyrophosphatase/ phosphodiesterase 1 (*ENPP1*), protein tyrosine phosphatase, non-receptor type 1 (*PTPN1*) and MODY gene *HNF4A* (Hanis et al., 1996; Ghosh et al., 1999; Vionnet et al., 2000; Meyre et al., 2005). Yet, recent meta-analysis comprising 52 linkage projects showed that there is clear tendency of linkage peak clustering within the certain loci like on 6q, 1q, 18p, 2q, 20q, 17pq, 8p, 19q and 9q. That not only supports the role of *ENPP1, CAPN10, HNF4A* and *PTPN1* representing 6q, 2q and 20q, but also suggests existence of additional T2D risk genes. According to the authors of the study these loci may contain multiple rare variants with moderate effects, which may explain difficulties in replication and failure of association studies to detect these sites (Lillioja and Wilton, 2009).

However, despite the complications, linkage studies have provided one of the most successful finds in T2D genetic research. Within the 10.5Mb 10q linkage region positional cloning localized transcription factor 7 like 2 (*TCF7L2*) gene (Reynisdottir et al., 2003; Grant et al., 2006). Common variants within this gene show the strongest correlation with T2D detected so far with comparable effects among populations with distinct ancestral background.

Initially scanning for T2D susceptibility variants within the biological candidate genes has revealed two SNPs, which have withstood validation by large scale association studies within different populations (Saxena et al., 2007; Scott et al., 2007; Zeggini et al., 2007). Common missense variant Glu23Lys (rs5219) is located within *KCNJ11* previously described in context of monogenic disease (Gloyn et al., 2004), whereas the second SNP rs1801282 leads to the amino acid change Pro12Ala within the peroxisome proliferator-activated receptor gamma (*PPARG*) gene, that codes for nuclear receptor relevant for regulation of adipogenesis and transcription of several genes involved in insulin signalling (Beamer et al., 1998). Later SNPs robustly associated with T2D were detected in two additional candidate genes known from studies of monogenic forms of the disease, *WFS1* and *HNF1B* (Sandhu et al., 2007; Winckler et al., 2007).

Breakthrough in genetic research of T2D came in 2007 along with the publishing of first GWA studies for this common disease (Figure 1.6). Besides validating correlation with variants within *TCF7L2*, *PPARG* and *KCNJ11*, initial genome wide screens for association signals discovered six novel T2D susceptibility loci comprising solute carrier

family 30 (zinc transporter), member 8 (*SLC30A8*), hematopoietically expressed homeobox (*HHEX*), CDK5 regulatory subunit associated protein 1-like 1 (*CDKAL1*), cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*), insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2*), and *FTO* also found among the best findings in GWA studies for obesity (Saxena et al., 2007; Scott et al., 2007; Sladek et al., 2007; Steinthorsdottir et al., 2007; Zeggini et al., 2007). Newly discovered loci displayed even more moderate effects on T2D risk than the undoubtedly the most potent signal represented by *TCF7L2* with OR above 1.30. According to data from the largest GWA study comprising more than 3000 and 5000 controls among novel loci the strongest signal was representred by *FTO* (OR 1.22 (1.12-1.32)), whereas the weakest effects were observed for *HHEX* (OR 1.08 (1.01-1.15)) and *IGF2BP2* (OR 1.09 (1.01-1.16)) loci (Zeggini et al., 2007).

Shortly afterwards a better powered study incorporating more than 10 000 individuals resulted in identification of additional variants associated with T2D in loci representing JAZF zinc finger 1 (*JAZF1*), notch 2 (*NOTHC2*), thyroid adenoma associated (*THADA*), ADAM metallopeptidase with thrombospondin type 1 motif (9*ADAMTS9*), tetraspanin 8/leucine-rich repeat containing G protein-coupled receptor 5 (*TSPAN8*/*LGR5*) and cell division cycle 123/calcium/calmodulin-dependent protein kinase ID (*CDC123*/*CAMK1D*) genes (Zeggini et al., 2008).

Figure 1.6 T2D susceptibility loci discovered in GWA studies for T2D risk and related quantitative traits.

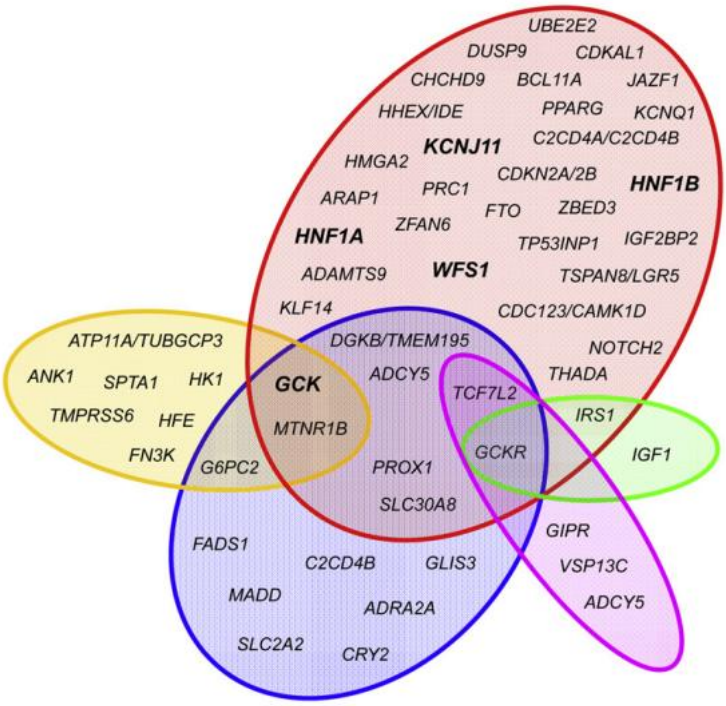

Table and table legends adapted from Vaxillaire et al. (2012). GWA studies for T2D (red); for fasting glycaemia and/or HOMA-B index (blue), for fasting insulinaemia and/or HOMA-IR index (green), for glycaemia 2h after or during OGTT (pink), for glycated haemoglobin (yellow). The genes/loci involved in both monogenic diabetes and polygenic/multifactorial forms of adult T2D are shown in bold.

These findings were followed by discovering association of SNP proximal to *IRS1* with increased T2D risk, insulin resistance and hyperinsulinemia and novel susceptibility locus for T2D near melatonin receptor 1B (*MTNR1B*) associated with increased fasting plasma glucose (Lyssenko et al., 2009; Prokopenko et al., 2009; Rung et al., 2009). Association of variants near *IRS1* and *MTNR1B* with T2D was further supported in a large

association analysis based on up to 42 542 cases and 98 912 controls along with mapping of 12 novel susceptibility loci. These included locus containing risk gene for monogenic diabetes *HNF1A* and dual specificity phosphatase 9 (*DUSP9*) located on X chromosome. Estimated allele specific ORs for 13 autosomal loci were small and ranged from 1.06 to 1.14 (Voight et al., 2010). Concurrently Qi et al. successfully validated novel association signal for T2D risk at 2q24 with the best SNP located within the intronic region of the RNA binding motif, single stranded interacting protein 1 (*RBMS1*) gene (Qi et al., 2010).

In studies focused on diabetes related quantitative traits association with glucose levels after OGTT, fasting glucose and/or HOMA-B was demonstrated for eight novel loci like adenylate cyclase 5 (*ADCY5*) and GLIS family zinc finger 3 (*GLIS3*) and several established T2D risk genes such as gastric inhibitory polypeptide receptor (*GIPR*), glucokinase (hexokinase 4) regulator (*GCKR*), *GCK, TCF7L2* and *SLC30A8* (Dupuis et al., 2010; Saxena et al., 2010). However, only one association signal was found for fasting insulin and indices of insulin resistance represented by variant near insulin-like growth factor 1 (*IGF1*) (Dupuis et al., 2010). Manning et al. in his study demonstratred that inclusion of BMI in the estimations may significantly improve power to discover variation involved in pathogenesis of T2D by revealing six loci linked to fasting insulin and seven novel association signals for fasting glucose including variants in *PDX1* and *GRB14* previausly linked to adiposity related traits (Manning et al., 2012).

Recently number of known susceptability loci involved in pathogenesis of T2D was increased up to 63 loci by two large meta-analysis comprising more than 100 000 participants with European ancestry each (Morris et al., 2012; Scott et al., 2012). Among these were *MC4R* locus previously associated with BMI and variants near cyclin D2 (*CCND2*) and *GIPR* showing gender specific effects (Morris et al., 2012).

Although with some minor discrepancy due to differences in allele frequencies, heterogeneity of allelic effects or LD patterns, most of the finds in Europeans have been confirmed in other populations with distinct ethnic background, particularly Asians (Waters et al., 2010; Kooner et al., 2011; Chen et al., 2012; Long et al., 2012). Thus several from association peaks detected in genome wide analysis like potassium voltage-gated channel, KQT-like subfamily, member 1 (*KCNQ1*), high mobility group 20A (*HMG20A*), phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (*PIK3R1*, also *GRB1*) and paired box 4 (*PAX4*) show stronger association in Asian populations compaired to Europeans (Yamauchi et al., 2010; Cho et al., 2011; Kooner et al., 2011; Ma et al., 2013). Apart from that, studies in non-European populations have highlighted novel associations such as those shown for ubiquitin-conjugating enzyme E2E 2 (*UBE2E2*), WW domain containing oxidoreductase (*WWOX*) and zinc finger, AN1-type domain 3 (*ZFAND3*) loci in Asians or variants near Rho family GTPase 3 (*RND3*) in African Americans that probably are population specific (Yamauchi et al., 2010; Kooner et al., 2011; Palmer et al., 2012). So, dissection of T2D genetics within distinct ethnic groups may further reveal novel aspects of T2D pathogenesis (Cooke et al., 2012; Palmer et al., 2012).

Most of the genes within discovered loci have been suggested to play a role in the regulation of beta cell functions rather than insulin sensitivity (Dupuis et al., 2010; Hart et al., 2010; Simonis-Bik et al., 2010; Voight et al., 2010; Morris et al., 2012; Scott et al., 2012). Attenuated first phase of GSIS associated with common variants in *KCNJ11, SLC30A8* and *IGF2BP2* loci, impaired beta cell turnover due to variants in genes of cell cycle regulators *CDKAL1* and *CDKN2A*/B and aberrations in circadian rhythms linked to variation in *MTNR1B* locus are examples of the suggested beta cell related defects associated with common variants from GWA studies (Dupuis et al., 2010; Voight et al., 2010; Morris et al., 2012; Scott et al., 2012). Exceptions include *PPARG, IRS1, KLF14, GRB14, AKNRD55* and *ADAMTS9* whose impact on T2D pathogenesis is likely mediated

by effects on insulin signalling (Voight et al., 2010; Morris et al., 2012). Although there is some certainty towards functional mechanisms providing link between development of T2D and well established candidate genes like *KCNJ11* and mediator of insulin signalling cascades *IRS1*, in general exact molecular pathways connecting most of the susceptibility loci with pathogenesis of T2D still remain largely unexplained (Voight et al., 2010).

Together common variants within 60 T2D susceptibility loci explain about 10% of familial clustering of the disease (Morris et al., 2012; Vaxillaire et al., 2012) (Figure 1.6). Like in the case of common type obesity, the "missing" heritability might be attributed to existence of a numerous variants with moderate effects, detection of which will require sample size exeeding 200 000 cases and controls (Morris et al., 2012). It also may be partly explained by "synthetic association", when common variant incompletely reflects effects of linked rare variant, or existence of additional independent variants within the same loci as suggested for *CDKNA2* and *FTO* masked by dominant signal (Voight et al., 2010; Morris et al., 2012). Besides, non-additive effects of gene-gene interactions and gene environment interactions particularly with lifestyle factors may modify contribution of genetic factors to the susceptibility of the disease (Qi et al., 2009; Bell et al., 2011).

1.6 Genetic studies of obesity and T2D in Latvia

Thus far there are only few studies that have been performed with the aim to investigate genetic risk factors contributing to development of obesity and T2D in the population of Latvia. These studies have been predominantly designed to evaluate impact of genetic variants that were located in particular candidate genes on specific phenotypes.

Five low frequency non-synonymous variantsVal103Ile, Ile121Thr, Ser127Leu, Val166Ile and Ile251Leu were found by sequencing the entire coding region of the *MC4R* gene in 380 subjects with severe obesity and 380 lean control subjects that were matched for age and gender. Both groups were selected from population of Latvia. In addition, *in silico* functional analysis was carried out to confirm contribution of variants found within *MC4R* to weight variation in the population (Rovite et al., 2014).

Carrier of Ile121Thr had normal weight. Variants V103I and I251L were found in both groups - cases and controls and Ile251Leu was more frequent among controls. The carriers of Val166Ile and Ser127Leu represented the case group(Rovite et al., 2014). Two of Ser127Leu carriers were also carrying Val103Ile. Obtained results mostly agreed with reports of studies that were performed in other populations with different ethnic background thus supporting protective effect of Ile125Leu and probably Val103Ile against obesity (Stutzmann et al., 2007) and confirming the prevalence of Ser127Leu and Val166Ile in obese subjects (Hinney et al., 2003; Wang et al., 2006a; Fan and Tao, 2009). As exception must be mentioned Ile121Thr, which in contrast to the current study was linked to obesity by other authors (Hinney et al., 2003). Since this variant had no effect it was not included in the functional analysis performed within the framework of the study under discussion (Rovite et al., 2014). Reported data are inconsistent making it difficult to evaluate the actual functional impact of Ile121Thr on weight gain difficult (Hinney et al., 2003; Xiang et al., 2010; Mo et al., 2012). Similar contradictions exist in the case of Val166Ile and Val103Ile. Despite the fact that the only carrier of the Val166Ile was obese, functional analysis showed that amino acid change at this particular position may affect ligand binding properties of MC4R rendering it more sensitive towards αMSH while lowering its affinity towards AGRP. Such effects would be expected to protect against weight gain (Rovite et al., 2014). Although neutral towards αMSH mediated signalling in various studies, Val103Ile has been demonstrated to decrease as well as to increase binding of the AGRP (Xiang et al., 2006; Rovite et al., 2014). Impaired binding of the AGRP in the

presence of Val103Ile would offer a straightforward explanation of observed protective effect in association studies. On the other hand it has been suggested that intensified inhibition of MC4R by AGRP may trigger mechanisms compensating for increased food intake (Rovite et al., 2014). The protective effects of Ile251Leu might be due to increased basal activity of MC4R (Xiang et al., 2006), although this theory is not supported by the current study as well as other authors reporting neutral effects of this amino acid substitution (Hinney et al., 2003; Rovite et al., 2014). Functional analysis of Ser127Leu demonstrated impaired transport of receptor to plasma membrane, which is in agreement with altered signal transduction in the presence of the rare allele observed by Fan and colleagues (Fan and Tao, 2009; Rovite et al., 2014). It must be noted, that negative impact of Ser127Leu was mitigated in presence of Val103Ile indicating that Val103Ile may modulate effects of Ser127Leu (Rovite et al., 2014). Considering supportive evidence from functional studies the amino acid change Ser127Leu found in three obese cases was proposed to be one of the most frequent causes of severe obesity associated with genetic variation within the *MC4R* locus among residents of Latvia (Rovite et al., 2014).

The same controls and cases were also used to evaluate effects of rs17782313, which is located in close proximity to *MC4R* gene and rs9939609, rs11642015 and rs62048402 within *FTO* gene on risk of obesity in the population of Latvia. Association of the common variants representing loci comprising *MC4R* and *FTO* genes with obesity has been described by GWA studies performed on obesity related traits (Frayling et al., 2007; Loos et al., 2008; Rovite et al., 2014). Risk alleles of SNPs located within *FTO* gene were more common in cases than in controls with difference in frequencies reaching significance for rs11642015 and rs62048402, whereas rs17782313 showed no association with severe obesity in the population of Latvia (Rovite et al., 2014). Although common variants within *FTO* gene are likely to have stronger impact on obesity in our population of than those representing *MC4R* locus, lack of association in the current case could be attributed to the study design which is categorical analysis of relatively small sample including severe cases of obesity. Subsequently additional studies including larger sample and BMI measurements could be valuable to evaluate the effects of common variants within the *MC4R* gene locus(Rovite et al., 2014).

Another candidate gene study which included the residents of Latvia has provided evidence supporting association of polymorphism within the promoter region of *IL6* gene with insulin resistance and T2D previously suggested by other authors (Tretjakovs et al., 2007). Its role in inflammation renders *IL6* as an eligible risk gene involved in pathogenesis of T2D (Weisberg et al., 2003). Although correlation of these variants with insulin resistance or T2D has not been unanimously accepted, more recent studies support the link between variants representing genomic region comprising *IL6* gene and T2D related traits with stronger effects observed in obese patients (Qi et al., 2006; Underwood et al., 2012; Yin et al., 2013).

There were also genetic studies that were conducted on the population of Latvia, which have promoted nomination of novel genes with possible relevance to the development of metabolic diseases. Thus analysis of microsatellite as well as SNP markers, namely rs2277460 (c.-110C>A) and rs1048990 (c.-8C>G), in the sample comprising healthy controls and T2D cases representing residents of Latvia has revealed proteasome (prosome, macropain) subunit, alpha type, 6 (*PSMA6*) gene encoding 20S core alpha subunit of proteasome complex as a plausible candidate region linked to T2D (Sjakste et al., 2007a; Sjakste et al., 2007b). Evidence towards correlation between markers representing genomic region comprising *PSMA6* and T2D or related traits has been also obtained in studies that included the samples from Botnia population and Chinese population (Sjakste et al., 2007a; Liu et al., 2012). Potential functional mechanisms linking

dysfunction of the ubiquitin-proteasome system with pathogenesis of T2D include impaired glucose sensing by pancreatic beta cells, amplification of ER stress and defects in insulin induced signalling due to dysregulated degradation of involved proteins (Rome et al., 2004; Hofmeister-Brix et al., 2013; Otoda et al., 2013).

Subsequently, considering contribution of obesity to the development of T2D the effects of rs2277460 and rs1048990 in *PSMA6* gene and rs2348071 (c. 543+138G>A) in proteasome (prosome, macropain) subunit, alpha type, 3 gene (*PSMA3*) coding for another component of proteasome complex were evaluated in the context of childhood obesity. There was no association between polymorphisms in *PSMA6* and obesity. On the contrary, frequency of rs2348071 which is located in *PSMA3* was significantly higher among cases who have reported family history of obesity than among normal weighted controls. The molecular pathways that could explain association of genetic variation in genes coding for proteins forming proteasomes are not yet described in detail. Recent studies have highlighted at least two distinct mechanisms. Increased activity of proteasome system has been positively associated with adipogenesis, whereas impaired function has been demonstrated to result in damage of neurons in hypothalamic feeding centres (Sakamoto et al., 2010; Ignacio-Souza et al., 2014).

Apart from the research done to investigate the genetic background of T2D, some studies have concentrated on deciphering genetic factors contributing to development and severity of complications of diabetes. One of the genes which is implicated in development of complications that are typical to T2D is glyoxalase 1 gene (*GLO1*). This gene codes for an enzyme neutralizing AGEs and protecting cells from damage caused by these agents. Polymorphisms within the promoter region of *GLO1* and gene itself have been associated with development of complications of T2D like retinopathy, nephropathy and neuropathy, although association results from different studies are controversial (Engelen et al., 2009; Wu et al., 2011; Groener et al., 2013). Nevertheless, recently conducted study comprising over 300 participants representing population of Latvia has described correlation of common single nucleotide variants within *GLO1* locus with reduced activity of the enzyme (Peculis et al., 2013). Considering that expression and activity of glyoxalase 1 has been shown to be altered in the presence of late diabetes complications, these findings add to the evidence asserting the role of genetic variation in *GLO1* locus in development of complications of *diabetes mellitus* (Wu et al., 2011; Skapare et al., 2013).

1.7 Characteristics of the studied genes

1.7.1 Agouti related protein homolog (*AGRP*) gene

Endogenous antagonist of MC3R and MC4R AGRP is coded by Agouti related protein homolog (*AGRP*) gene located on chromosome 16q22. The AGRP was identified as a hypothalamic protein displaying high homology with the mouse Agouti protein and human ASIP (Ollmann et al., 1997; Shutter et al., 1997). Besides expression in hypothalamus, human and murine protein has been detected in subthalamic nucleus and several sites in periphery like adrenal gland, testis lung and kidney (Ollmann et al., 1997; Shutter et al., 1997; Ilnytska and Argyropoulos, 2008). The *AGRP* gene gives rise to two different transcripts. Within the CNS AGRP is expressed in its longer form, while short transcript is found in periphery. Shorter transcript is probably produced through induction of an alternative promoter region located within the first non-coding exon of the gene known to be active in peripheral tissues (Brown et al., 2001). In addition, AGRP can be post-translationally cleaved by PCSK1 into mature carboxyl-terminal AGRP(83-132) with high affinity to MC4R and MC3R and two amino-terminal peptides AGRP(25-51) and

AGRP(54-82) lacking ability to inactivate melanocortin receptors (Quillan et al., 1998; Creemers et al., 2006).

Expression of AGRP by neurons in hypothalamic ARC is upregulated in mice lacking leptin or functional leptin receptors and increases during fast (Ebihara et al., 1999). Accordingly, increase in the expression is reversed by intracerebroventricular (icv) administration of leptin (Ebihara et al., 1999; Mizuno et al., 1999; Korner et al., 2000). When administered icv, carboxyl-terminal form of AGRP blocks αMSH effects increasing long term food intake and body weight gain as well as decreases energy expenditure (Rossi et al., 1998; Ebihara et al., 1999; Lu et al., 2001; Small et al., 2001; Goto et al., 2003), which is in line with obese hyperphagic phenotype of transgenic animals overexpressing AGRP (Graham et al., 1997; Ollmann et al., 1997). Furthermore, administration of AGRP(83-132) changed choice of diet in rats enhancing intake of food with high fat content through mechanisms supposedly involving interaction with opioid system (Hagan et al., 2001). It is possible that the amino terminal region of AGRP is also functionally active in regulation of energy turnover, hence, although no impact was observed on amount of consumed food, administration of AGRP(25-51) and AGRP(54-82) induced long lasting decrease in energy expenditure. Since both peptides do not bind melanocortin receptors, AGRP may induce pathways distinct from melanocortin system (Goto et al., 2003). Opposite to pronounced phenotypic effects induced by overexpression of the neuropeptide, initial studies of *AGRP* knockout mice revealed no significant aberrations in the regulation of food intake, total energy expenditure, body composition or response to starvation suggesting existence of compensatory mechanisms operative in the absence of AGRP signalling (Qian et al., 2002). However, following research showed that animals lacking active AGRP develop lean phenotype with age due to increased metabolic rate and physical activity as well as display impaired response towards stimulation of preference of high fat diet mediated by opioid receptors (Wortley et al., 2005; Barnes et al., 2010).

Apart from its role in control of calorie intake, AGRP may also participate in regulation of energy metabolism and substrate utilisation in peripheral tissues independently of its impact on food consumption mediated by central melanocortin pathway (Joly-Amado et al., 2012).

In humans, levels of AGRP in circulation positively correlated with parameters of adiposity and have been shown to increase in response to prolonged fast in lean individuals coincidentally with changes in level of leptin (Katsuki et al., 2001; Hoggard et al., 2004). There is evidence that circulating AGRP may antagonise inhibitory effects of αMSH on secretion of leptin from adipocytes and influence release of hormones from adrenal glands (Dhillo et al., 2003; Hoggard et al., 2004). Yet, functions of peripheral AGRP particularly in regard to control of energy homeostasis and its source need further clarification.

Evidence from genetic studies in human populations further support the role of AGRP as an important regulator of energy turnover. Although thus far only few variants linked to variation in measures of body fatness have been described in *AGRP* locus including population specific variants -3019G>A (rs8047574), -38C>T (rs5030981) and Ala67Thr (rs5030980). Two variants in complete LD -38C>T and -3019G>A located within the minimal promoter region of *AGRP* gene were associated with leanness, lower intake of proteins and decreased risk of T2D in populations of African descent (Mayfield et al., 2001; Argyropoulos et al., 2002; Loos et al., 2005; Bonilla et al., 2006). Adiposity lowering effects of -38T allele were likely mediated by decreased expression of the protein, for it was found to significantly alter promoter activity of *AGRP* gene in both hypothalamic and adrenal cell lines (Mayfield et al., 2001; Bai et al., 2004). Despite positive association with body leanness, -3019A increased *AGRP* promoter activity. However, these contradiction were explained by overwhelming negative impact of -3019A

linked -38T allele, since the latter displayed significantly stronger effects and therefore most likely was the driving force of the observed associations (Bai et al., 2004). Low frequency missense variant Ala67Thr (rs5030980) in the third exon of *AGRP* inducing allelic substitution Ala67Thr has been associated with reduced body fatness and *anorexia nervosa* in Caucasians, whereas its major allele Ala67 has been linked to late onset obesity (Argyropoulos et al., 2002; Marks et al., 2004; Li et al., 2013). Higher preference towards fat rich versus carbohydrate rich diet was suggested as the one of the contributors to weight gain observed in carriers of Ala67 (Loos et al., 2005). Computational analysis suggested that this missense variant may affect three dimensional structure of the protein, though these findings were not supported by functional studies showing no changes in binding activity of mutant AGRP towards melanocortin receptors (Argyropoulos et al., 2002; de Rijke et al., 2005).

Sozen and colleagues described another variant within the promoter region of the *AGRP* gene rs34018897 (+79G>A) in two individuals of European descent. Elderly female carrier of rs34018897 had reduced resting metabolic rate and was obese, whereas younger male, also carrying 67Thr allele, displayed only lower resting metabolic rate compared to controls from general population (Sozen et al., 2007). Authors suggested that male carrier of +79G>A could be protected from becoming obese by 67Thr associated with lean phenotype or possibly would develop obesity later in life (Argyropoulos et al., 2002; Sozen et al., 2007). The risk allele +79A led to decreased activity of promoter in adrenocortical cells, but no significant impact was observed in hypothalamic cell line. Thus, it is possible that particular phenotype could be induced by impaired expression of the gene in the peripheral tissues, although underlying mechanisms are not clear (Sozen et al., 2007).

Vink et al. within their study besides Ala67Thr found two additional variants 526G>A (rs34123523) located within second exon and in complete LD with Ala67Thr and 650C>T (rs11575892) in second intron by performing direct sequencing of the coding region of *AGRP* in 100 patients with *anorexia nervosa*. SNP 650C>T showed no association with the trait, when further investigated in 145 cases and 244 controls (Vink et al., 2001).

In common with other candidate genes, region harbouring *AGRP* is not represented by significant finds in GWA studies for obesity in European populations (Speliotes et al., 2010; Kilpelainen et al., 2011; Bradfield et al., 2012; Loos, 2012). However, there is still considerable lack of data on African populations, therefore it cannot be excluded, that large scale studies within these populations may bring forward also AGRP locus.

1.7.2 Adiponectin, C1Q and collagen domain containing (*ADIPOQ*) gene

Adiponectin, discovered almost a decade ago, is the most abundant hormone produced by adipose tissue (Scherer et al., 1995; Hu et al., 1996; Nakano et al., 1996). This adipocyte produced signalling protein or adipokine is coded by adiponectin gene (*ADIPOQ*) consisting of three exons that covers 15.8kb on chromosome 3q27. Protein containing a collagenous structure at the N-terminus and a globular C-terminal domain bears homology with members of complement factor C1q and tumor necrosis factor protein superfamily (Hu et al., 1996; Nakano et al., 1996). Adiponectin is permanently released into the circulation, where it is found as homotrimers, hexamers and high weight molecular complexes formed from multiple trimers (Scherer et al., 1995; Nakano et al., 1996; Pajvani et al., 2003). Adiponectin signalling appears to be predominantly mediated by widely expressed adiponectin receptors 1 and 2 (ADIPOR1 and ADIPOR2) (Yamauchi et al., 2003). Other suggested mediators include cell surface protein T-cadherin and

adaptor protein phosphotyrosine interaction PH domain and leucine zipper containing 1 (APPL1) located downstream of ADIPOR1/2 (Hug et al., 2004; Mao et al., 2006).

Adiponectin acts as an anti-inflammatory, anti-atherogenic, anti-apoptotic agent and insulin sensitizer in periphery and is thought to be involved in central regulation of energy homeostasis (Ouchi et al., 2001; Matsuda et al., 2002; Vasseur et al., 2002; Spranger et al., 2003; Qi et al., 2004; Coope et al., 2008; Park et al., 2011; Breitfeld et al., 2012; Turer and Scherer, 2012). Pathophysiological conditions like metabolic syndrome, obesity, T2D and cardiovascular diseases are characterised by low levels of adiponectin in the circulation (Weyer et al., 2001; Kumada et al., 2003; Matsuzawa et al., 2003; Iwashima et al., 2004; Pischon et al., 2004; Chen et al., 2005; Heid et al., 2006; Tabak et al., 2009; Breitfeld et al., 2012). Adiponectin deficient mice are glucose intolerant mainly due to hepatic insulin resistance, which is intensified during prolonged challenge with high fat diet (Maeda et al., 2002; Nawrocki et al., 2006). Besides, these animals exhibit impaired uptake of fatty acids by muscle and high levels of TNFα in adipose tissues and circulation (Maeda et al., 2002). Accordingly, transgenic overexpression of the hormone in adipose tissue correlated with higher basal metabolic rate, improved metabolic profile and decreased weight (Bauche et al., 2007; Kim et al., 2007).

Beneficial effects of adiponectin can be partly explained by its mediated abolishment of adipocyte differentiation. That leads to formation of adipocyte population exhibiting lower production rate of inflammatory cytokines and higher capacity of fatty acid uptake, thus decreasing inflammation and ectopic fat accumulation (Bauche et al., 2007). Along with its local anti-inflammatory action in adipose tissue, adiponectin promotes change from production of TNFα and IL6 towards release of anti-inflammatory cytokine IL10 in macrophages and suppresses proliferation and growth of macrophage precursors (Yokota et al., 2000; Wulster-Radcliffe et al., 2004).

In addition to secondary improvement in insulin sensitivity resulting from reduced inflammation and fat accumulation in non-adipose tissues, adiopnectin exerts direct beneficial effects on insulin signalling and glucose metabolism in the liver and skeletal muscle (Kim et al., 2007; Polyzos et al., 2011). The hormone activates ceramidase preventing accumulation of ceramides and enhances phosphorylation of insulin receptor and its downstream effectors in both, skeletal myocytes and hepatocytes (Stefan et al., 2002; Kim et al., 2007; Holland et al., 2011). In skeletal muscle adiponectin promotes glucose uptake and utilisation, stimulates fatty acid oxidation, induces mitochondrial biogenesis and enhances mitochondrial oxidative capacity (Yamauchi et al., 2002; Civitarese et al., 2006). In liver the hormone potentiates insulin mediated reduction in hepatic glucose output by suppressing activity and expression of major enzymes regulating gluconeogenesis (Combs et al., 2001; Nawrocki et al., 2006; Kim et al., 2007). Apart from amelioration of insulin action, adiponocetin is thought to improve insulin secretion and protect beta cells from stress induced apoptosis via Akt and ERK mediated pathways (Brown et al., 2010; Wijesekara et al., 2010).

The role of adiponectin in hypothalamic pathways regulating energy metabolism is less clear than its effects in periphery and and both positive and negative impact on food intake and energy expenditure has been attributed to the hormone (Qi et al., 2004; Kubota et al., 2007; Coope et al., 2008). Possible mechanisms underlying central action of adiponectin may involve interaction with leptin and insulin signalling pathways, melanocortin system and regulation of CRH (Qi et al., 2004; Coope et al., 2008; Park et al., 2011).

Protective effects of adiponectin on cardiovascular system are likely linked to its ability to distract formation of atherosclerotic plaques and enhance functions of endothelial cells by attenuating damage from inflammation and oxidative stress as well as increasing

production of nitric oxide (Ouchi et al., 2001; Plant et al., 2008; Tian et al., 2009). Furthermore, adiponectin also has antiapoptotic effects on cardiomyocytes and protects from damage caused by acute myocardial infarction (Shibata et al., 2005; Wei et al., 2012). However, during the further progression of tissue damage high circulating adiponectin positively correlates with cardiovascular disease and mortality rate (Shimano et al., 2008; Chang et al., 2009). Mechanisms of this paradoxical reversal from protective to damaging impact of adiponectin action are not yet clear (Cavusoglu et al., 2006; Turer and Scherer, 2012).

Pleiotropic physiological functions of adiponectin have aroused interest about genetic factors influencing adiponectin action and its link to metabolic diseases in human populations. The first evidence linking the genetic variation in chromosomal region containing *ADIPOQ* gene with the metabolic traits comes from linkage studies searching for novel susceptibility loci for metabolic syndrome, T2D and cardiovascular diseases (Vionnet et al., 2000; Francke et al., 2001). Afterwards correlation between variants representing *ADIPOQ* locus and levels of adiponectin in blood was confirmed (Vasseur et al., 2002; Gonzalez-Sanchez et al., 2005; Hivert et al., 2008).

Genetic factors are responsible for high proportion of variability in circulating levels of adiponectin with estimated heritability from 30 up to 70% across different populations (Vasseur et al., 2002; Menzaghi et al., 2007). The *ADIPOQ* locus comprises the strongest association signals for adiponectin levels in the European populations, yet common SNPs within the gene and its regulatory region appear to explain only 5% to 6.7% of observed variation in the circulating hormone (Heid et al., 2010; Breitfeld et al., 2012; Dastani et al., 2012). In Asians variants within the cadherin 13 (*CDH13*) gene share this position explaining up to 6.5% of variation in circulating adiponectin (Wu et al., 2010b). Other loci showing correlation with the concentration of the hormone in blood include ADP-ribosylation factor-like 15 (*ARL15*), kininogen 1 (*KNG1*), c-Maf inducing protein (*CMIP*), peptidase D (*PEPD*) and others (Richards et al., 2009; Wu et al., 2010b; Dastani et al., 2012).

The most replicated association signals within the *ADIPOQ* locus between changes in adiponectin and genetic variation among more than 17 variants linked to phenotype belong to SNPs rs17300539 (−11391 G>A), rs16861194 (-11426 G>A), rs266729 (−11377 C>G), rs2241766 (+45 T>G) and rs1501299 (+276 G>T) (Kyriakou et al., 2008; Enns et al., 2011). Variants rs17300539, rs16861194 and rs266729 represent the haplotype block covering the promoter of the gene and the most part of the first intron, while rs2241766 and rs1501299 found within the haplotype comprising the rest of the *ADIPOQ* gene (Heid et al., 2006; Breitfeld et al., 2012). However, no strong evidence has been found so far to confirm the two of the most studied SNPs, rs1501299 and rs2241766, as being causal variants, although the latter has been associated with allele specific expression of *ADIPOQ* (Yang et al., 2003; Bouatia-Naji et al., 2006; Hivert et al., 2008; Enns et al., 2011). On the contrary, presence of rs16861194 or rs266729 in *ADIPOQ* promoter resulted in significant decrease of its activity in adipocytes, whereas minor allele of rs17300539 was suggested to display positive effects (Bouatia-Naji et al., 2006; Laumen et al., 2009). Promoter harbouring three minor alleles was almost completely inactive further supporting functional significance of these variants (Laumen et al., 2009).

Some proportion of variation in adiponectin levels can be explained by low frequency variants. For example, in Hispanic Americans variants with frequency below 5% where demonstrated to explain 18% from variation in circulating adiponectin attributed to inherited factors compared to 5% for common variants (An et al., 2012). The most important variant specific to Hispanic Americans was functional missense variant rs200573126 (Gly45Arg) explaining 16.7% of varia (Waki et al., 2003; An et al., 2012).

Although rare variants associated with adiponectin levels such as rs62625753 (Gly90Ser) and rs17366743 (Tyr111His) have been described in Europeans, none of these have so strong effect as Gly45Arg, indicating that impact of different classes of variants may vary from population to population (Vasseur et al., 2002).

Variants associated with adiponectin levels also have been demonstrated to correlate with components of metabolic syndrome like increased BMI, concentration of blood lipids, insulin resistance, hypertension and other traits including several cardiovascular diseases, T2D and fatty liver disease (Vasseur et al., 2002; Gonzalez-Sanchez et al., 2005; Bouatia-Naji et al., 2006; Hegener et al., 2006; Li et al., 2007b; Menzaghi et al., 2007; Hivert et al., 2008; Melistas et al., 2009; Avery et al., 2011; Gupta et al., 2011). Yet, despite the vast evidence supporting link between metabolic diseases like T2D and adiponectin released in circulation, there are notable discrepancies among studies in different populations investigating the association between variants linked to adiponectin release and other metabolic traits (Xita et al., 2005; Heid et al., 2006; Mackevics et al., 2006; Menzaghi et al., 2007; Potapov et al., 2008; An et al., 2012). Reasons for observed discrepancy could be unaccounted effects of other loci, strong impact of non-genetic factors or development of compensatory mechanisms in strongly affected individuals (Heid et al., 2006; An et al., 2012; Warren et al., 2012). Indeed, recent large scale studies, less prone to bias due to numerous cofactors, support the link between genetic factors correlating with circulating adiponectin and development of metabolic disorders (Richards et al., 2009; Dastani et al., 2012; Scott et al., 2012).

1.7.3 Transcription factor 7 like 2 (*TCF7L2*) gene

The first evidence linking region on chromosome 10q with T2D came from the study by Duggirala and colleagues in Mexican American pedigrees where markers within this region showed strong correlation with T2D and age at onset of the disease (Duggirala et al., 1999). Later, the same chromosomal region was highlighted by study in Icelandic population (Reynisdottir et al., 2003). More detailed analysis using higher density of markers located the strongest association signal with T2D within the 92.1kb LD block overlapping third and fourth introns of the gene encoding the transcription factor 7 like 2 (TCF7L2), also known as T cell specific transcription factor 4 (TCF4) (Castrop et al., 1992). The rs7903146 has been recognised as a plausible functional variant or at least its closest proxy among other variants rs12255372, rs7901695, rs11196205 and rs7895340 in LD with a microsatellite DG10S478 originally associated with T2D (r^2 ranging from 0.95 for rs12255372 to 0.42 for rs7895340 according to Grant et al.) (Grant et al., 2006; Helgason et al., 2007; Palmer et al., 2010).

Since then, association between variation in *TCF7L2* and T2D has been consistently replicated across populations of European, Asian, North African and South American origin (Cauchi et al., 2006; Groves et al., 2006; Cauchi et al., 2007; Mayans et al., 2007; Wang et al., 2007; Herder et al., 2008; Marquezine et al., 2008; Sanghera et al., 2008; Ezzidi et al., 2009; Takeuchi et al., 2009; Billings and Florez, 2010; Bouhaha et al., 2010). With pooled OR of 1.46 estimated in meta-analysis combining published results from different populations it has been confirmed as the most significant common T2D risk locus identified to date (Cauchi et al., 2007). Still, Saudi Arabs and Pima Indians may be an exception since studies within these two populations have failed to show cogent correlation between the locus and risk of T2D (Guo et al., 2007; Alsmadi et al., 2008).

Despite the convincing finds from association studies, the functional role of the TCF7L2 in the regulatory network managing glucose metabolism is still obscure, although some of the possibly involved pathways have been already highlighted.

High-mobility group box-containing transcription factor encoded by the *TCF7L2* in the complex with β-catenin regulates expression of genes in the final stage of canonical wingless-type MMTV integration site family (Wnt) receptor signalling pathway (Liu and Habener, 2008; Welters and Kulkarni, 2008; Ip et al., 2012). It has been shown that canonical Wnt signalling pathway along with TCF7L2 is involved in development of pancreas as well as in the regulation of functions of mature pancreatic cells, though some disagreements exist towards whether this regulation is positive or negative (Lyssenko et al., 2007; Boj et al., 2012; Shu et al., 2012).

The *Tcf7l2* knockout mice displayed improved glucose tolerance and were protected from T2D, while overexpression of TCF7L2 in transgenic animals induced marked glucose intolerance (Savic et al., 2011). Similarly, increased expression of TCF7L2 in human pancreatic islets was accompanied by reduction in glucose stimulated insulin release, which is in concordance with higher total *TCF7L2* mRNA levels found in pancreatic islets from patients having T2D, with even more pronounced increase in mRNA for those carrying rs7903146 risk allele T (Lyssenko et al., 2007; Le Bacquer et al., 2012). Moreover homozygous T/T islets displayed morphological changes and reduced beta cell ratio (Le Bacquer et al., 2012). Correspondingly, the T allele of rs7903146 has been repeatedly associated with impaired insulin secretion, reduced stimulatory action of incretin hormones on GSIS as well as impaired response to insulin secretagogues sulphonylureas (Damcott et al., 2006; Florez et al., 2006; Saxena et al., 2006; Lyssenko et al., 2007; Pilgaard et al., 2009; Villareal et al., 2009; Schroner et al., 2011; Le Bacquer et al., 2012). As another defect underlying observed association with T2D in carriers of risk allele of rs7603146 has been proposed decreased proinsulin processing, which is supported by the fact that key prohormone convertases in proinsulin processing, PCSK1 and PCSK2, contain TCF-binding sites in their promoters (Loos et al., 2007; Gjesing et al., 2011).

Still, relation of TCF7L2 to disease might be more complex than that, for in other studies depletion of TCF7L2 in human or mouse pancreatic islets was found to lower GSIS and decrease β-cell proliferation, while increased expression protected against glucose and inflammation induced apoptosis (Shu et al., 2009; da Silva Xavier et al., 2012). Pancreas-specific knockout of *Tcf7l2* resulted in impaired glucose tolerance and inability of beta cells to proliferate in response to insulin resistance and, on the contrary, conditional knockout of *Tcf7l2* in mouse beta cells did not affect their functions (Savic et al., 2011; Boj et al., 2012; da Silva Xavier et al., 2012).

Apart from direct role in beta cell, TCF7L2 binds the promoter of the proglucagon gene and reduced insulin release may be as a consequence of altered expression of potent insulin secretagogue GLP1 in intestinal L cells (Yi et al., 2005). Indeed, functional inactivation of *Tcf7l2* in proglucagon expressing cells in mice decreased levels of GLP1 in gut and brain leading to defects in GSIS (Shao et al., 2012). Nonetheless, it seems that secretion levels of the GLP1 remain unaffected by *TCF7L2* genotype (Schafer et al., 2007; Pilgaard et al., 2009; Villareal et al., 2009). Considering that TCF7L2 depleted pancreatic islets from human and mice displayed decline in incretin hormone receptors, GLPR and GIPR, decreased augmentation of GSIS may be due to impaired action rather than impaired release of the both hormones (Shu et al., 2009; Gjesing et al., 2011; da Silva Xavier et al., 2012).

Outside the pancreas *TCF7L2* is expressed in central nervous system, liver, skeletal muscle and adipose tissue (Cauchi et al., 2006; Lyssenko et al., 2007). Therefore it cannot be excluded that, besides its role in endocrine pancreas, *TCF7L2* dysfunction in other tissues may have impact on the development of T2D as well. For example, liver specific removal of TCF7L2 resulted in severe hypoglycaemia, while overexpression accordingly resulted in increased hepatic glucose production (Boj et al., 2012). This is in line with

findings that carriers of *TCF7L2* risk genotype had increased rates of the endogenous glucose production compared with non-carriers (Lyssenko et al., 2007; Pilgaard et al., 2009).

Explanation to observed pleiotropic effects of TCF7L2 as well as contradictions among various studies may lay in highly variable alternative splicing of its mRNA. Some of transcripts were demonstrated to display tissue specific distribution, distinct physiological functions in various tissues and differ in their ability to activate Wnt signalling (Osmark et al., 2009; Prokunina-Olsson et al., 2009; Mondal et al., 2010; Weise et al., 2010; Kaminska et al., 2012). For instance mRNA levels of short *TCF7L2* splice variant lacking exons 12, 13 and 13a, thought to be unable to activate Wnt target promoters, positively correlated with impaired insulin action on adipocytes (Kaminska et al., 2012). Higher levels of the same splice variant increased beta cell apoptosis, whereas longer splice variants improved beta cell survival. Interesting, that these splice variants were shown to be differently regulated post transcriptionally with mRNA levels inversely correlating with protein expression (Le Bacquer et al., 2011). However, no convincing evidence supporting association between different splice forms and T2D risk genotype has been found so far within any of investigated tissues, although failure to observe such correlation could partly attributable to low power of mentioned studies (Prokunina-Olsson et al., 2009; Mondal et al., 2010; Kaminska et al., 2012).

Besides alternative splicing, variation in *TCF7L2* intronic region may influence activity of the gene transcription. The 92kb region containing polymorphisms associated with T2D is rich in enhancer sequences (Savic et al., 2012). Indeed, minor allele of rs7903146 was shown to alter the binding of regulatory transcription factors and to influence the expression patterns of *TCF7L2* gene in pancreatic islets (Gaulton et al., 2010; Savic et al., 2012). Genetic variation within the disease associated locus may also have an impact on the transcription in tissues like skeletal muscle and adipose tissue (Cauchi et al., 2008b; Savic et al., 2012). Taking into account the wide expression of *TCF7L2*, resolving of molecular pathways explaining the strong association between the locus and the trait may be promoted by further studies focusing also on other tissues than endocrine pancreas.

1.7.4 Fat mass and obesity associated (*FTO*) gene

FTO gene was first described as one of the genes located within 1.6 Mb region on chromosome 8 deleted in the Fused toes mutant mice (Peters et al., 1999). The human *FTO* gene spans more than 400 kb on chromosome 16q12.2 and contains nine exons. Initially captured by GWA studies as a signal linked to higher risk of T2D, variation within *FTO* gene has proved to be the most cogent risk factor for common type obesity in populations of European, Asian and African descent (Frayling et al., 2007; Andreasen et al., 2008; Do et al., 2008; Freathy et al., 2008; Grunnet et al., 2009; Zabena et al., 2009; Adeyemo et al., 2010; Wing et al., 2010; Binh et al., 2012).

SNPs associated with BMI and obesity risk are located in 47kb haplotype block comprising the part of the first two introns of the gene and are most commonly tagged by two closely linked markers rs9939609 and rs8050136 (Frayling et al., 2007; Andreasen et al., 2008; Do et al., 2008; Freathy et al., 2008; Jacobsson et al., 2008; Grunnet et al., 2009; Zabena et al., 2009; Wing et al., 2010; Robiou-du-Pont et al., 2012). Recent resequencing of the entire *FTO* gene has highlighted a cluster of SNPs closely correlated with rs9939609 in the first intron including rs62048402, rs55872725 and rs11642015, which showed stronger association with obesity in European subjects (Almen et al., 2012). Meanwhile fine-mapping of the *FTO* in African Americans proposed rs56137030 as the leading SNP within the same haplotype block (Peters et al., 2013). Other studies have reported

association between BMI and variants located in the region comprising parts of second and third intron, in eighth intron as well as alternative signals within the first intron of *FTO* gene, although these finds are not widely replicated (Rampersaud et al., 2008; Tonjes et al., 2009; Adeyemo et al., 2010).

Correlation between increased BMI and common variants in *FTO* gene was confirmed to be mediated dominantly by increased accumulation of fat mass and could be observed already in postnatal period (Dina et al., 2007; Frayling et al., 2007; Lopez-Bermejo et al., 2008). Furthermore, weight increasing effects were shown to be more pronounced during childhood and gradually weaken with age (Frayling et al., 2007; Qi et al., 2008; Jacobsson et al., 2009). Physical inactivity and high fat diet were shown to amplify effects of SNPs in *FTO* on accumulation of fat mass (Andreasen et al., 2008; Rampersaud et al., 2008; Hennig et al., 2009; Sonestedt et al., 2009).

Although obesity related variants in the first intron of *FTO* have been consistently associated with higher risk of T2D, this association has been largely thought to be attributable to negative impact of increased adiposity, since it was abolished after adjustment for BMI (Frayling et al., 2007; Freathy et al., 2008; Renstrom et al., 2009; Meyre, 2012; Robiou-du-Pont et al., 2012; Thomsen et al., 2012). However, this hypothesis has not been unanimously accepted. Some studies in European and Asian populations showed that only small proportion of observed effects is explained by increased BMI (Legry et al., 2009; Hertel et al., 2011; Binh et al., 2012; Li et al., 2012). Additionally obesity susceptibility SNPs have been associated with insulin resistance, insulin secretion, serum triglycerides, low and high density lipoprotein cholesterol, fasting leptin and glucose. However, whether observed correlations are solely byproduct of increased adiposity seen in the carriers of risk genotype is not yet clear (Andreasen et al., 2008; Do et al., 2008; Freathy et al., 2008; Jacobsson et al., 2008; Grunnet et al., 2009; Zabena et al., 2009; Tan et al., 2010; Wing et al., 2010; Meyre, 2012; Robiou-du-Pont et al., 2012).

Mice with inactivated *FTO* gene had reduced fat mass most likely explained by increased basal metabolic rate due to sympathetic activation and were protected from weight gain induced by high fat diet (Church et al., 2009; Fischer et al., 2009). Apart from that, impaired function of *FTO* associated with enhanced expression of genes involved in carbohydrate and lipid metabolism and reduced inflammation (Church et al., 2009). Overexpression of *FTO*, on the other hand, led to hyperphagia-induced obesity and glucose intolerance, while no changes in energy expenditure were detected (Church et al., 2010). In humans inactivating mutation R316Q in highly conserved position in *FTO* causes an autosomal recessive lethal syndrome characterized by multiple malformations and developmental delay. Of note, authors of the study hypothesized that heterozygous loss of function mutations in *FTO* might protect against becoming obese (Boissel et al., 2009).

FTO belongs to the superfamily of Fe(II) and 2-oxoglutarate (2OG) oxygenases (Gerken et al., 2007). The protein localizes to the cell nucleus and may serve as a transcriptional co-activator as well as regulate RNA processing and stability via nucleic acid demethylation (Gerken et al., 2007; Berulava and Horsthemke, 2010; Wu et al., 2010a). FTO is widely expressed across different types of tissues like skeletal muscle, adipose tissue, liver and pancreas, though the highest levels of its mRNA have been observed in the brain including hypothalamic nuclei essential for energy homeostasis (Frayling et al., 2007; Gerken et al., 2007; Fredriksson et al., 2008). Role of FTO in central regulation of feeding is further supported by evidence showing that expression of hypothalamic *FTO* vary depending on starvation state (Gerken et al., 2007; Fredriksson et al., 2008; Stratigopoulos et al., 2008; Wang et al., 2011b). In line with these findings, carriers of risk alleles of rs9939609 or linked SNPs have reduced responsiveness to satiety

signals and show higher preference for energy dense, fat rich foods (Tanovsky-Kraff et al., Cecil et al., 2008; Speakman et al., 2008; Wardle et al., 2008; Olszewski et al., 2011; Timpson et al.,). To control central mechanisms of energy homeostasis FTO may influence expression of oxytocin and interact with melanocortin and leptin signalling pathways (Fischer et al., 2009; Olszewski et al., 2011; Wang et al., 2011b).

Other studies have focused on investigation of FTO functions in periphery. Thus expression of FTO in adipose tissue was demonstrated to correlate with obesity level, although obesity has been associated with both downregulation and upregulation of FTO expression (Kloting et al., 2008; Wahlen et al., 2008; Zabena et al., 2009). Decline in mRNA levels of FTO has been linked to changes in glucose to fat oxidation ratio in muscle (Grunnet et al., 2009). Increased expression of FTO stimulated lipogenesis and oxidative stress in liver in rat model (Guo et al., 2013), whereas overexpression in cultured pancreatic beta cells promoted glucose induced release of insulin (Russell and Morgan, 2011). Patients with T2D displayed higher rate of FTO and its mRNA in skeletal muscle than diabetes free lean and obese controls, which was associated with mitochondrial dysfunction, enhanced lipid accumulation and ROS production as well as impaired insulin signalling due to enhanced basal phosphorylation of Akt (Bravard et al., 2010). Carriers of rs9939609 risk allele had decreased lipolysis, increased efficiency of energy usage in skeletal muscle and hepatic insulin resistance (Wahlen et al., 2008; Grunnet et al., 2009).

However, despite intensive studies there is still no incontestable evidence confirming true functional variant or explaining mechanisms of its action. Variants within the obesity risk haplotype like rs62048402, rs8050136 and rs1421085 are located in the potential regulatory sequences and thus may modify transcription of the gene (Stratigopoulos et al., 2008; Almen et al., 2012; Peters et al., 2013). Impact on the expression is supported by fact that transcripts containing risk allele of rs9939609 were found to be more abundant than those with major allele (Berulava and Horsthemke, 2010). Apart from that, higher methylation rate associated with risk haplotype was observed within the first intron in the region including long-range enhancer potentially regulating transcription of iroquois homeobox 3 (*IRX3*) gene involved in pancreatic function (Ragvin et al., 2010).

1.7.5 Transmembrane 18 (*TMEM18*) gene

Cluster of highly correlated common variants, rs7561317, rs6548238, rs2867125 and rs4854344, located approximately 30kb downstream of transmembrane 18 (*TMEM18*) gene on chromosome 2p25.3 represent another well replicated susceptibility locus for common type obesity discovered by GWA studies displaying comparable association to that estimated for SNPs found in the first intron of *FTO* (Yamashita et al., 2008; Thorleifsson et al., 2009; Willer et al., 2009).

Variation in *TMEM18* locus has been also associated with several other anthropometric measures such as body weight, height, waist circumference and skinfold measures (Thorleifsson et al., 2009; den Hoed et al., 2010; Haupt et al., 2010; Rask-Andersen et al., 2011). In common with variants found within *FTO* impact of rs7561317 and linked SNPs on body adiposity manifests already in early childhood and persists through adulthood, although its effects may be weaker in older cohorts (Thorleifsson et al., 2009; Willer et al., 2009; Zhao et al., 2009; Almen et al., 2010; den Hoed et al., 2010; Holzapfel et al., 2010; Dorajoo et al., 2011; Rask-Andersen et al., 2011; Takeuchi et al., 2011; Graff et al., 2012). Apart from the positive correlation with BMI, in GIANT consortium based GWA study rs6548238 representing this obesity related locus associated with increased risk of T2D (Willer et al., 2009). Although some large scale studies have

failed to show such relationship (Thorleifsson et al., 2009), further investigation supported possible impact of obesity risk alleles found at the *TMEM18* locus on T2D, but suggested these effects to be entirely mediated by BMI (Renstrom et al., 2009; Sandholt et al., 2011; Thomsen et al., 2012). Yet, studies in Danish and Japanese populations cast some doubt on the role of BMI as a dominant mediator in observed relation showing that association between the SNPs and T2D that retained its significance independently of BMI (Takeuchi et al., 2011; Thomsen et al., 2012).

The *TMEM18* gene is coding for highly conserved nuclear membrane protein with three transmembrane domains and intranuclear C-terminus (Jurvansuu et al., 2008; Yamashita et al., 2008; Almen et al., 2010). TMEM18 has a capability to bind sequence specific double stranded DNA and may serve as transcription silencer by docking chromatin close to the nuclear membrane (Jurvansuu and Goldman, 2011). Although the high conservation rate may be indicative of physiological significance, the functions of protein in the organism are still poorly characterized. TMEM18 has been expressed in tissues throughout the body, albeit with variable levels. Its mRNA is found also in all parts of the central nervous system, including those involved in the regulation of energy homeostasis like hypothalamus and brainstem (Willer et al., 2009; Almen et al., 2010).

The first indication towards its possible role in the organism comes from the study aimed to discover candidate genes that may regulate neuronal stem cell tropism for glioma cells, where overexpression of the *TMEM18* enhanced cell mobility (Jurvansuu et al., 2008). Whether these findings are significant or not in the context of regulation of energy turnover is not yet clear. Its expression in the brain regions responsible for regulation of food intake may suggest that TMEM18 may be involved in central pathways of energy homeostasis, although its regulation may differ from that of MC4R and FTO. No correlation of transcription levels with changes in feeding profile like starvation, calorie rich diets, preference of palatable foods and ingested food or body weight were observed in murine hypothalamus, brain stem, amygdala and hipoccampus (Almen et al., 2010; Rask-Andersen et al., 2011). However, high fat diet induced weight gain may lead to the decrease in hypothalamic mRNA levels of *TMEM18* over a longer period (Gutierrez-Aguilar et al., 2011). Interesting that increase in rates of *TMEM18* expression with higher body weight were observed in rat prefrontal cortex thus supporting its involvement in higher cognitive processes regulating feeding. Nevertheless, no association of rs6548238 and rrs4854344 with self-reported 24 hour energy and macronutrient intake was observed within the same study in children (Rask-Andersen et al., 2011).

Increased BMI observed in carriers of risk genotype at the *TMEM18* locus is largely attributable to general accumulation of excess adipose tissue unrestricted to particular fat deposition compartment (Renstrom et al., 2009; Willer et al., 2009; Haupt et al., 2010). Furthermore, inhibition of expression of TMEM18 resulted in impaired adipocyte differentiation that may propose it as one of the regulators of adipogenesis. Levels of *TMEM18* mRNA were decreased in the adipose tissue from obese subjects, although no allele-specific changes in the expression of the gene have been observed (Bernhard et al., 2012). TMEM18 may also regulate metabolic functions in other tissues like liver and skeletal muscle as expression of *TMEM18* was demonstrated to be impaired by diet induced obesity in rats (Gutierrez-Aguilar et al., 2011).

It is still not clear, how polymorphisms found in *TMEM18* loci may affect functions of the transcription factor coded by the gene in metabolism. These variants were suggested unlikely to be in linkage with functional proxy within the gene itself and thus rather may lead to impaired transcription (Rask-Andersen et al., 2011). However, at least in tissues examined so far no evidence for correlation between genotype and expression levels were found (Bernhard et al., 2012).

2 MATERIALS AND METHODS

2.1 Selection of study subjects

Subjects included in the studies accomplished within the framework of current doctoral thesis were selected from the Latvian Genome Data Base (LGDB) (shortly described in Ciganoka et al., (2011)), a government funded biobank, and Latvian State Research Program Database (LSRPD). Participants of the LGDB were recruited by medical personnel in hospitals or general practices using both disease based and population based approaches. All participants willing to participate in the LGDB were required to be older than 18 years, and to provide information on their ethnic, social, environmental background and familial health status. The anthropometric measurements (including weight and stature) were obtained during a questionnaire based interview. Body mass index (BMI) (kg/m^2) was calculated as weight (kg) divided by the squared height (cm) x 10,000. Health status of the participants was affirmed by physicians using International Classification of Diseases (ICD-10) codes. LSRPD collection was carried out following the same protocol, applying identical questionnaires and sample treatment techniques, as used by LGDB. Signed informed consent was asked from all participants of the both biobanks. Employed protocols were approved by Central Medical Ethics Committee of Latvia (Protocol Nr. 22.03.2007 A-7). Approval of Central Medical Ethics Committee of Latvia was also received for studies included in the current thesis (Protocols Nr.24.05.2011.Nr.01-29.1/10, Nr.22.03.2007. A-5 and Nr.26.05.05. A-33).

In general, only the subjects that provided complete basic phenotypic information, including gender, age, weight and height, were considered for inclusion in the study groups. Further specific criteria for each particular study were postulated.

2.1.1 Description of study specific selection of participants

2.1.1.1 Participants included in the study of variation within the locus comprising AGRP gene

One of the strategies to increase the probability to discover genetic variants linked to the trait is to use extreme cases, hence for initial assessment of variation in the *AGRP* gene and its 5' untranslated region, 95 obese subjects having the highest BMI values were selected from the total pool of 1612 participants (1173 from LGDB and 439 from LSRPD). None of the obese cases had BMI below 35 kg/m^2. Patients with all types of cancers and thyroid gland diseases were rejected, as progression and treatment of these conditions may strongly alter BMI. Yet, patients having other metabolic diseases like T2D and cardiovascular diseases were retained. After subject filtration, 1135 subjects were selected for inclusion in the study. This group also included 73 of 95 subjects from initial sample of extreme obesity cases. The rs5030980 was analysed in total of 789 samples, comprising 350 samples available from LGDB and all 439 from LSRPD.

2.1.1.2 Study participants selected to investigate the impact of SNPs in ADIPOQ gene locus

The investigation of SNPs representing *ADIPOQ* gene and its promoter region was carried out in 835 subjects from LGDB who provided relevant phenotypic information. Similarly as in the *AGRP* study, patients with malignant tumors and thyroid gland diseases were excluded from the study group, while patients with cardiovascular diseases and T2D were retained. In total 740 out of 835 participants (which included 97 patients with T2D) were diagnosed with cardiovascular diseases like hypertension (ICD-10 code I10), heart

failure (I50), atrial fibrillation (I48) and/or myocardial infarction (I21 and I25). The T2D cases included all patients (in total 170 subjects) with respective diagnosis (ICD-10 code E11.0 – E11.9) available within records of LGDB at the time, when selection of the subjects for the current study was performed.

2.1.1.3 The study population collected to evaluate impact of SNPs in TCF7L2 on T2D susceptibility

T2D cases and controls for estimation of the effects of common variants in *TCF7L2* on T2D susceptibility in the population of Latvia were selected from a total number of 14047 participants of LGDB recruited during the time period from January 2003 to June 2010, including 1783 patients diagnosed with T2D (ICD-10 code E11.0 – E11.9). After exclusion of participants with incomplete relevant phenotype data (missing information on gender, age, weight or height) 1043 T2D cases and 9795 potential control subjects (T2D free at the time of recruitment to the LGDB), remained. All 1043 patients with T2D were included in the study and 1093 controls were randomly selected from 9795 T2D free subjects maintaining the same gender proportion as in the T2D cases. Description of subjects is given in Table 2.1.

Table 2.1 Characteristics of the patients with T2D and control subjects included in the analysis of *TCF7L2*

Characteristic	Patients with T2D	Controls	p-value[a]
Number of subjects	1043	1093	-
Males/Females n (%)	331/712(31.7/68.3)	354/739(32.4/67.6)	0.7641
Mean age, years ± SD	60.27 ± 10.84	53.62 ± 12.99	<0.0001
Mean BMI, kg/m^2 ± SD	32.44 ± 6.87	27.18 ± 5.19	<0.0001
Mean age at T2D diagnosis, years ± SD	51.54 ± 11.18	-	-

SD – standard deviation; BMI – body mass index; [a]p-value for differences between diabetic patients versus controls (*t*-test).

2.1.1.4 Description of subjects included in the study of common variants in FTO and near TMEM18

Both control group and T2D patient group used for the association study of common variants representing loci comprising *FTO* and *TMEM18* genes were selected following the same procedure as described in the previous section. In brief, the final group included 2073 participants with valid data on the main phenotypic traits enrolled to LGDB during the time period from January 2003 till June 2010 It was composed of 993 cases with T2D (selected from 1783 diabetics) and 1080 controls (selected from 9795 suitable participants). Although 252 out of 993 patients had not reported their age at diagnosis they were still included in the study to retain larger number of T2D cases for statistical purposes. The smaller replication sample group was created from 199 patients that were not included in the main group and were recruited to the LGDB during the period from 2010 to 2011 by endocrinologists from 13 health care institutions throughout Latvia. These were further filtered by availability of the data on age at the start of initial medical therapy and thus 171 subjects were selected. Short description of replication sample is given in the Table 2.2.

Table 2.2 Characteristics of the subjects included in the replication sample included in the study relationship between polymorphisms near *TMEM18* and in *FTO* and phenotype

Characteristic	Patients with T2D
Number of subjects	171
Males/Females n (%)	66/105
Mean age, years ± SD	58 ± 10.68
Mean BMI, kg/m^2 ± SD	34.05 ± 5.89
Mean age initial medical therapy of T2D ± SD	58 ± 10.68
Subjects with positive family history of T2D n (%)	47 (27.5)

BMI – body mass index; T2D – type 2 diabetes; SD – standard deviation; n –number.

2.2 Sequencing of the *AGRP* gene and its promoter region

For assessment of the variations within the *AGRP* gene and its promoter region directed sequencing was selected as the best approach since it provides the most detailed information on variations found within region of interest (ROI). Amplification of *AGRP* gene promoter region and its coding part (from -1102bp to +1027bp respective to start codon) was accomplished by two separate polymerase chain reactions (PCR). The region containing *AGRP* gene was amplified in total volume of 25µl. The reaction mixture contained 25ng of genomic DNA, 1x PCR buffer, 1,5mM $MgCl_2$, 0,2mM dNTP mix, 8% DMSO (Serva, Germany), 1mM 0.188Fw and 0.344Rs primers (ThermoHybaid, Germany) and 0,5u of recombinant *Taq* DNA polymerase (Fermentas, Lithuania), and it was carried out in the following conditions: 2min at 95°C followed by30 cycles of three steps: 10s at 95°C, 15s at 50°C and1min at 72°C, and an additional extension for 2min at 72°C. Amplification of region containing promoter of *AGRP* was performed in similar conditions using AGRPprom-PCR-Fw and AGRPprom-PCR-Rs primers, only during this reaction primer annealing temperature was set to 55°C.

Acquired PCR products were purified with PEG-precipitation. Samples were incubated for 15min at 37°C in solution containing 10% PEG8000 (Merck, Germany) and 1,25M NaCl (Merck, Germany). After incubation samples were centrifuged at room temperature for 15min at 14.000 rpm. Supernatant was removed and samples were washed with 70% ethanol, centrifuged for 5min at 14.000 rpm and then vacuum dried for 15min. Purified samples were dissolved in 15µl of sterile H_2O.

Both strands of purified amplification products were sequenced using 0.188 Fw and 0.344Rs primes for protein coding sequence, and AGRPprom-Seq1-Fw, AGRPprom-Seq2-Rs, AGRPprom-Seq3-Fw and AGRPprom-Seq4-Rs primers for promoter region. Sequencing reaction was carried out in total volume of 10µl and it contained 80mM Tris-Cl pH 9.0 and 2mM $MgCl_2$, 1mM primer, 1µl Big Dye Terminator v3.1 Mix (Applied Biosystems) and 1µl of purified PCR product. The reaction conditions were following: 1min at 95°C followed by 30 cycles, each consisting of three steps: 10s at 95°C, 15s at 50°C and 4min at 60°C. Acquired sequencing products were precipitated in 0.75M ammonium acetate and 96% ethanol at room temperature for 10min. At the end of incubation samples were centrifuged at 14.000 rpm for 10min, supernatant was discarded and samples were washed with 70% ethanol and vacuum dried. DNA fragments were analysed on Applied Biosystems (USA) 3130 XL Genetic Analyser system. The Sequences of the primers used for PCR and sequencing reactions are given in Table 2.3. Obtained sequences were manually analysed with ContigExpress software following VectorNTI guidelines (Invitrogen, USA).

Table 2.3 Primers used in the study of variants in the *AGRP* gene locus.

Primer ID	Sequences of primers
AGRP gene	
0.188Fw	5'- GGATAAGGAGGGACTAAGTGGTACA -3'
0.344Rs	5'- CTGACGTTGGCCAGCTAGGT -3'
AGRP-Alu Fw	5'- CCCTCCCCTGGGAGGTGGG**(T^A)**G -3'
AGRP-Alu Rs	5'- GGTGAGGGAGTTTGGAGCTGG -3'
AGRP promoter region	
AGRPprom-PCR-Fw	5'- TGGAATTACAGGTGTGAGC-3'
AGRPprom-PCR-Rs	5'- AGGTGCCCAGATAGAGAC -3'
AGRPprom-Seq1-Fw	5'- GCAGGAGTGGGCGGTCTT -3'
AGRPprom-Seq2-Rs	5'- GGACATTGGAGCGGGTGAT -3'
AGRPprom-Seq3-Fw	5'- GTTTGTGCACTGGGGCCTTG -3'
AGRPprom-Seq4-Rs	5'- TCTGCCTCCGGGATTCTTGC -3'

2.3 Genotyping

2.3.1 Selection of SNP

Selection of SNPs for association analysis aiming to assess probable correlation between variants representing *AGRP* locus and phenotype was based on the information derived from direct sequencing of the particular region in the obese individuals (see Chapter 2.2). From three variants, rs34123523 (c.123G>A), rs5030980 (c.199G>A p.Ala67Thr) and rs11575892 (c.131-42C>T), detected by sequencing analysis rs34123523 and rs5030980 were in complete LD and therefore only the missense variant rs5030980 and rs11575892 were included in genotyping.

Selection of tagging SNPs (tagSNPs) representing variation within *ADIPOQ* locus was performed with Haploview software, version 3.3 (Barrett et al., 2005) using SNP information provided in HapMap (Public Release #21, July 2006, http://hapmap.ncbi.nlm.nih.gov/). Relative coordinates of the fragment uploaded in respect to the first position of starting codon ATG in Haploview for search of tagSNP from HapMap were from -12541 to 5459bp (total length of 18,000bp). Number of variants included was limited to maximum nine tags with minimal allele frequency 0.01 and r^2 threshold set at ≥ 0.8. Variants according to data published at the time of selection of SNPs showing association with adiponectin levels in blood were force included in the final set of tagSNPs.

In the case of *TCF7L2* gene four common variants, rs12255372, rs7903146, rs11196205 and rs7901695 representing T2D susceptibility locus spanning from third intron to fourth intron of the gene were selected according to the strength of previously reported associations with the disease (Grant et al., 2006).

To evaluate impact of the genetic variation in the loci overlapping *FTO* and *TMEM18* genes on T2D, four polymorphisms in *FTO* gene, rs11642015, rs62048402, rs9939609 within the 1th and rs57103849 within 4th intron of the gene and rs7561317 near *TMEM18* gene, were selected for genotyping. The rs9939609 and rs7561317 were selected as the two adiposity related signals most commonly associated with T2D risk (Frayling et al., 2007; Willer et al., 2009). The rs11642015 and rs62048402 represent the same haploblock including rs9939609 within the 1th intron, but were recently shown to have stronger association with obesity than rs9939609 (Almen et al., 2012). The rs57103849 located outside the obesity associated cluster within 1th intron, was included due to its probable association with insulin resistance (Jacobsson et al., 2008).

2.3.2 Aliquoting of DNA samples

Prior genotyping on MALDI-TOF MS or Real-Time PCR systems DNA samples from LGDB were diluted to final concentration of 7ng/µl and aliquoted in 96-well or 384-well PCR plates (only for rs11642015 and rs62048402). 4µl of DNA solution were placed in each well using the Freedom Evo robotic workstation (Tecan, Männedorf, Switzerland). Aliquoted samples were allowed to air-dry and thus prepared PCR plates with 28ng of genomic DNA in each well were further used for genotyping.

2.3.3 PCR based restriction fragment length polymorphism analysis

2.3.3.1 Nested - PCR reaction

The rs11575892 - SNP that is located within *AGRP* gene was screened using PCR based restriction fragment length polymorphism (RFLP) assay. Yields of PCR product that were gained from direct amplification of genomic DNA were too low for further restriction and polyacrylamide gel electrophoresis, therefore nested-PCR amplification was performed.

Mastermix of the first PCR contained 1x PCR -$MgCl_2$ buffer, 1,5mM $MgCl_2$, 8% DMSO, 0,2mM dNTP mix, 0,47mM forward and reverse primers, 0,5u recombinant Taq DNA polymerase (all reagents from Fermentas, Lithuania) and 25ng of genomic DNA. The total volume of reaction mix was 15µl and amplification conditions were as follows: denaturation 2 min at 95°C, 40 cycles of three following steps: 10s at 95°C, 15s at 55°C, 1min at 72°C, and additional extension for 1min at 72°C.

Reaction mix for the second PCR was prepared in similar manner, the exceptions were concentrations of primers (1.636Fw and 1.637Rs), that were increased to 0.6mM each, and addition of 0,2µl of PCR product from the first reaction as matrix. Cycling conditions were: 2min at 95°C, 40 times: 10s at 95°C, 15s at 55°C,30s at 72°C, followed by 2min at 72°C. Sequence of forward primer 1.636Fw used in the second PCR contained T→A nucleotide exchange to introduce an artificial AluI cleavage site. Primer sequences are shown in Table 2.3.

2.3.3.2 Restriction with AluI

Products from second PCR were cleaved with *AluI* in 37°C overnight containing 1x buffer Y^+/TANGO™, 4u of restriction enzyme *AluI* (Fermentas, Lithuania) and 2µl of PCR product. Total volume of reaction mixture was 10µl. Restriction fragments were analysed with 6% polyacrylamide gel electrophoresis and visualized by incubation in ethidium bromide - water solution. As the result only wild type fragments were cut (forming 217bp and 21bp long products) as the presence of rs11575892 allele T within the fragment disrupted artificially introduced *AluI* cleavage site. Additional wild type cleavage *AluI* site in the PCR products enabled control for false positive results and samples with minor T allele were verified by direct sequencing performed as described in Section 2.2.

2.3.4 MALDI-TOF MS based genotyping

Genotyping using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed for the rs7903146 in the 3th intron of *TCF7L2* gene and polymorphisms located in the *ADIPOQ* gene.

2.3.4.1 Amplification of ROI

PCR based amplification of each of the specific regions containing one of the SNPs was performed in 12µl of total reaction mix per well in 0.2ml ThermoFast 96 well non-skirted plate (Thermo Scientific, UK). The reaction mix contained 1x PCR buffer BD, both primers - 0.3pmol/µl each (Metabion, Germany), dNTP mix –0.2mM each, $MgCl_2$ - 2.5mM, 0.1 unit/µl of HOT FIREPol (SolisBioDyne, Estonia) and 28ng of genomic DNA (aliquotation described in Section 2.4.1). Sequences of employed PCR primers are given in Table 2.4. Amplification was performed in following conditions: 95ºC for 5min, followed by 40 cycles of 95 ºC for 30s, 54ºC (SNPs in *ADIPOQ*) or 55ºC (SNP in *TCF7L2*) for 30s, 72 ºC for 30s and ending with additional extension step 72ºC for 5min. In order to dephosphorylate excess of nucleotides acquired PCR products were incubated at 37ºC for 60min with 0.1unit/µl of shrimp alkaline phosphatase (Fermentas, Lithuania). Phosphatase was inactivated by heating at 94ºC for 20min.

2.3.4.2 Single base extension (SBE)

The PCR clean-up products were used as templates in single base extension reaction (SBE). SBE reaction primers were designed using CalcDalton software. They were biotinylated at their 5' end and contained internal photo-cleavage linker (BioTez, Germany) (summarised in Table 3). SBE reactions were performed in total volume of 10µl per well in 96 well plate (described previously). Reaction mixture contained 0.5x buffer C, 1,25 mM $MgCl_2$, 0.2U/µl TERMI Pol DNA polymerase (SoliBioDyne, Estonia), SNP allele specific pair of ddNTP 0,2 pmol/µl each (Fermentas, Lithuania) and SBE reaction primer 1 pmol/µl. Reaction was carried out in following conditions: 2min at 95 ºC, followed by 100 cycles of 95 ºC for 10s, 55 ºC for 10s, 72 ºC for 10s with additional synthesis step at 72 ºC for 5min.

2.3.4.3 Purification of SBE reaction products

Prior purification 10µl of Mili-Q H_2O water (demineralized water with specific resistance better than 18 MΩcm, purified using Milli-Q Synthesis A10 System by Millipore) were added to SBE reaction products. 16 µl of diluted products were transferred to the streptavidin coated 96 V-well polypropylene plate with maximum biotin binding capacity (BioTez, Germany). Afterwards 4µl of BB buffer (2M NaCl, 1mM EDTA, 20mM Tris-HCl pH7.0) were added to each well and samples were incubated for 30 min at a room temperature. The incubation was followed by liquid removal and washing of immobilized products using wash buffer (100 mM ammonium acetate, 20 mM Tris acetate pH 7.0, 1mM EDTA, 0.1% Tween 20) and Mili-Q H_2O water. After removal of all wash solutions, 20µl Mili-Q H_2O water were added to each well and photo cleavage was performed by exposure of the sample plate to UV light for 15min using UV Unit CL-366 (Bruker Daltonik, Germany).

2.3.4.4 MALDI-TOF MS analysis

1µl of purified and cleaved products were spotted onto air dried MALDI matrix - 3-hydroxypicolinic acid (Bruker Daltonik, Germany) crystals that were prespoted on AnchorChip 400/384TF plate (Bruker Daltonik, spot size 400µm), and dried at room temperature. Each sample was spotted in 4 repeats. AnchorChip plate with dried samples was inserted into the MALDI-TOF mass spectrometer for SBE product mass determination (Autoflex; Bruker Daltonik). Parameters of the instrument were set to be suitable for mass determination in range between 1-12kDa and analysis was performed with maximum of 40 laser shots (frequency 16.7 Hz) per sample spot. Results were inspected manually using Genotools^{TM} 2.0 software (Bruker Daltonik, Germany).

Table 2.4 Sequences of PCR and minisequencing primers that were used for genotyping of rs7903146 in *TCF7L2* and SNPs located in the *ADIPOQ* gene.

SNP	Sequence of primer
TCF7L2	
rs7903146	Fw ACCGTTGGATGTGTTATTTACTGAACAATTAGAGAGCTAAG Rs ACGTTGGATGGCCCAAGCTTCTTCAGTCAC B-CAATTAGAGAGCTL*AGCACTTTTTAGATA_ [T/C]
ADIPOQ	
rs16861194	Fw ACGTTGGATGGGATGTCTTGTTGAAGTTGG Rs ACGTTGGATGATTCAACACCTTGGACTTTCT B-AGCCCTTGCTL*GGGTCGTA_[A/G]
rs17300539	Fw ACGTTGGATGGGATGTCTTGTTGAAGTTGG Rs ACGTTGGATGATTCAACACCTTGGACTTTCT B-AGAATGTGTGGCTTGL*AAGAACC_[A/G]
rs266729	Fw ACGTTGGATGGGATGTCTTGTTGAAGTTGG Rs ACGTTGGATGATTCAACACCTTGGACTTTCT B-ACGCTCATGTTTTGTTTL*TGAAG_[G/C]
rs182052	Fw ACGTTGGATGGGGTTATTTCAGACATTCCTT Rs ACGTTGGATGAATTGGACTTCATCTGTGGA B-TGCTACL*GAGCGAACTGGG_[T/C]
rs16861210	Fw ACGTTGGATGCCTGTTTTGACTCTAATGTG Rs ACGTTGGATGCAAGAACGGACTCTGATTG B-GCTCTGL*CGCTAGCATCTTG_[T/C]
rs822396	Fw ACGTTGGATGAATACTGAAGTTTGGGGACA Rs ACGTTGGATGTCAGGGGTCTCTGGAGTT B-TCTGGL*TCCTCCCCCTTTGT_[T/C]
rs2241766	Fw ACGTTGGATGTTGCTGGGAGCTGTTCTA Rs ACGTTGGATGGGCCAGAAACATTCTTACCT B-TACTGCTATTAL*CTCTGCCCGG_[G/T]
rs1501299	Fw ACGTTGGATGAGAATGTTTCTGGCCTCTTT Rs ACGTTGGATGCAAAGCCAAAGTCTTGGTTA B-GTCTAGGCCTTL*GTTAATAATGAATG_[A/C]
rs2241767	Fw ACGTTGGATGAGAATGTTTCTGGCCTCTTT Rs ACGTTGGATGCAAAGCCAAAGTCTTGGTTA B-GGAAGACCL*ACCCCAAATCAC_[T/C]
rs3774261	Fw ACGTTGGATGACCACATTTACTGCACACC Rs ACGTTGGATGATTCAAGGCCTAGCTGAAG B-ATTCAAAGTATGGAGCL*TAGAGAAAAT_[A/G]

[ ] – indicates the bases by which each product is extended during minisequencing reaction; MiniSeq primers – primers for the minisequencing (single base extension reaction), L* - photosensitive linker, B - biotin cap at 5' end.

2.3.5 Real-Time PCR system based genotyping with TaqMan SNP assays

Variants located within *AGRP* (rs11575892 (437 samples) and rs5030980), the intronic region of *TCF7L2* (rs7901695, rs11196205 and rs12255372), near *TMEM18* (rs7561317) and within *FTO* (rs11642015, rs62048402, rs9939609 and rs57103849) genes were genotyped using TaqMan SNP genotyping assays (Applied Biosystems, USA). List of the corresponding IDs of pre-designed TaqMan SNP genotyping assays and sequence information for probes and primers of custom designed assays are summarised in Table 2.5.

The PCR reaction mix for genotyping of all listed polymorphisms contained 4.75 µl of 2x TaqMan Genotyping Mix (final concentration 1×) (Life Technologies, ASV), 0.25µl of 40×SNP genotyping assay (final concentration 1×) (Life Technologies, ASV), 5µl of Millipore H_2O and 28ng of genomic DNA. The cycling conditions were set as recommended by manufacturer: 10min at 95ºC followed by 40 cycles of 15s at 95ºC and 60s at 60ºC. The genotyping was carried out on 7500 Real-Time PCR system or Viia7

Real-Time PCR system for rs11642015 and rs62048402 (Life technologies, USA) (description of genotyping procedure may be found also in (Peculis et al., 2011)).

Table 2.5 List of TaqMan SNP genotyping assays.

SNP	Assay ID	Sequence of the probe [VIC/FAM]
Pre-designed assays		
AGRP		
rs5030980	C_29708280_10	GCAGTTACCTCTGCCAAGGCCTGAG[C/T]CTCCTGCAACAGATCCT CTTCTGCC
rs11575892	C_32373505_10	GAGGGCAGGAACCCCCATGCACAAA[A/G]CACCCACCTCCCAGGG GAGGGTTTG
TCF7L2		
rs7901695	C_384583_10	CATATAAATGGTATCATAAAATCTA[C/T]GGGCTTTTGTGTCTGTCT GCTTTCA
rs11196205	C_27432600_10	GAAAGTTCTCAACATTTATAACTAC[C/G]AGCAGTATGTAAGAGA GTTATGGTT
rs12255372	C_291484_20	TGCCCAGGAATATCCAGGCAAGAAT[G/T]ACCATATTCTGATAATT ACTCAGGC
TMEM18		
rs7561317	C_11804554_10	GAGATGACAAGTGACACTTCCTGTC[A/G]TCTGCCTACAAGTTCCC AAAGATCC
FTO		
rs11642015	C_2031268_20	CTTTTGATGCACAAAGGGGCAACTC[C/T]GTGTAGCAGGACCTTGG TGGGATGG
rs9939609	C_30090620_10	GGTTCCTTGCGACTGCTGTGAATTT[A/T]GTGATGCACTTGGATAG TCTCTGTT
Custom designed assays		
SNP	**Assay ID**	**Primers Fw/Rs Sequences of the probe [VIC/FAM]**
FTO		
rs62048402	-	Fw ACCTTGATTCTCCGCATTTATAGCA Rs AGAGCTAGTACTCAAGCCCAAGT TCTGGCAG[T]CATGCTT / TGGCAG[C]CATGCTT
rs57103849	-	Fw GAGGTGGAAACGGCAATGAG Rs TGAGATGGAGCTTCACTCTTGTTG CAGTGGC[G/A]CAATC

2.4 Statistical analysis

2.4.1 Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium (HWE) testing was used to detect possible genotyping errors that may lead to erroneous conclusions. Correspondence of observed genotype frequencies to theoretical HWE distribution was verified by performing exact test described by Wiggington et al using Plink software (available at http://pngu.mgh.harvard.edu/purcell/plink/) (Wigginton et al., 2005; Purcell et al., 2007).

2.4.2 Estimation of statistical power

Statistical power which is provided by sample size of each study was calculated using Quanto v1.2.3 (available at http://hydra.usc.edu/gxe/) (Gauderman and Morrison, 2006). For all calculations we assumed an additive mode of inheritance and used previously published frequencies for genotyped variants in populations of European descent. In the case of quantitative traits, like BMI and age at diagnosis of T2D, necessary mean values were calculated within the each study sample. Significance level α was set at 0.05 (Loos et al., 2005; Heid et al., 2006; van Rossum et al., 2006; Abecasis, 2010) (http://www.ncbi.nlm.nih.gov/snp/).

2.4.3 Assessment of linkage disequilibrium and its graphical representation

Pairwise LD measured with r^2 between variants found in *AGRP*, *TCF7L2* and *FTO* genes was assessed as indicated in Plink. HaploView version 4.2 (available at http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) (Barrett, 2009) was used to create LD plots which represented linkage between proximal variants. The measure r^2 is one of the two estimates of LD. It shows the statistical correlation between two sites and ranges from 0 (no disequilibrium) to 1 ('complete' disequilibrium – only two haplotypes are present).

2.4.4 Correction for multiple testing

The probability of false significant findings increases proportionally to increase of number performed of tests. To avoid type I error related to multiple testing Bonferroni correction and permutation tests were performed, the choice of these calculations was made according to Plink guidelines (available at http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007). Permutation analysis was performed for each comparison by Max(T) permutation test based on label-swapping specifying 100,000 permutations to estimate p-values (EMP2) corrected considering the number of SNPs included in the analysis.

2.4.5 Normal distribution and data normalization

To apply parametric statistical methods like linear regression, parametric ANOVA and others normal distribution of quantitative data is required. Therefore initially non-normally distributed data were logarithmically transformed and obtained values were included in further analysis. Deviation from normal distribution in data set was evaluated with non-parametric Kolmogorov-Smirnov test (SPSS Standard versions 12.0.0 -13.0.0; Chicago, IL, USA).

2.4.6 Assessment of association between genetic variants and traits

Statistical analysis of obtained date was performed using Plink software (versions 1.00 and 1.06) available at http://pngu.mgh.harvard.edu/purcell/plink/ (Purcell et al., 2007) and SPSS statistical software (Standard version 12.0.0; SPSS, Chicago, IL, USA).

2.4.6.1 Statistical analysis of variants found in AGRP locus

Within the framework of the study that was focused on SNPs found in *AGRP* gene, the statistical analysis was carried out using SPSS statistical analysis software. Univariate one-way ANOVA was used to test association of non-genetic categorical factors (gender, cardiovascular diseases, T1D, T2D and dyslipidemia) with logarithmically transformed BMI (logBMI) and relationship between logBMI and age was estimated by linear regression analysis. General linear model (GML) approach adjusted for age, gender and factors showing correlation with BMI in ANOVA tests was applied to investigate association between variants in *AGRP* and logBMI. Association of polymorphisms with categorical variables was estimated with logistic regression corrected for age and gender. For categorical analysis median BMI value 27.34 kg/m^2 was used to sort study subjects in two categories: normal <27.34 kg/m^2 and overweight >27.34 kg/m^2. An omnibus haplotype test was performed with Plink software for assessment of haplotype association.

2.4.6.2 Association study of tagSNPs in ADIPOQ gene

In the association study of tagSNPs within *ADIPOQ* with logBMI, the associacion was estimated with linear regression adjusted for age and gender. Logistic regression adjusted for age, gender, BMI and/or T2D, factors showing significant correlation with dependent variable under multivariate logistic regression, was performed to assess association of variants with dichotomous traits like T2D, hypertension, heart failure, atrial fibrillation and myocardial infarction. Additive, dominant and recessive models were tested using the exact Fisher's test. Statistical analysis software Plink was used for estimation of impact of SNPs on phenotype. Haplotype analysis was performed using omnibus haplotype test according to guidelines given in Plink manual.

2.4.6.3 Evaluation of correlation between SNPs in TCF7L2, FTO and near TMEM18 with T2D

Within the framework of the studies aiming to assess effects caused by common variants within *TCF7L2* and SNPs representing loci of *FTO* and *TMEM18* genes, the phenotypic data between case and control groups were compared using Chi-square and Student's t-test. Logistic regression adjusted for age, gender and/or BMI was applied to investigate association of SNPs with T2D. As a control for possible confounding effects of *TCF7L2*, the genotypes of rs12255372 were treated as ordinal data. Impact of SNPs in *TCF7L2* on T2D were also evaluated in non-obese (BMI<30kg/m^2) and obese (BMI≥30kg/m^2) subjects separately with logistic regression corrected for age and gender, excluding BMI from the list of cofactors. Analysis for correlation with quantitative traits: logBMI, age at diagnosis and biochemical measurements, was performed using linear regression adjusted for gender, age and/or BMI. Association with traits was estimated assuming additive, dominant and recessive models of inheritance and in all calculations a p-value < 0.05 was considered significant.

Haplotype specific conditional tests (selected haplotype versus all other haplotypes) adjusted for gender, age and/or BMI were performed to estimate association of haplotypes formed by variants in *FTO* and *TCF7L2* with phenotype. This analysis was done according to Plink guidelines.

Allelic score was calculated by summing risk alleles of each subject for examination of joint effects of following SNP combinations: rs7561317 (*TMEM18*) and rs62048402 (*FTO*) and rs57103849 (*FTO*), rs7561317 (*TMEM18*), rs12255372 (*TCF7L2*) and rs62048402 (*FTO*); rs7561317 (*TMEM18*), rs57103849 (*FTO*) and rs12255372 (*TCF7L2*). Subjects were divided in groups according to risk allele score: with zero to one (assumed as reference), two, three, four or five to six risk alleles. Logistic or linear regression adjusted for covariates as described before was used to test association with trait for formed categories in SPSS. Only subjects with complete genotypes for examined SNPs were included in the analysis. Theoretical and estimated ORs were compared using Wilcoxon test.

2.4.7 Analysis of probable functional effects of rs11575892

Probable functional impact of rs1157892 within *AGRP* gene was investigated by modelling of the possible effects the nucleotide exchange might inflict on the secondary structure of mRNA and screening for binding sites of regulatory elements that might br involved in the regulation of alternative splicing using Mfold software from Vienna RNA package (available at http://www.tbi.univie.ac.at/RNA/) and online RegRNA software (available at http://regrna.mbc.nctu.edu.tw).

3 RESULTS

3.1 Association between a rare SNP in the second intron of human Agouti related protein gene and increased body mass index

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Association between a rare SNP in the second intron of human Agouti related protein gene and increased BMI

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Abstract

Background: The agouti related protein (AGRP) is an endogenous antagonist of the melanocortin 4 receptor and is one of the most potent orexigenic factors. The aim of the present study was to assess the genetic variability of *AGRP* gene and investigate whether the previously reported SNP rs5030980 and the rs11575892, a SNP that so far has not been studied with respect to obesity is associated with increased body mass index (BMI).

Methods: We determined the complete sequence of the *AGRP* gene and upstream promoter region in 95 patients with severe obesity (BMI > 35 kg/m^2). Three polymorphisms were identified: silent mutation c.123G>A (rs34123523) in the second exon, non-synonymous mutation c.199G>A (rs5030980) and c.131-42C>T (rs11575892) located in the second intron. We further screened rs11575892 in a selected group of 1135 and rs5030980 in group of 789 participants from the Genome Database of Latvian Population and Latvian State Research Program Database.

Results: The CT heterozygotes of rs11575892 had significantly higher mean BMI value (p = 0.027). After adjustment for age, gender and other significant non-genetic factors (presence of diseases), the BMI levels remained significantly higher in carriers of the rs11575892 T allele (p = 0.001). The adjusted mean BMI value of CC genotype was 27.92 ± 1.01 kg/m^2 (mean, SE) as compared to 30.97 ± 1.03 kg/m^2 for the CT genotype. No association was found between rs5030980 and BMI.

Conclusion: This study presents an association of rare allele of *AGRP* polymorphism in heterozygous state with increased BMI. The possible functional effects of this polymorphism are unclear but may relate to splicing defects.

Background

The central melanocortin system and its components play an important role in regulation of food intake and energy balance and it is known as a major pathway of leptin signaling (reviewed in [1,2]). Agouti related protein (AGRP) is predominantly located in ARC neurons that co-express neuropeptide Y (NPY), also a strong mediator of regulation of energy balance. *AGRP* is evolutionary conserved gene [3] that acts as endogenous melanocortin 4 receptor (MC4R) and melanocortin 3 receptor (MC3R) antagonist

(inverse agonist) with strong orexigenic effect (for review see [4]). Both ICV injection [5,6] and transgenic overexpression [7] of AGRP leads to increased food consumption and obesity in mice. Elevated plasma levels of AGRP have been reported in obese men [8] in comparison to non-obese men. In addition, AGRP levels are increased in humans as a result of fasting [9], indicating a possible role of AGRP in periphery.

The *AGRP* gene is located on chromosome 16q22, consists of four exons and expresses two alternatively spliced variants that differ in presence or absence of the 5' non-coding exon. The long transcript variant is predominantly expressed in the hypothalamus while the shorter transcript lacking the 5' untranslated exon is found in peripheral tissues such as testis, lung and kidney [10,11]. A few single nucleotide polymorphisms (SNPs) have previously been identified in *AGRP* gene that are associated with energy homeostasis disorder phenotypes. Two SNPs in the promoter region -3019G>A and -38C>T have been found in individuals of African origin but not in Caucasians [12]. Both these SNPs are in complete linkage disequilibrium (LD) and are associated with leanness, decreased risk for type two diabetes (T2DM) and reduced macronutrient intake [12-14]. Another SNP in the third exon of the *AGRP* gene that changes the alanine at position 67 to a threonine (rs5030980) has been identified, and is so far, found in Caucasians only. This SNP has been shown to be associated with anorexia nervosa [15]. Homozygosity of Ala67 did not have any association to a metabolic phenotype at mean age of 25 years but it was associated with late onset obesity at mean age of 53 years [16]. Patients homozygous for the Thr67 allele had significantly reduced body fatness [17]. In the present study we demonstrate association of a C>T polymorphism at position c.131-42C>T (rs11575892) with increased BMI in cohort of patients from the Latvian Genome Database.

Methods

Study subjects

The study was based on data and samples from the Genome Database of Latvian Population (Biobank 1), the disease based biobank comprising 1173 subjects and 439 subjects from population based Latvian State Research Program Database (Biobank 2). Both biobanks were collected by using the same protocols, questionnaires and sample treatment techniques. Briefly; individuals were required to be over the age of 18; the health status was recorded, the diagnoses was based on approved clinical criteria according to the ICD-10 codes (International Classification of Diseases); anthropometric measurements (including weight and stature), ethnic, social, environmental information and familial health status was acquired based on self-reported questionnaire. Written informed consent was obtained from all participants. We selected 95 of the most obese (BMI > 35 kg/m^2) subjects from the database for initial screening using sequencing. The study group for the subsequent genotyping of rs11575892, was selected from all available subjects with validated health records and questionnaires in biobank by exclusion of patients with diseases whose outcome or treatment may influence BMI, including all types of cancer and diseases of thyroid gland, but not excluding patients with cardiovascular diseases. Patients with metabolic diseases were included in the study as well. In total 1135 subjects were selected based on these criteria. This sample group included 73 individuals from the 95 that were sequenced. rs5030980 was genotyped subsequently in total of 789 samples comprising 350 samples from Biobank 1 and all 439 samples included in study from Biobank 2. The 348 samples from initial selection were not available for genotyping of rs5030980. Study protocol was approved by Central Medical Ethics Committee of Latvia.

Genetic analysis

The *AGRP* gene containing genomic DNA region including 5' upstream region and the entire coding region (positions from -1102 bp to +1027 respective to start codon) were amplified in two individual PCRs. Both strands of amplification products were directly sequenced using set of six primers (primer sequences and reaction conditions can be provided upon request from authors). In total 95 overweight individuals (BMI > 35 kg/m^2) were sequenced with 100% success rate. All chromatograms were manually inspected using Contig Express software of Vector NTI Advance 9.0 package. Presence of polymorphisms was confirmed by opposite strand analysis. Restriction enzyme fragment polymorphism analysis of rs11575892 was used for genotyping the 698 samples from Biobank 1. Primer with a sequence mismatch in 3' part; 5'-CCCTC-CCCTGGGAGGTGGG AG-3' (mismatched position underlined, wild type cuts only) was used to create mutation conditional AluI restriction sites in PCR reaction with reverse primer; 5'-GGTGAGGGAGTTTGGTGCTGG-3'. The amplified PCR product contained additional wt AluI site allowing to control the restriction reaction for false positive results. The AluI restriction products were visualized on PAAG gels. The samples with polymorphisms were verified by direct sequencing. Genotyping of the rs11575892 in all other samples and genotyping of rs5030980 was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, California, USA) on ABI 7500 Real-Time PCR system (Applied Biosystems) according to the supplier's recommendations. In addition, the 5' upstream region of the *AGRP* sequence for all carriers of the rs11575892 from Biobank 1 was determined by sequencing. The designations of the SNP's are based on recommendation by the Human Genome Variation Society.

Statistical analysis

Statistical analyses were performed with the statistical package SPSS (Standard Version 12.0.0;SPSS, Chicago, IL, USA). In contrast to the natural BMI values, the logarithmically transformed BMI values displayed normal distribution and were further used for all quantitative analysis. A one-way ANOVA was used in univariate analysis to test for association of log-BMI values with other categorical variables. Linear regression analysis was used to study relationship between log-BMI and age. General linear model (GLM) approach was applied to study association between each SNP and log-BMI adjusting for main effects of age, gender and those factors that showed association with logBMI under separate ANOVA tests (presence of hypertension, angina pectoris, myocardial infarction, heart failure, dyslipidemia and T2DM). Adjusted mean logBMI values, corresponding CI intervals and significance of association were estimated according to the GLM model. Logistic regression analysis adjusting for age and gender was used for dichotomous trait analysis. Power calculations were performed using Quanto v.1.2.3 [18]. Our sample size provided 80% power (at $\alpha = 0.05$) to detect difference between rs11575892 genotypes in log-BMI of >0.040 assuming minor allele frequency of 0.015 and to detect difference between rs5030980 genotypes in logBMI of >0.028 assuming minor allele frequency of 0.044. PLINK 1.00 software http://pngu.mgh.harvard.edu/purcell/plink/[19] was used to perform Hardy-Weinberg test, LD calculations, haplotype based association and permutation test for ANOVA and multivariate linear regression using label-swapping between SNP and logBMI values but leaving intact correlation with other covariates. 100000 permutations were performed for each analysis and we used corrected (EMP2) p-values. These values are corrected based on calculation of the proportion of permutations in which any of the test statistics exceeds the particular observed statistic and are more stringent then uncorrected p-values.

Results and Discussion

Direct sequencing of the coding and 5' untranslated regions of the human *AGRP* gene in 95 obese patients with BMI > 35 kg/m^2 revealed three polymorphisms: silent mutation c.123G>A (rs34123523) in second exon (minor allele frequency MAF = 0.042), non-synonymous mutation c.199G>A (rs5030980) in third exon (MAF = 0.042) and c.131-42C>T (rs11575892) located in second intron (MAF = 0.016). As reported previously [15] rs34123523 and rs5030980 were in complete linkage disequilibrium (LD) with each other (D' = 1.00, $r^2 = 1.00$) while none of these subjects with both SNPs had rs11575892. The allele frequency of rs5030980 was in agreement (MAF from 0.036 to 0.05) with previous reports [13,20]. None of the SNPs currently present in the SNP databases or any new SNP were found in the 5' untranslated region of the *AGRP* gene. The SNPs reported for this region are however with low frequency (MAF = 0.005 for rs34731556 and rs34018897) or found in subjects of African origin (rs5030981), that may explain the low number of SNPs in our study.

The rs11575892 had not previously been associated with any phenotype and we tested the association between this SNP and BMI in a cohort of 1135 individuals from the biobank collections that were available to us. Characteristics of the study population for all subjects are presented in Table 1. The estimated minor allele frequency of rs11575892 was 0.015 and no significant departure from Hardy-Weinberg equilibrium was observed (p = 1.0). When tested for non-genetic factors, we found that hypertension, angina pectoris, heart failure, myocardial infarction, type 2 diabetes (T2DM) and dyslipidemia were significantly ($p < 0.05$) associated with increased logBMI. We did not find significant difference in logBMI between females and males (p = 0.34). The results of association between non-genetic factors and BMI are displayed in Additional File 1. The age of the patients correlated significantly with increased BMI ($r = 0.204$; $P < 0.001$), when analyzed using linear regression. In order to remove bias from confounding factors, we adjusted association of rs11575892 with BMI using general linear model including only those factors and covariate that showed significant association with BMI (Table 2). rs11575892 CT heterozygotes displayed significantly higher (p = 0.001) mean BMI value compared to the CC genotype (Table 2). To minimize the false-positive results given the relatively small sample size and SNP frequency, we repeated the above mentioned analysis performing permutation test with 'label swapping' method for both analysis using exactly the same parameters. Corrected empirical P values

Table 1: Characteristics of the study population.

Variable	Number	Percentage	Mean ± SD
Total number	1135	-	-
Females	613	54.0	-
Males	522	46.0	-
Patients with:			
Hypertension	375	33.0	-
Angina pectoris	381	33.6	-
Myocardial infarction	246	21.7	-
Heart failure	292	25.7	-
Atrial fibrillation	24	2.1	-
T1DM	53	4.7	-
T2DM	146	12.9	-
dyslipidemia	136	12.0	-
Age (years)	1135	-	56.68 ± 12.52
BMI (kg/m^2)	1135	-	28.07 ± 5.36
logBMI	1135	-	1.4407 ± 0.0804

T1DM, type 1 diabetes; T2DM, type 2 diabetes
SD, standard deviation; BMI, body mass index

Table 2: Association of BMI with two SNPs

GLM analysis[a]					
SNP					P value
rs11575892	Genotype	C/C	C/T	T/T	0.001
	Number	1101	34	-	
	Mean logBMI ± SE	1.446 ± 0.005	1.491 ± 0.014	-	
	(95% CI)	(1.436–1.456)	(1.463–1.518)	-	
	Mean BMI[c] (kg/m2)	27.92	30.97	-	
rs5030980	Genotype	G/G	G/A	A/A	0.986
	Number	720	68	1	
	Mean logBMI ± SE	1.458 ± 0.007	1.458 ± 0.011	1.471 ± 0.076	
	(95% CI)	(1.445–1.472)	(1.436–1.481)	(1.322–1.620)	
	Mean BMI[c] (kg/m2)	28.70	28.70	29.58	

Categorical analysis [b]					
SNP		Genotype distribution (n) BMI <= median	BMI > median	OR (95% CI)	P value
rs11575892	C/C	558	543	2.59 (1.24–5.37)	0.011
	C/T	12	22		
rs5030980	G/G	368	352	1.08 (0.65–1.78)	0.767
	G/A	33	35		
	A/A	0	1		

SE, standard error of mean; CI, confidence interval; OR, odds ratio.
a- values from general linear model adjusted for gender, age, presence of hypertension, angina pectoris, myocardial infarction, heart failure and T2DM
b- values from logistic regression adjusted for age and gender
c- BMI values back-transformed from mean logBMI values

generated by permutation test were significant (p = 0.0016) in multivariate linear regression analysis. We also used median BMI (27.34 kg/m^2) as a cut off value for overweight threshold in a categorical analysis. A significant difference in genotype distribution between the normal and the overweight groups was found in logistic regression analysis adjusting for age and sex (p = 0.011) (Table 2).

These results indicate that association of rs11575892 with increased BMI is not due to possible confounding by specific diseases or other factors in study cohort. Nevertheless we cannot completely exclude the role of different environmental variables such as social background, diet and physical activity that could have impact on BMI, but such parameters were not accessible for present analysis. Due to low frequency of the rs11575892 polymorphism, we could only estimate the effect of this genetic variant in its heterozygous state, as we did not detect any homozygous individuals who may have pronounced effect of this SNP on BMI. The power analyses show that the present sample size had >80% power to detect changes in our logBMI of 0.045. Since rs11575892 has not previously been studied with respect to obesity and no association for this polymorphism has been found with anorexia by Vink et al[15], further studies with increased sample size or case-control studies with stratification for environmental factors such as social background, diet and physical activity would be valuable to verify the effects of rs1157892. There are reports that show effects of other *AGRP* polymorphisms on body weight are expressed in age dependant fashion [16]. This study group consists mostly of elderly people (mean 59.0 years) and this could contribute to enhance the effect of this particular SNP on BMI.

In order to test previously reported associations of rs5030980 with decreased body fatness we subsequently performed genotyping in available samples (n = 789) that were used for genotyping of rs1157892. The estimated minor allele frequency of rs11575892 was 0.044 and we did not observe significant departure from Hardy-Weinberg equilibrium (p = 1.0). Pairwise LD analysis showed absence of LD between both SNPs (D' = 1.00, r^2 = 0.001). No association of rs5030980 with BMI was found neither in GLM or logistic regression analysis (Table 2). We did not find any difference in mean values between GG and

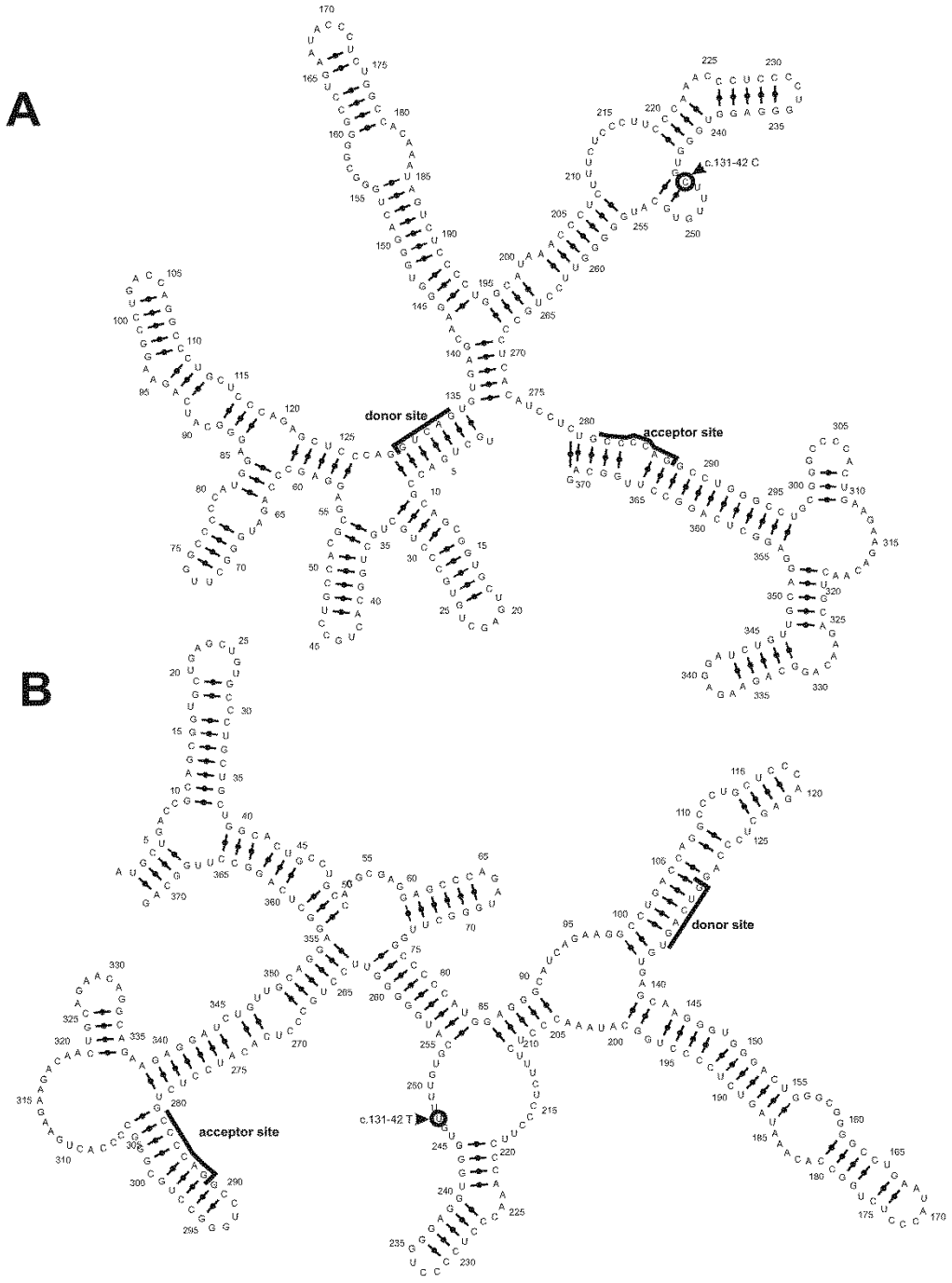

Figure 1
Secondary RNA structure model of AGRP gene fragments containing either C (A) or T (B) at position c.131-42. Donor and acceptor sites of second AGRP intron are marked with bold line. Nucleotide positions corresponding to rs1157892 are circled. Modeling was done using Mfold program from Vienna RNA package http://www.tbi.univie.ac.at/RNA/.

GA genotypes as opposite to previous report [16]. We found only one AA genotype carrier in our study group with slightly increased BMI value (29.58 kg/m^2). This is in disagreement with results of Marks et.al where carriers of AA displayed significantly lower BMI [17]. Relatively low frequency of this SNP however permits to compare these effects with sufficient power. Haplotype analysis did not return significant difference in BMI values between three possible haplotypes (data not shown).

The previously studied SNPs in the promoter region and the coding sequence of *AGRP* have been considered to be functional mutations [21-23], while functional importance of rs1157892 is unclear. Analysis of secondary RNA structure models of this region shows significant change when this SNP is present (see Figure 1) that could influence a splicing efficiency or promote an alternative splicing. However, if rs1157892 induces production of truncated or defective AGRP peptide, it would result in lower stimulation of food intake and lower BMI what is opposite to the effects found in our study. Our findings may however be explained by action of the N-terminal part of AGRP. It has recently been shown that AGRP is posttranslationally cleaved after Arg82 [24] and it is only the C-terminal peptide that acts on the melanocortin receptors. Even though the ICV injection of N-terminal parts of AGRP (aa25-51) and (aa54-82) did not affect food consumption in rats, it caused increase of body weight and epididymal/mesenteric fat weight [25]. It is therefore possible that rs1157892 may cause splicing defects resulting in an increased fraction of the N-terminal AGRP peptide subsequently affecting body weight. Alternatively the polymorphism may promote the formation of more active AGRP isoform as a result of modified splicing reaction. We also performed screening of *AGRP* gene sequence for putative gene regulatory factor and microRNA (miRNA) binding sites using online RegRNA software (available at http://regrna.mbc.nctu.edu.tw). This search revealed a sequence that overlaps rs1157892 and is highly complementary to human miR-330. This complementarily is significantly decreased in presence of rs1157892. Even though most of the miRNA functions so far have been assigned to regulation at mature RNA level it is shown that miRNA can act in nucleus regulating gene expression [26]. We can thus speculate that if role of miR-330 is to suppress *AGRP* expression, the presence of rs1157892 could lead to increase of AGRP synthesis and subsequent weight gain. It is also possible that rs1157892 may be in linkage disequilibrium with other functional mutations in regulatory regions of *AGRP* gene or even in neighboring genes. It should be noted however that no polymorphisms where found in the 5' untranslated region, 1102 bp upstream of *AGRP* gene neither in carriers of rs1157892 or other samples included in sequencing.

Conclusion

In summary, our results show novel association of a SNP in the second intron of *AGRP* with increased BMI suggesting that naturally occurring mutations in *AGRP* may be associated with enhanced effect of this peptide in the human population. This SNP could provide valuable information on the regulation of *AGRP* gene.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IvK and InK carried out genetic studies, participated in statistical analysis and drafted the paper, VP supervised clinical part of the study and performed clinical characterization, VI participated in genetic studies and performed statistical analysis, HBS participated in study design and has been involved in the approval of the final version, JK coordinated the study, participated in drafting and gave final approval of version to be published.

Additional material

Additional file 1

Table. Association between BMI and non-genetic factors. *The data provided represent the results of ANOVA analysis between BMI and other factors used in the study.*

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3.1 SUPPLEMENTARY DATA

Supplementary table: Association between BMI and non-genetic factors

Characteristic	Mean logBMI (SE)	Mean BMI [a] (kg/m^2)	P-value[b]
Gender			
Male	1.438 (0.003)	27.41	0.342
Female	1.443 (0.004)	27.73	
Hypertension			
No	1.428 (0.003)	26.79	<0.001
Yes	1.468 (0.004)	29.37	
Angina pectoris			
No	1.436 (0.003)	27.28	0.002
Yes	1.451 (0.004)	28.24	
Myocardial infarction			
No	1.438 (0.003)	27.41	0.011
Yes	1.452 (0.004)	28.31	
Heart failure			
No	1.435 (0.003)	27.22	<0.001
Yes	1.457 (0.004)	28.31	
Atrial fibrillation			
No	1.442 (0.002)	28.01	0.112
Yes	1.466 (0.010)	29.26	
T1D			
No	1.441 (0.002)	27.61	0.120
Yes	1.424 (0.010)	26.54	
T2D			
No	1.433 (0.002)	27.10	<0.001
Yes	1.492 (0.007)	31.04	
Dyslipidemia			
No	1.439 (0.003)	27.47	0.023
Yes	1.457 (0.006)	28.64	

T1D - type 1 diabetes; T2D - type 2 diabetes; SE - standard error of mean; BMI - body mass index (kg/m^2); [a]BMI values back-transformed from logBMI; [b]p values based on logBMI comparison.

3.2 Analysis of polymorphisms at the adiponectin gene locus in association with type 2 *diabetes mellitus*, body mass index and cardiovascular traits in Latvian population

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ANALYSIS OF POLYMORPHISMS AT THE ADIPONECTIN GENE LOCUS IN ASSOCIATION WITH TYPE 2 DIABETES, BODY MASS INDEX AND CARDIOVASCULAR TRAITS IN LATVIAN POPULATION

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Despite the number of recently conducted studies seeking to determine the association between genetic variants of adiponectin gene and susceptibility to type 2 diabetes (T2D) and increased body mass index (BMI), the results obtained are often inconsistent. To determine the impact of common polymorphisms in promoter and coding regions of adiponectin gene on these conditions in Latvian population, we selected ten SNPs (rs2241767, rs1501299, rs3777261, rs16861210, rs2241766, rs822396, rs182052, rs17300539, rs16861194, rs266729) based on haploblock structure and previously reported association studies. The selected SNPs were screened in a study group of 835 participants from the Genome Data Base of Latvian Population and mainly consisted of patients with T2D and coronary heart disease. None of the individual polymorphisms were significantly associated with T2D status or BMI when analysed using logistic or linear regression and adjusted for gender, age and other significant covariates. Frequency of rs2241766 T allele homozygotes however was significantly increased in T2D patients compared to controls (uncorrected P = 0.007). When analysed with other traits, the rs182052 G allele was found to be less frequent in patients suffering from myocardial infarction (P = 0.02; OR = 0.76, CI95% [0.61–0.92]) compared to others. Haplotype analysis revealed significant association of one haplotype with atrial fibrillation (uncorrected P = 0.01). In summary, we conclude that SNPs in adiponectin gene are unlikely to represent the risk for T2D, but may be involved in pathogenesis of CHD in the Latvian population.

Key words: ADIPOQ, *tagSNP, BMI, T2D, CHD.*

INTRODUCTION

Adiponectin is an adipose tissue-derived plasma protein that plays an important role in energy homeostasis regulation, glucose and lipid metabolism as well as anti-inflammatory responses in the vascular system (Hu *et al.*, 1996; Ouchi *et al.*, 2003). Low plasma adiponectin concentrations are linked to type 2 diabetes (T2D) (Weyer *et al.*, 2001), obesity (Matsuzawa *et al.*, 2003) and coronary heart disease (CHD) related traits (Matsuda *et al.*, 2002; Kumada *et al.*, 2003). Possible mechanisms of adiponectin action in relation with these inflammatory diseases may include its inhibition of smooth muscle cell proliferation, monocyte adhesion to endothelium, and macrophage uptake of LDL (Chen *et al.*, 2005).

Adiponectin is a product of the *ADIPOQ* gene consisting of three exons that occupies 16 kb on chromosome 3q27. During the last five years, a large number of association studies have been performed to search for polymorphisms that may influence metabolic conditions (reviewed in Vasseur *et al.* (2006) and Menzaghi *et al.* (2007)). Even though the number of polymorphisms and haplotypes are repeatedly associated with increased or decreased blood adiponectin levels (Heid *et al.*, 2006; Mackevics *et al.*, 2006; Li *et al.*, 2007; Kyriakou *et al.*, 2008), association analysis of these SNPs

with metabolic and cardiovascular phenotypes give inconsistent results across different studies.

The aim of our study was to search for the association between systematically selected adiponectin gene polymorphisms and T2D, body mass index (BMI) as well as CHD phenotypes in the Latvian population.

MATERIALS AND METHODS

Study subjects. The study was based on data and samples from the Genome Data Base of Latvian Population, the disease based biobank. Briefly: participating individuals were over the age of 18; the health status was recorded, the diagnoses were based on approved clinical criteria according to the ICD-10 codes (International Classification of Diseases); andanthropometric measurements (including weight and stature), ethnic, social, environmental information and familial health status was acquired based on a self-reported questionnaire. Written informed consent was obtained from all participants. The study group for the subsequent genotyping of selected markers was selected from all available subjects with validated health records and questionnaires in the biobank. We excluded patients with diseases where outcome or treatment may influence BMI, including all types of cancer and diseases of the thyroid gland, but not excluding patients with cardiovascular diseases. All patients with T2D were included in the study. In total, 835 subjects were selected based on these criteria. The study protocol was approved by the Central Medical Ethics Committee of Latvia.

SNP genotyping. TagSNPs in the adiponectin gene locus were selected using Haploview software (Barrett *et al*., 2005). The SNPs with previously published effect on adiponectin plasma concentration were force included in the tagSNP set. MALDI TOF-based genotyping platform (Bruker Daltonics) was used for genotyping all SNPs (primer sequences and reaction conditions available upon request). Genotype calling and quality control was performed using GenoTools software (Bruker Daltonics).

Statistical analysis. Statistical analyses were performed with the PLINK 1.06 software (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al*., 2007). In contrast to the natural BMI values, the logarithmically transformed BMI values displayed normal distribution and were further used for all quantitative analyses. Linear regression was applied to examine the association between tagSNPs and log-BMI adjusting for age and gender. The allelic association test (additive model), dominant and recessive model analysis was performed using the exact Fisher's test. Logistic regression analysis adjusting for age and those factors that showed significant association with trait under multivariate logistic regression analysis were used for dichotomous trait analysis. Bonferroni correction was used to adjust for multiple testing. The permutation test was performed using label-swapping between SNP and trait condition or logBMI values but leaving intact correlation with other covariates. 10,000 permutations were performed for each analysis and corrected (EMP2) *P*-values were used. These values are corrected based on calculation of the proportion of permutations in which any of the test statistics exceeds the particular observed statistic and are more stringent then uncorrected *P*-values. Odds ratios (OR) and CI (95%) values were obtained from logistic regression analysis (Exp(B) value), unadjusted mean logBMI values were calculated and back-transformed for each genotype.

RESULTS

Description of the study group is summarised in Table 1. A total of ten SNPs in the adiponectin gene locus were genotyped in all or part of the participants. SNP information, observed minor allele frequency (MAF) and results of deviation from the Hardy–Weinberg equilibrium test are shown in Table 2. None of the SNPs were in disagreement with Hardy-Weinberg equilibrium after correction for multiple testing. The exact number of successfully genotyped samples for each SNP in the study group is displayed in Tables 3 and 4, along with the results of association with T2D and logBMI values, respectively. The SNP rs2241766 was asso-

Table 1

CHARACTERISTICS OF THE STUDY POPULATION

Parameter	Values
Total number of patients	835
Percentage of females (n)	66.7 (557)
Percentage of males (n)	33.3 (278)
Percentage of patients (n) with:	
Type 2 diabetes	20.3 (170)
Hypertension	55.9 (467)
Heart failure	52.2 (435)
Atrial fibrillation	4.6 (39)
Myocardial infarction	46.2 (390)
Age (years) [mean ± SD]	62.82 ±10.12
BMI [mean ± SD]	28.77 ± 4.71

Table 2

CHARACTERISTICS OF TEN SNPs GENOTYPED IN 835 SUBJECTS

SNP No.	rs code	Genome position[a]	Gene position[b]	Common allele	Rare allele	MAF	H-W test *P* value
1	rs16861194	188042119	-11422	A	G	0.12	0.29
2	rs17300539	188042154	-11387	G	A	0.05	0.11
3	rs266729	188042168	-11373	C	G	0.28	0.01
4	rs182052	188043476	-10065	G	A	0.43	0.35
5	rs16861210	188049192	-4349	G	A	0.05	0.27
6	rs822396	188049571	-3970	A	G	0.22	1
7	rs2241766	188053586	45	T	G	0.05	0.66
8	rs1501299	188053817	276	A	C	0.28	1
9	rs2241767	188053890	349	A	G	0.05	1
10	rs3774261	188054253	712	G	A	0.33	0.93

MAF, minor allele frequency; H-W, Hardy-Weinberg equilibrium

[a] - according to the NCBI build 36; [b] - relating to the first position of the starting codon ATG

Table 3

ASSOCIATION OF T2D WITH TEN tagSNPs

SNP	Genetic model	Number of subjects [a]		*P* value (Fisher test)	*P* value [b] (logreg)	OR [95% CI][b]
		T2D	Control			
rs16861194	additive	3/29/110	10/115/439	0.869	0.778	0.93 [0.57–1.51]
	dominant	32/110	125/439	0.924	0.653	0.89 [0.52–1.52]
	recessive	3/139	10/554	0.788	0.646	1.49 [0.28–8.08]
rs17300539	additive	0/18/129	4/48/517	0.407	0.377	1.35 [0.7–2.59]
	dominant	18/129	52/517	0.258	0.302	1.45 [0.72–2.9]
	recessive	0/147	4/565	0.308	-	-
rs266729	additive	13/49/72	50/182/271	0.988	0.959	0.99 [0.7–1.4]
	dominant	62/72	232/271	0.976	0.986	1.01 [0.64–1.6]
	recessive	13/121	50/453	0.934	0.884	0.95 [0.44–2.07]
rs182052	additive	31/57/51	101/270/183	0.95	0.306	0.85 [0.62–1.16]
	dominant	88/51	371/183	0.415	0.114	0.69 [0.43–1.1]
	recessive	31/108	101/453	0.274	0.983	1.01 [0.58–1.76]
rs16861210	additive	0/18/143	4/59/576	0.804	0.793	1.09 [0.58–2.06]
	dominant	18/143	63/576	0.62	0.676	1.16 [0.59–2.26]
	recessive	0/161	4/635	0.314	-	-
rs822396	additive	9/55/98	30/221/395	0.761	0.518	1.12 [0.79–1.6]
	dominant	64/98	251/395	0.879	0.558	1.14 [0.74–1.75]
	recessive	9/153	30/616	0.628	0.666	1.23 [0.49–3.14]
rs2241766	additive	2/13/141	0/50/516	0.443	0.314	1.46 [0.7–3.02]
	dominant	15/141	50/516	0.763	0.562	1.26 [0.59–2.7]
	recessive	2/154	0/566	**0.007**	-	-
rs1501299	additive	13/52/71	47/254/315	0.887	0.545	1.11 [0.78–1.58]
	dominant	65/71	301/315	0.821	0.641	1.12 [0.71–1.76]
	recessive	13/123	47/569	0.452	0.572	1.26 [0.57–2.81]
rs2241767	additive	1/13/133	0/49/508	0.607	0.924	1.04 [0.47–2.32]
	dominant	14/133	49/508	0.784	0.987	1.01 [0.44–2.33]
	recessive	1/146	0/557	0.051	-	-
rs3774261	additive	5/34/32	61/235/249	0.673	0.815	0.95 [0.62–1.46]
	dominant	39/32	296/249	0.922	0.613	1.16 [0.66–2.03]
	recessive	5/66	61/484	0.288	0.177	0.47 [0.16–1.42]

Logreg- logistic regression;

a- Distribution of subjects according to genotypes/models: additive 22/12/11; dominant: 12+22/11; recessive 22/12+11 (1, common allele; 2, rare allele)

b- From logistic regression analysis adjusted for gender, age and logBMI

ciated with T2D under the recessive model (P = 0.007). This association remained significant after applying the permutation test (P = 0.049), but lost its significance after correction for multiple comparisons. Neither of the SNPs showed any association with T2D when tested using logistic regression with adjustment for age, sex and BMI under different genetic models (Table 3). Similarly, no difference in mean values of logBMI was found between genotypes of any SNP included in analysis (Table 4). We also tested for association of SNPs with other traits present in our study group. The results are shown in Figure 1A. Allele A of SNP rs182052 was less frequent in patients with myocardial infarction (39%) compared to controls (45%). This association remained significant using multiple logistic regression analysis adjusting for sex, age, BMI and T2D (P = 0.02; OR = 0.76, CI95%[0.61–0.92]). The permutation test on this SNP gave a corrected P value of 0.028. The association did not retain its significance after Bonferroni correction.

In order to identify possible effects of individual haplotypes on traits analysed in our study we performed haplotype-based association. In total, 14 haplotypes were reconstructed with frequency exceeding 1%. Statistical analysis of the distribution of probabilistically-inferred set of haplotypes for different traits is shown in Figure 1B. Haplotype determined by C and A alleles of rs1501299 and rs3774261, respectively, and common alleles for other SNPs was more frequent (26%) in patients with atrial fibrillation compared to others (14%), P = 0.009.

DISCUSSION

We report in this study the first evaluation of adiponectin gene polymorphisms in relation with T2D, BMI and cardiovascular traits in the Latvian population. First we performed systematic selection of tagSNPs in *ADIPOQ* gene locus in

Table 4

ASSOCIATION OF BMI WITH TEN tagSNPs

SNP	Number of subjects	Mean [± SE] BMI[a]			*P* value[b]		
	11/12/22	11	12	22	Additive	Dominant	Recessive
rs16861194	539/143/13	29.47 [0.23]	29.84 [0.39]	30.23 [1.23]	0.244	0.266	0.536
rs17300539	635/66/4	29.7 [0.22]	29.27 [0.55]	33.22 [2.21]	0.891	0.848	0.175
rs266729	340/227/60	29.79 [0.28]	29.59 [0.32]	29.58 [0.59]	0.427	0.447	0.615
rs182052	232/320/131	29.48 [0.32]	29.57 [0.29]	30.28 [0.41]	0.094	0.358	0.053
rs16861210	706/77/4	29.65 [0.21]	29.71 [0.52]	30.61 [2.24]	0.737	0.757	0.825
rs822396	487/270/38	29.71 [0.24]	29.54 [0.3]	29.45 [0.73]	0.436	0.455	0.670
rs2241766	647/62/2	29.62 [0.21]	29.85 [0.58]	33.15 [3.14]	0.373	0.510	0.137
rs1501299	379/302/60	29.82 [0.26]	29.81 [0.3]	29.47 [0.58]	0.696	0.700	0.836
rs2241767	631/61/1	29.59 [0.22]	30.63 [0.57]	34.78 [4.4]	0.110	0.137	0.256
rs3777261	276/265/66	29.68 [0.34]	29.3 [0.35]	30.09 [0.59]	0.926	0.628	0.328

1, common allele; 2, rare allele

a- BMI values back-transformed from unadjusted mean logBMI values

b- *P* values from linear regression analysis adjusted for gender and age

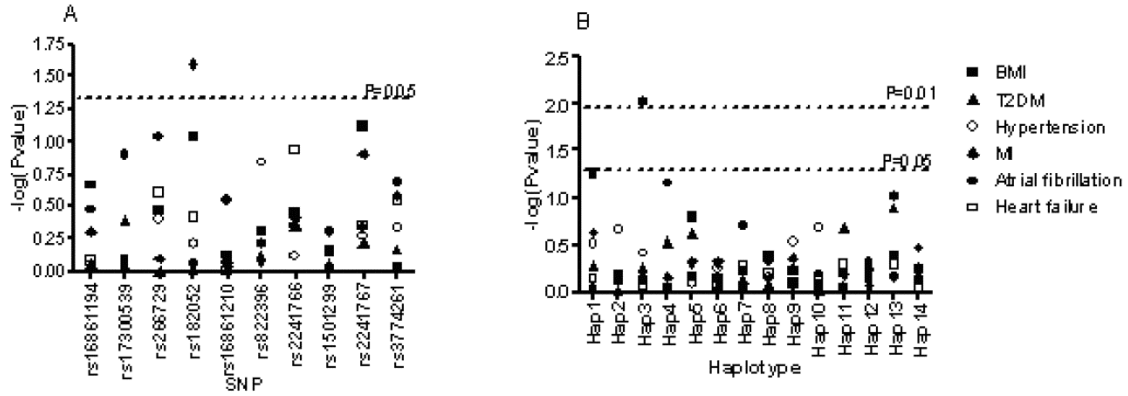

Fig. 1. Significance values depicted as negative logarithm of *P* value from adjusted linear or logistic regression analysis for SNP (A) and haplotype (B) association with all phenotypes.

order to ensure maximal capture of all known alleles. In addition, the forced inclusion of variants shown to influence plasma adiponectin levels was performed. Estimated minor allele frequencies (Table 2) of all ten SNPs were higher than 0.05 in the Latvian population and similar to those reported in the SNP database (www.ncbi.nlm.nih.gov/SNP) or other publications (Heid *et al*., 2006). We believe that altogether this ensures the high informativeness of the selected set of ten markers.

In the present quantitative trait analysis we found no evidence for an association of the tested SNPs with changes in BMI value. Other studies have previously reported that at least three of the SNPs also included in this study (rs266729, rs2241766, rs1501299) might modulate body weight (reviewed in Vasseur *et al*. (2006)). However, recent meta-analysis did not confirm the association of these or other markers with BMI (Menzaghi *et al*., 2007). The failure to identify a stable association between *ADIPOQ* polymorphisms and BMI, despite the repeatedly confirmed relationship between the levels of plasma adiponectin and BMI (Yang *et al*., 2001; Spranger *et al*., 2003), can be explained by the variation in genetic background determining the predisposition to obesity. Stratification of the study group based on common genetic variants influencing body mass regulation could help to estimate the role of adiponectin SNPs more precisely.

The only SNP that was significantly associated with T2D in our study was rs2241766, under the recessive genetic model. The relatively low frequency of this SNP and absence of GG homozygotes in the control group, however, do not allow to precisely determine the relative risk of this variation. G allele of rs2241766 was found to be associated with decreased levels of serum adiponectin (Xita *et al*., 2005; Li *et al*., 2007;) as well as T2D (Li *et al*., 2007). This result is in agreement with finding, that low plasma levels are associated with insulin resistance and T2D. It should be noted, however, that a substantial number of studies failed to replicate these results (Mackevics *et al*., 2006; Potapov *et*

al., 2008; Szopa *et al.*, 2009). Since previous studies did not support the recessive model for association of rs2241766 with T2D our result may be as well a false positive finding.

Adiponectin mediates vascular inflammation as an inhibitor of smooth muscle cell proliferation and macrophage uptake and has been associated with decreased risk of hypertension (Iwashima *et al.*, 2004), miocardial infarction (Pischon *et al.*, 2004) and ischemic stroke (Chen *et al.*, 2005). We, therefore, tested the association of all SNPs analysed with presence of different cardiovascular traits. In our study, rs182052 was associated with decreased risk of miocardial infarction. Allele A of rs182052 was previously associated with a decreased adiponectin serum level (Heid *et al.*, 2006; Kyriakou *et al.*, 2008) and, thus, would be expected to have negative effect on cardiovascular functions which is opposite to our finding. Nevertheless, Hegener *et al.* (2006) reported a decreased risk of ischemic stroke for carriers of this allele. One of the imputed haplotypes was found to be more frequent in another heart disease trait, atrial fibrillation. To our knowledge this is the first report of genetic variants of adiponectin associated with this trait. The two SNPs, rs1501299 and rs3774261, determining this haplotype, have been associated with increased adiponectin level (Heid *et al.*, 2006; Kyriakou *et al.*, 2008). Interestingly, plasma adiponectin is higher in patients with persistent atrial fibrillation (Shimano *et al.*, 2008), which is in opposite to the general tendency of a high adiponectin level being protective to most of the CHD-related pathogeneses. Similarly, high blood adiponectin was found to be a predictor for mortality in heart failure (Chang *et al.*, 2009). It has been speculated that this could be explained with lower sensitivity of adiponectin receptors in atrial fibrillation resulting in increased adiponectin secretion (Shimano *et al.*, 2008). In general, however, the situation with CHD and adiponectin polymorphisms is similar to obesity and T2D, where associations found in some studies can not be consistently replicated in other studies (Menzaghi *et al.*, 2007; Vasseur *et al.*, 2006). It should also be mentioned that none of the genome wide association studies have identified the *ADIPOQ* locus as a candidate for any trait (based on a database search at https://gwas.lifesciencedb.jp and http://www.genome.gov/gwastudies/).

The main drawback of our study is an increased proportion of patients with metabolic and cardiovascular traits in study group. The relatively low number of healthy participants in the study may have increased Type II error, which would mask some existing association or decrease the statistical power. From this respect, additional study with a large control group is needed to fully evaluate the impact of adiponectin polymorphisms on T2D and BMI in the Latvian population. An important problem in all studies on a large number of genetic variations is multiple testing. In our study, none of the discovered associations retained its significance ($P < 0.05$) after applying the Bonferroni test. It has been suggested, however, that study-wide adjustments are not appropriate in the context of genetic association analysis and that permutation based significance testing can be used instead. All associations found in this study remained significant after the permutation test.

In summary, although adiponectin plasma levels are linked with many metabolic traits, our study does not provide strong support for the hypothesis that SNPs in the *ADIPOQ* locus play an important role in development of T2D, obesity and CHD.

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ADIPONEKTĪNA GĒNA LOKUSA ASOCIĀCIJAS ANALĪZE SAISTĪBĀ AR OTRĀ TIPA DIABĒTU, ĶERMEŅA MASAS INDEKSU UN KARDIOVASKULĀRĀM PAZĪMĒM LATVIJAS POPULĀCIJĀ

Neraugoties uz daudzajiem adiponektīna gēna variantu asociācijas pētījumiem saistībā ar otrā tipa diabētu (T2D) un paaugstinātu ķermeņa masas indeksu (ĶMI), līdz šim iegūtie rezultāti bieži ir pretrunīgi. Lai noskaidrotu adiponektīna gēna promotera un kodējošā rajona bieži sastopamo polimorfismu ietekmi uz šīm pazīmēm Latvijas populācijā, balstoties uz haplobloku struktūru un iepriekš zināmiem asociācijas pētījumiem, tika izvēlēti desmit SNP (rs2241767, rs1501299, rs3777261, rs16861210, rs2241766, rs822396, rs182052, rs17300539, rs16861194, rs266729). Izvēlētie SNP tika genotipēti pētījuma grupā, kas sastāvēja no 835 Valsts iedzīvotāju genoma datu bāzes gēnu donoriem. Šajā grupā bija pārsvarā no T2D un koronārās sirds slimības (KSS) pacienti. Neviens no individuāliem polimorfismiem nebija būtiski asociēts ar T2D vai ĶMI, lietojot daudzfaktoru loģistiskās vai lineārās regresijas analīzes un koriģējot rezultātus pēc dzimuma, vecuma un citiem būtiskiem līdzfaktoriem. Tomēr tika atrasts, ka rs2241766 T alēles homozigotu frekvence T2D pacientu vidū bija būtiski lielāka nekā kontroles grupā (nekoriģēta $P = 0.007$). Analizējot saistībā ar citām pazīmēm, rs182052 G alēle tika biežāk atrasta pacientiem ar miokarda infarktu ($P = 0.02$; OR = 0.76; CI95%[0.61–0.92]), salīdzinot ar pārējo pētījuma grupu. Haplotipu analīze atklāja viena haplotipa būtisku asociāciju ar mirdzaritmiju (nekoriģēta $P = 0.01$). Kopumā var secināt, ka adiponektīna gēna polimorfismi nav uzskatāmi par būtiskiem T2D riska faktoriem, bet varētu būt iesaistīti koronārās sirds slimības patoģenēzē Latvijas populācijā.

3.3 Stronger association of common variants in *TCF7L2* gene with nonobese type 2 *diabetes mellitus* in the Latvian Population

Stronger Association of Common Variants in TCF7L2 Gene with Nonobese Type 2 Diabetes in the Latvian Population

Authors

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Key words
- association study
- polymorphism
- T2D

Abstract

Polymorphisms in the gene coding for transcription factor 7 like 2 (TCF7L2) are recognized as the strongest common genetic risk factors for type 2 diabetes (T2D) across multiple ethnicities. This study was conducted to evaluate an association between TCF7L2 variants and diabetes susceptibility in the population of Latvia. We genotyped 4 single nucleotide polymorphisms (SNP) rs7901695, rs7903146, rs11196205 and rs12255372 in 1093 controls and 1043 diabetic subjects. Association with T2D was found for 3 SNPs rs7901695, rs7903146 and rs12255372 in the whole sample (under an additive genetic model, the adjusted odds ratios (OR) were 1.26, 95% CI [1.08–1.48], P=0.003; OR=1.32, 95% CI [1.12–1.55], P=0.001 and OR=1.35, 95% CI [1.15–1.60], P=0.0004 respectively). In addition observed effects on T2D susceptibility for analysed SNPs were higher among subjects with BMI under 30 kg/m^2. The impact of TCF7L2 variation on T2D risk in Latvian population is compatible with that demonstrated by a range of studies conducted in various ethnic groups.

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Introduction

Type 2 diabetes mellitus (T2D) is a common multifactorial disease with rapidly increasing prevalence. Like many other complex traits, it is triggered by interaction of multiple genetic variants and diverse environmental factors. Only recently genetic studies including genome wide associations (GWAs) have led to successful discovery of several common T2D susceptibility gene loci [1]. From those, the transcription factor 7 like 2 gene (TCF7L2) has shown the highest effect and this association has been replicated across different populations of European, Asian and African descent [2].

In this study we investigate 4 SNPs rs7901695, rs7903146, rs11196205 and rs12255372 to study for the first time the impact of TCF7L2 gene polymorphic variants on T2D risk in Latvian population.

Methods

Case-control study groups were selected from the Latvian Genome Data Base (LGDB), a government funded biobank (shortly described in [3]). Subjects included in this study were selected from 14047 participants of LGDB recruited during the period from January 2003 to June 2010. In total 1783 T2D cases (ICG-10 code E11.0) were identified from the database. From this group we excluded all patients with insufficient relevant phenotype data, leaving 1043 cases in the final sample. 9795 participants who had not been diagnosed with T2D at the time of recruitment to the database with complete phenotype data set were considered for selections of controls. From this group 1093 subjects were randomly selected as controls maintaining the same gender proportion as in T2D cases. Genotyping was performed using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) for the rs7903146 as described previously [3] and TaqMan SNP genotyping assays on 7500 Real-Time PCR system in the case of rs7901695, rs11196205 and rs12255372 according to recommendation of manufacturer (Applied Biosystems, USA). Genotyping success rate was 99.1%, 97.5%, 99.1% and 98.8% for rs7901695, rs7903146, rs11196205 and rs12255372 respectively. Statistical analysis was performed using Plink (1.06) software [4] (http://pngu.mgh.harvard.edu/purcell/

* Both authors contributed equally.

SNP	Number (MAF)		OR [CI-95%][a]	P-value[a] (P-perm)[b]
	T2D patients	Controls		
rs7901695 (CC/ CT/TT)	83/400/548 (0.28)	50/406/629 (0.23)	1.264 [1.083–1.476]	**0.003 (0.009)**
rs7903146 (TT/TC/CC)	65/362/588 (0.24)	39/344/685 (0.20)	1.318 [1.118–1.552]	**0.001 (0.003)**
rs11196205 (CC/CG/GG)	251/502/279 (0.49)	237/514/333 (0.46)	1.127 [0.987–1.287]	0.077 (ns)
rs12255372 (TT/TG/GG)	55/361/616 (0.23)	37/322/720 (0.18)	1.354 [1.147–1.599]	**0.0004 (0.001)**

OR – odds ratio; CI – confidence interval; MAF – minor allele frequency, ns – non significant

[a] OR and P-values estimated using logistic regression adjusted for age, sex and BMI

[b] P-value obtained from 100 000 permutation and corrected for multiple testing (EMP2)

Table 1 Association of SNPs in TCF7L2 gene with T2D.

plink/). Association with T2D was estimated by logistic regression assuming additive, dominant and recessive genetic models of inheritance. Logistic regression analysis was adjusted for other non-genetic covariates including age, gender and BMI. In all calculations a P-value <0.05 was considered significant. To adjust for multiple testing a permutation tests with 100 000 permutations were performed for each analysis and we used corrected (EMP2) P-values. The sample population was separated into 2 groups: non-obese (BMI<30 kg/m^2) and obese (BMI≥30 kg/m^2). Association between SNPs and T2D in 2 groups separately was analyzed as described above but excluding the BMI from the list of cofactors.

Results

4 SNPs rs7901695, rs7903146, rs11196205 and rs12255372 in TCF7L2 gene were genotyped in 1043 T2D patients (331 males and 712 females) and 1093 controls (354 males and 739 females). The mean age±standard deviation (SD) at T2D diagnosis was 51.54±11.18 years while mean age of the control group was 53.62±12.99. The mean BMI±SD differed significantly between cases (32.44±6.87) and controls (27.18±5.19). Distribution of the genotype frequencies for all SNPs was in accordance with Hardy-Weinberg equilibrium. Our sample size provided sufficient power (>95%) for all SNPs except rs11196205. LD pattern in our sample was compatible with that given by other authors and in Hapmap database for caucasians (Rel#28; www.hapmap.org). Although the rare allele frequencies for all 4 SNPs were higher in cases, significant association with T2D was observed only for 3 polymorphisms, rs7901695 (P=0.002), rs7903146 (P=0.0005) and rs12255372 (P=0.0004) with the following crude ORs and 95% confidence intervals (CI) respectively: 1.25 [1.08–1.43]; 1.297 [1.12–1.5] and 1.31 [1.13–1.53]. Association remained significant after correction for age, gender and BMI (**Table 1**). The significant association for 3 positive SNPs was observed under all genetic models (data not shown). 3 SNPs displayed higher ORs [95%CI] for association with T2D in the non-obese subgroup in comparison with obese patients: rs7901695 1.36 [1.11–1.66]; rs7903146 1.42 [1.14–1.76] and rs12255372 1.42 [1.15–1.76]. Only association between rs12255372 and T2D remained significant in the obese subgroup of patients: 1.34 [1.03–1.75].

Discussion

This is the first report from the Baltic countries demonstrating significant association of genetic variants within third and fourth intronic regions of TCF7L2 gene with T2D. 2 polymorphisms demonstrating the strongest association with T2D risk are rs12255372 and rs7903146 followed by rs7901695. These results agree with previous findings in European populations and other ethnic groups [2,5]. Our study reveals similar results. In the case of rs11196205 we did not find significant association with the disease risk in the whole sample. Similarly, no association for this SNP was observed in another study performed on population from northern Sweden [5]. It is likely that association of this SNP with T2D in other studies is due to LD with other variants and it is unlikely that rs11196205 could be functional variation. Since rs12255372 and rs7903146, the variants with higher OR are in strong LD it is difficult to point which of these might be the causal variant based on the data obtained in our study. Attempts to find other potentially functional variants have been unsuccessful so far [6]. Evidence of rs7903146 being a causal variant is further supported by functional studies were the risk allele T was shown to alter enhancer activity in this gene locus [7]. In our sample, the previously associated rs7903146 and rs7901695 remained significant only among non-obese subjects having BMI under 30 kg/m^2 and only the rs12255372 showing the strongest association was statistically significant in the obesity group, though with weaker effects. The risk allele of rs7903146 is shown previously to be associated with T2D in subjects with severe obesity, albeit with the clearly weaker OR than in lean population [8]. Obesity is an essential factor contributing to T2D development and it is well known that several SNPs show variable significance of association according to obesity status. Analysing several T2D susceptibility variants in large groups of obese and non-obese subjects it was proposed that variants acting on insulin resistance have more pronounced effects in the presence of obesity while variants affecting insulin secretion have a greater impact in lean subjects [8]. TCF7L2 is an important regulator of beta cell functions and intronic variants in its gene are shown to be modifiers of insulin secretion [9] and response to sulfonylurea treatment [10].

In summary this study demonstrates that TCF7L2 is important T2D susceptibility gene in Latvian population in particular among non-obese individuals. Our data support associations observed across population of European origin.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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3.4 Polymorphisms in *FTO* and near *TMEM18* associate with type 2 *diabetes mellitus* and predispose to younger age at diagnosis of diabetes.

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Polymorphisms in *FTO* and near *TMEM18* associate with type 2 diabetes and predispose to younger age at diagnosis of diabetes

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ABSTRACT

Variations in the *FTO* gene and near the *TMEM18* gene are risk factors for common form of obesity, but have also been linked with type 2 diabetes (T2D). Our aim was to investigate the contribution of these variants to risk of T2D in a population in Latvia. Four single nucleotide polymorphisms (SNP) in the first and fourth intronic regions of *FTO* and one close to *TMEM18* were genotyped in 987 patients with T2D and 1080 controls selected from the Latvian Genome Data Base (LGDB). We confirmed association of SNPs in the first intron (rs11642015, rs62048402 and rs9939609) of *FTO* and rs7561317 representing the *TMEM18* locus with T2D. Association between SNP in *FTO* and T2D remained significant after correction for body mass index (BMI). The rs57103849 located in the fourth intron of *FTO* and rs7561317 in *TMEM18* showed BMI independent association with younger age at diagnosis of T2D. Our results add to the evidence that BMI related variants in and near *FTO* and *TMEM18* may increase the risk for T2D not only through secondary effects of obesity. The influence of variants in the fourth intron of the *FTO* gene on development of T2D may be mediated by mechanisms other than those manifested by SNPs in the first intron of the same gene.

1. Introduction

Modern lifestyle has led to the rapid increase in prevalence of obesity which is one of the most important risk factors of type 2 diabetes (T2D) (Vazquez et al., 2007). Moreover genetic variants predisposing to obesity have been shown to associate with increased risk of developing T2D, although this is mostly mediated by the impact on the body mass regulation (Li et al., 2011; Robiou-du-Pont et al., 2012). The fat mass and obesity associated (*FTO*) gene and the transmembrane 18 (*TMEM18*) gene are among the strongest common obesity linked loci identified thus far by genome wide association (GWA) studies (Frayling et al., 2007; Scuteri et al., 2007; Thorleifsson et al., 2009; Willer et al., 2009).

Association of a number of highly correlated single nucleotide polymorphisms (SNP) within a 47 kb region of the *FTO* gene, comprising parts of first two introns, with adult and childhood obesity has been proven in multiple populations (Dina et al., 2007; Do et al., 2008; Frayling et al., 2007; Meyre, 2012; Renstrom et al., 2009; Takeuchi et al., 2011; Wing et al., 2010). Gender, age and lifestyle factors such as diet and physical activity may interact with the effects of the *FTO* genotype on body fat accumulation (Andreasen et al., 2008; Jacobsson et al., 2008a; Jacobsson et al., 2009; Qi et al., 2008; Sonestedt et al., 2011). At the same time, the alleles predisposing for obesity have been found to correlate with higher risk of T2D. These effects are thought to be secondary due to increased adiposity (Frayling et al., 2007; Freathy et al., 2008; Meyre, 2012; Renstrom et

Abbreviations: T2D, type 2 diabetes; SNP, single nucleotide polymorphisms; BMI, body mass index; logBMI, logarithmically transformed body mass index values; *FTO*, the fat mass and obesity associated; *TMEM18*, transmembrane 18; GWA, genome wide association; LD, linkage disequilibrium; LGDB, Latvian Genome Data Base; ICD-10, International Statistical Classification of Diseases 10th Revision; OR, odds ratio; CEU, Utah residents with ancestry from northern and western Europe; *IRX3*, iroquois homeobox 3; SD, standard deviation; CI, confidence interval; freq, frequency; n, number; RAF, risk allele frequency; p-perm, p - values derived performing permutation tests specifying 100000 permutations and corrected for multiple testing (EMP2); EMP2, corrected empirical p-value; ns, non-significant; Add, additive; Rec, recessive.

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al., 2009; Robiou-du-Pont et al., 2012; Thomsen et al., 2012), however some studies provide evidence that this may only partly be true (Hertel et al., 2011; Legry et al., 2009; Li et al., 2012; Meyre, 2012). In addition, variation in *FTO* has been associated with insulin sensitivity, glucose homeostasis and changes in blood lipid profile, but there is no clear coherence to what extent these findings can be explained by BMI (Andreasen et al., 2008; Do et al., 2008; Freathy et al., 2008; Grunnet et al., 2009; Meyre, 2012; Robiou-du-Pont et al., 2012; Wing et al., 2010; Zabena et al., 2009).

FTO encodes a nucleic acid demethylase (Gerken et al., 2007) expressed in several tissues relevant for maintaining energy balance including the brain, particularly the hypothalamus, skeletal muscle, adipose tissue, liver and pancreas (Frayling et al., 2007; Fredriksson et al., 2008; Gerken et al., 2007). The *FTO* risk genotype has been linked with altered central regulation of energy homeostasis in the hypothalamus and metabolic dysfunction in peripheral tissues, which further supports its role in metabolic disease (Cecil et al., 2008; Grunnet et al., 2009; Meyre, 2012; Olszewski et al., 2011; Speakman et al., 2008; Wahlen et al., 2008; Zabena et al., 2009).

Common SNPs downstream of *TMEM18* represent the second best association signal next to the *FTO* locus for anthropometric measures of adiposity and this effect is already pronounced in childhood (Almen et al., 2010; Holzapfel et al., 2010; Rask-Andersen et al., 2011; Thorleifsson et al., 2009; Willer et al., 2009). No association between obesity risk alleles near *TMEM18* and T2D were observed in a GWA study by Thorleifsson and colleagues (Thorleifsson et al., 2009), while the GIANT consortium study showed positive correlation of this variation with the trait (Willer et al., 2009). Further investigations in populations of European origin showed that association with T2D disappeared after adjustment for BMI (Renstrom et al., 2009; Sandholt et al., 2011), but this effect was only partly attenuated in studies comprising Japanese and Danish subjects respectively (Takeuchi et al., 2011; Thomsen et al., 2012).

TMEM18 is coding for nuclear transmembrane protein which may play a role in regulation of neuronal stem cell mobility (Jurvansuu et al., 2008). TMEM18 mRNA has been detected in tissues throughout the body including central nervous system (Almen et al., 2010; Willer et al., 2009), but its functions in the organism are poorly characterized.

In the current study we investigated the impact of genetic variation in the loci overlapping *FTO* and *TMEM18* genes on the presence of T2D and to what extent this effect is BMI dependent in a population in Latvia. For this purpose we analysed polymorphism in close proximity to *TMEM18* (rs7561317) and four polymorphisms in *FTO*. In the *FTO* gene, besides the commonly replicated rs9939609, we selected two novel potentially causative variants rs11642015 and rs62048402 representing the first intron (Almen et al., 2012) and rs57103849 within the fourth intron previously associated with insulin resistance (Jacobsson et al., 2008b) as the information on association of these variants with phenotype is relatively scarce.

2. Materials and methods

2.1. Study population

Subjects included in the current association study were selected from the Latvian Genome Data Base (LGDB), a government funded biobank shortly described in (Ciganoka et al., 2011). Case and control groups were selected from 14,047 participants of LGDB recruited during the period from January 2003 to June 2010. 993 patients with T2D were included in the study sample after exclusion of all subjects lacking the data on gender, age, weight or height from in total of 1783 T2D cases (ICD-10 code E11) originally identified from the database. Availability of information on age at diagnosis of T2D was not used as an inclusion criterion for cases in order to retain larger case group. From 993 selected cases age at diagnosis of T2D has been reported by 741 subjects. Age at onset of T2D was not available for the current study, because collection of the data on age at onset of the disease is complicated by the lack of regular monitoring of glucose tolerance in the general population. The control group included 1080 subjects selected from 9795 participants who had not been diagnosed with T2D at the time of recruitment to the database and had complete data on above mentioned traits.

All participants gave signed informed consent and the protocol of the study was approved by the Central Medical Ethics Committee of Latvia (Protocols Nr. 24.05.2011. Nr.01-29.1/10 and 2007 A-7).

2.2. SNP selection and genotyping

For this study we selected SNP rs7561317 located 23 kb downstream of the *TMEM18* and four SNPs rs11642015, rs62048402, rs9939609 and rs57103849 in the *FTO* gene. The rs9939609 and rs7561317 were selected as the two most commonly adiposity associated signals related to T2D risk identified by GWA studies (Frayling et al., 2007; Willer et al., 2009). rs11642015 and rs62048402 were added to the study as these variants were recently found to have stronger association with obesity than previously described proximal SNPs in the first intron of *FTO* including rs9939609 (Almen et al., 2012). We included rs57103849 which is located in the fourth intron and lies outside the obesity associated cluster as this SNP was shown to correlate with insulin resistance independently of BMI (Jacobsson et al., 2008b).

Genotyping was performed using pre-designed TaqMan SNP genotyping assays for the rs11642015, rs9939609, rs7561317 with corresponding assay IDs: C_2031268_20, C_30090620_10, C_11804554_1 and custom designed assays for the rs62048402 and rs57103849 (Applied Biosystems, USA). Sequences of primers and probes for custom designed assays are available upon request. All genotyping reactions were carried out according to the protocol described elsewhere (Peculis et al., 2011). Genotyping entirely failed for six samples which were excluded from further analysis leaving the final sample of 2067 subjects.

In order to assess genotyping accuracy repeated analysis of SNPs under investigation following the same protocol was carried out for 95 randomly selected subjects (4.6%) included in the sample population.

2.3. Statistical methods

Statistical analysis was carried out using Plink (1.06) software (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007). Consistency of genotype distribution with the Hardy–Weinberg equilibrium was evaluated using exact test as given in Plink (Wigginton et al., 2005). Chi-square and Student's t-tests were performed to control for differences in phenotypes between case and control groups. In all calculations a p-value < 0.05 was considered significant. Logistic regression adjusted for gender, age and BMI assuming an additive, recessive and dominant mode of inheritance was used to assess association of SNP with T2D. BMI had non-normal distribution within the whole study population, therefore data were logarithmically transformed and obtained normally distributed values were included in the further analysis. Associations between polymorphisms and quantitative traits like BMI and age at T2D diagnosis were tested using linear regression. All three modes of inheritance were assumed and calculations were corrected correspondingly for gender, age and/or BMI. Following Plink guidelines haplotype specific conditional tests (given haplotype versus all other haplotypes) adjusted for gender, age and/or BMI were used to assess haplotype association with phenotype (Purcell et al., 2007). Considering the high linkage between the SNPs within the first intron of the *FTO* gene instead of Bonferroni correction Max(T) permutation test based on label-swapping specifying 100,000 permutations was performed for each analysis to estimate p-values corrected for multiple testing (p-perm) (EMP2 calculated considering the number of SNPs analysed) as implemented in Plink (Purcell et al., 2007).

In order to assess joint effects of rs7561317 and rs62048402 or rs7561317 and rs57103849 on the analysed traits number of risk alleles were summed for each subject. Logistic or linear regression adjusted for covariates as described before was used to test association between a trait and groups of subjects with two, three or four risk alleles. Regression analysis was performed with the statistical package SPSS (Standard version 13.0.0; SPSS, Chicago, IL, USA). Subjects having zero to one risk allele were considered as reference group. Those with missing genotypes for either of three examined SNPs were excluded from this analysis.

Power calculations were performed using Quanto v1.2.3 assuming additive mode of inheritance (available at http://hydra.usc.edu/gxe/) (Gauderman and Morrison, 2006). Significance level α was set at 0.05. Population risk for T2D was set at 0.05 and mean values for continuous traits were calculated in subjects included in the study. We used allele frequencies according to the 1000 genome project data on pilot 1 CEU population (Utah residents with ancestry from northern and western Europe) low coverage panel (Abecasis, 2010). LD plot representing linkage between variants within *FTO* was created using HaploView 4.2 (Barrett et al., 2005).

3. Results

We genotyped five SNPs rs7561317, rs11642015, rs62048402, rs9939609 and rs57103849 in the total sample of 2067 individuals composed from 987 patients with T2D and 1080 controls. Detailed description of subjects is given in Table 1. Briefly, the case and control groups comprised the same gender ratio, but the mean age and BMI differed significantly between the two groups. Proportion of subjects having family history of T2D was significantly higher among cases. The sample size was valid to detect significant associations between the SNPs in *FTO* and T2D with power ≥80% if odds ratios (OR) were 1.20 or higher and OR above 1.30 in the case of rs7561317 (Supplementary Fig. 1). For continuous traits the sample size provided 80% power to detect ≥0.0081 difference in logBMI and ≥1.66 in mean age at diagnosis between genotypes for the SNP with lowest frequency (Supplementary Table 1).

Variants within the first intron of the *FTO* were in strong linkage disequilibrium (LD) (pairwise $r^2 = 99.9$ between rs11642015 and rs62048402 and $r^2 = 0.93$ between the two SNPs and rs9939609), but not with the rs57103849 located in the fourth intron (pairwise $r^2 < 0.006$). Due to complete LD with rs62048402, results for rs11642015 are not further presented. Graphical representation of LD between SNPs located within *FTO* can be found in Supplementary Fig. 2. All SNPs followed the Hardy–Weinberg equilibrium and genotyping success rates exceeded 98%. Repeatedly analysed samples showed 100% genotype concordance for all SNPs. Information on analysed variants is summarised in Information on SNP is summarised in Supplementary Table 2.

Table 1
Characteristics of the subjects included in the study.

Characteristic	Patients with T2D	Controls	p-value[a]
Number of subjects	987	1080	–
Males/females n (%)	318/669 (32.2/67.8)	346/734 (32/68)	0.960
Mean age, years ± SD	60 ± 10.86	54 ± 12.96	<0.0001
Mean BMI, kg/m^2 ± SD	32.7 ± 6.79	27.2 ± 5.19	<0.0001
Mean age at diagnosis of T2D[b]	52 ± 11.04	–	–
Subjects with positive family history of T2D n (%)	321 (32.5)	79 (7.3)	<0.0001

Abbreviations: BMI — body mass index; T2D — type 2 diabetes; SD — standard deviation; n — number.
[a] Groups were compared using *t*-test.
[b] Information on the mean age at diagnosis of T2D was available for 738 cases.

Subjects carrying risk alleles of rs62048402, rs9939609 or rs7561317 had significantly higher BMI than non-carriers with unadjusted p-values 0.0036, 0.0069 and 0.015 respectively estimated in the whole study population assuming the additive model (respective p-values after 100,000 permutations (p-perm) 0.013, 0.024 and 0.05). Associations remained significant after adjustment for gender and age. In contrary, SNP rs57103849 showed no association with BMI (Supplementary Table 3).

Test results for the association of polymorphisms with T2D are shown in Table 2. All variants in *FTO* except rs57103849 also showed significant association with T2D producing unadjusted p-values 0.000087 for rs62048402 and 0.000075 for rs9939609 under the additive model. Inclusion of gender and age in the model did not significantly affect the observed results as well as permutation test adjusted for multiple comparisons. Adding BMI as a covariate resulted in slightly lower OR and raised p values, however this association still retained its significance. The rs7561317 gained marginally significant result after logistic regression was corrected for age and gender (unadjusted p = 0.11). However in this case adjustment for BMI removed the observed association. We found no relevant correlation between T2D and rs57103849 genotype.

Results of associations with age at diagnosis of T2D are summarised in Table 3. Risk allele carriers of SNPs previously correlated with T2D incidence and higher BMI tended to have T2D diagnosed earlier than non-carriers. Only association between SNP near *TMEM18* rs7561317 and age at diagnosis of the disease reached significance level under the additive model after correction for major covariates. The rs57103849 located in the fourth intron of *FTO* in contrast to rs62048402 and rs9939609 from the first intron was associated with younger age at diagnosis of T2D under the recessive model. Adjustment for the BMI had no influence on the relationship between neither rs7561317 nor rs57103849 and the trait. Association retained its significance after permutation test corrected for multiple comparisons.

While performing the haplotype based association including all SNPs in the *FTO* locus, we did not identify specific haplotypes that would display association effects stronger than identified by individual SNPs (data not shown).

We examined joint effects of variants representing *FTO* and *TMEM18* loci. As expected subjects with higher allelic score, taking into account rs62048402 and rs7561317, had increased T2D risk compared with those with no or only one risk allele. Subjects with four risk alleles had an OR almost twice (OR = 2.25, 95% CI 1.97–3.3) of that produced by the same SNPs individually (OR = 1.27, 95% CI 1.12–1.44, for rs62048402 and OR = 1.25, 95% CI 1.05–1.49, for rs7561317) (Fig. 1). Patients homozygous for the risk alleles of both rs7561317 and rs57103849 were diagnosed with diabetes almost six years earlier than subjects representing the reference group (Fig. 2). Weaker effects on the traits were observed for combination of rs62048402 and rs57103849 (Supplementary Table 4).

4. Discussion

In the current study we confirmed that SNPs in the obesity related loci comprising *FTO* and *TMEM18* genes are linked with higher T2D prevalence and younger age at the time of diagnosis of T2D in a Latvian population. We also show that association of these variants with T2D retained its significance after correction for BMI. Interestingly, rs57103849 found in the fourth intron of the *FTO* gene was associated with younger age at diagnosis of T2D while no correlation with T2D risk and BMI was observed.

Cluster of SNPs in the first intron of *FTO*, most commonly represented by rs9939609 and rs8050136, shows robust association with obesity related characteristics in Europeans (Dina et al., 2007; Do et al., 2008; Frayling et al., 2007; Jacobsson et al., 2008a; Renstrom et al., 2009; Thorleifsson et al., 2009; Wahlen et al., 2008; Willer et al., 2009; Wing et al., 2010). Recently a detailed study of *FTO*, using

Table 2
Association of polymorphisms near *TMEM18* and in *FTO* with type 2 diabetes.

Gene SNP		Patients with T2D		Controls		Unadjusted values		Adjusted for gender, age		Adjusted for gender, age, BMI	
		Genotype freq n(%)	RAF	Genotype freq n(%)	RAF	OR (95% CI)	p-value [p-perm]	OR (95% CI)	p-value [p-perm]	OR (95% CI)	p-value [p-perm]
TMEM18	GG	707 (72.1)	0.85	727 (68.1)	0.83	1.15	0.11	1.25	0.014	1.14	0.19
rs7561317	AG	251 (25.6)		318 (29.8)		(0.97–1.36)	[ns]	(1.047–1.49)	[0.045]	(0.94–1.38)	[ns]
	AA	23 (2.3)		22 (2.1)							
FTO 1st	TT	280 (28.4)	0.49	215 (19.9)	0.45	1.28	0.000087	1.27	0.00018	1.21	0.007
rs62048402	TC	457 (46.3)		548 (50.8)		(1.13–1.44)	[0.0003]	(1.12–1.44)	[0.00064]	(1.053–1.39)	[0.023]
	CC	250 (25.3)		316 (29.3)							
FTO 1st	AA	266 (27.3)	0.50	198 (18.4)	0.44	1.28	0.000075	1.27	0.00017	1.22	0.0053
rs9939609	AT	444 (45.6)		546 (50.8)		(1.13–1.45)	[0.00027]	(1.12–1.45)	[0.00056]	(1.06–1.40)	[0.018]
	TT	264 (27.1)		331 (30.8)							
FTO 4th	GG	91 (9.3)	0.32	118 (11.1)	0.34	0.93	0.29	0.96	0.56	0.96	0.64
rs57103849	AG	444 (45.4)		474 (44.8)		(0.82–1.062)	[ns]	(0.84–1.10)	[ns]	(0.83–1.12)	[ns]
	AA	444 (45.4)		467 (44.1)							

Abbreviations: T2D — type 2 diabetes; BMI — body mass index; freq — frequency; n — number; RAF — risk allele frequency; OR — odds ratio; CI — confidence interval; SD — standard deviation; p-perm — p-values derived performing permutation tests specifying 100,000 permutations and corrected for multiple testing (EMP2); ns — non-significant, used for clarity of presentation of p-perm in cases were original p value was already above 0.05. Underlining indicates homozygous risk genotypes. OR and p-values were calculated with logistic regression assuming an additive model.

massive parallel sequencing, has proposed a putative causative region represented by novel leading SNPs rs62048402 and rs11642015 within the same intron showing stronger association with obesity compared to rs9939609 (Almen et al., 2012). The association of these three SNPs with BMI is also replicated in our study group including the T2D patients.

We have found that alleles of the closely correlated rs62048402, rs11642015 and rs9939609, which are related to the increased BMI (Almen et al., 2012; Dina et al., 2007; Frayling et al., 2007; Renstrom et al., 2009; Wing et al., 2010), are also associated with higher risk of T2D even when adjusted for multiple testing. This association was only slightly attenuated and remained significant after controlling regression analysis with inclusion of BMI. Although this is in contrast with the majority of studies where adjustment for BMI completely abolished the observed association between SNPs within the first intronic region of the *FTO* and T2D (Andreasen et al., 2008; Frayling et al., 2007; Freathy et al., 2008; Meyre, 2012; Renstrom et al., 2009; Robiou-du-Pont et al., 2012) similar findings are reported by other authors in both European and Asian populations (Hertel et al., 2011; Legry et al., 2009; Li et al., 2012; Takeuchi et al., 2011).

Risk allele carriers of any of the three variants in the first intron of *FTO* gene tended to have younger age at diagnosis of T2D, but none of those associations reached significance. Still, the current study may be underpowered to detect weaker effects of the SNPs on the trait. On the contrary homozygotes of the rs57103849 risk allele located in the fourth intron of *FTO* were diagnosed with the disease on average four years earlier compared with heterozygotes and non-risk homozygotes, suggesting association with younger age at T2D diagnosis independent of BMI withstanding correction for multiple testing provided by Plink permutation test. This is in line with findings in Swedish adolescents where rs57103849 showed association with insulin levels and insulin resistance, which persisted after correction for BMI (Jacobsson et al., 2008b). Yet this SNP was not linked with the disease status itself in our study and there is no information on its association with T2D in other populations, although weak effects cannot be excluded. Jacobsson et al. found no association between rs57103849 and severe obesity in children. Still normal weighted risk allele carriers had slightly increased BMI (Jacobsson et al., 2008b). We observed no such trend in our study population including patients with T2D and diabetes free adults. This may be related to other findings demonstrating weaker obesity affecting effects of variation in *FTO* gene with increased age (Jacobsson et al., 2009; Qi et al., 2008).

The contribution of *FTO* to obesity has been linked to its role in the central regulation of metabolism (Cecil et al., 2008; Fredriksson et al., 2008; Speakman et al., 2008), but altered functions of the *FTO* gene in the periphery may propose valid mechanisms trough which it has impact on how independent the effect on T2D is towards obesity. Indeed increased expression of FTO has been shown to potentiate glucose stimulated insulin secretion in pancreatic beta cell line (Russell and Morgan, 2011). Protein and mRNA levels were found to be higher in skeletal muscle from patients with T2D compared to BMI-matched non-diabetic subjects and its overexpression led to defective energy turnover in these tissues (Bravard et al., 2010). Grunnet and colleagues observed enhanced mitochondrial oxidative phosphorylation in oxidative muscles, elevated fasting blood glucose and higher

Table 3
Association of polymorphisms near *TMEM18* and in *FTO* with the mean age at diagnosis of type 2 diabetes.

Gene SNP	Mean age at diagnosis ± SD			Unadjusted values	Adjusted for gender	Adjusted for gender, BMI
				p-value [p-perm]	p-value [p-perm]	p-value [p-perm]
TMEM18 rs7561317	GG (523)	AG (196)	AA (16)	0.0039 [0.013]^{Add}	0.0031 [0.01]^{Add}	0.0033 [0.011]^{Add}
	51.2 ± 10.9	53.7 ± 11.38	54.8 ± 8.20	0.0038 [0.013]^{Rec}	0.003 [0.0099]^{Rec}	0.0026 [0.0083]^{Rec}
FTO 1st rs62048402	TT (217)	TC (328)	CC (193)	0.06	0.089	0.19
	50.9 ± 11.84	52 ± 10.68	53 ± 10.67	[ns]	[ns]	[ns]
FTO 1st rs9939609	AA (207)	AT (323)	TT (198)	0.041	0.067	0.16
	50.7 ± 11.81	52.1 ± 10.74	52.9 ± 10.61	[0.13]	[ns]	[ns]
FTO 4th rs57103849	GG (67)	AG (330)	AA (336)	0.11 [ns]^{Add}	0.17 [ns]^{Add}	0.19 [ns]^{Add}
	48.1 ± 11.69	52.6 ± 10.49	52.1 ± 11.33	0.0027 [0.009]^{Rec}	0.005 [0.016]^{Rec}	0.0049 [0.016]^{Rec}

Abbreviations: SNP — single nucleotide polymorphism; SD — standard deviation; BMI — body mass index; Add — additive; Rec – recessive; p-perm — p-values derived performing permutation tests specifying 100,000 permutations and corrected for multiple testing (EMP2); ns — non-significant, used for clarity of presentation of p-perm in cases were original p value was already above 0.05. Table shows values calculated with linear regression assuming the additive model or recessive model were significance level was reached (indicated with Add or Rec). In total 738 cases having information on age at diagnosis of T2D were included in the analysis.

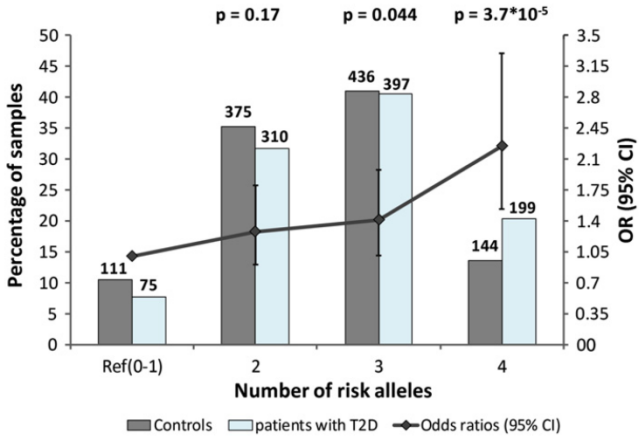

Fig. 1. Joint effects of rs62048402 in the first intron of *FTO* and rs7561317 near *TMEM18* on type 2 diabetes risk.

hepatic insulin resistance, defects characteristic to T2D, in homozygous carriers of rs9939609 minor allele and these correlations retained significance after correction for body fat percentage (Grunnet et al., 2009). Thus far functional studies involving rs9939609 have not revealed convincing mechanisms explaining how variation in the first intron of *FTO* influences functions of the gene on a molecular level, although impact on methylation rate and expression of the gene have been suggested (Bell et al., 2010; Berulava and Horsthemke, 2010). As the region containing risk variants is rich in regulatory elements, long-range impact on transcription of other genes, such as IRX3 involved in pancreatic function, cannot be excluded (Ragvin et al., 2010).

Besides the relationship of rs57103849 with insulin resistance (Jacobsson et al., 2008b), no other links with phenotypes have been found so far for this variant. Because this SNP is not in LD with the other *FTO* SNPs included in this study, which is in concordance with weak linkage observed between haploblocks comprising first and fourth intronic regions of *FTO* in the HapMap CEU population ($r^2 < 18$) (Rel#28; www.hapmap.org), and it showed different association pattern, this variant or tagged proxy may influence *FTO* functions only in the specific tissue or pathway in a different manner than the variation in the first intron of the gene. One of the possible explanations for this discrepancy could be a long range influence on the expression of other gene. Absence of the impact on the T2D risk may indicate necessity of coexistence of other genetic or environmental factors for the manifestation of rs57103849 effects on T2D onset. Likewise strong BMI associated signals in *FTO* distinct from those observed for cluster of SNP tagged by rs9939609 have been found in population of Sorbs in Germany within the third intron of the *FTO*, while another study revealed novel BMI associated polymorphisms in the first intron outside the traditionally replicated haplotype in Old Order Amish (Rampersaud et al., 2008; Tonjes et al., 2009). Furthermore the latter association was strongly augmented by physical inactivity (Rampersaud et al., 2008). Such interaction has been also observed for rs9939609 with diet and physical activity (Andreasen et al., 2008; Sonestedt et al., 2009). Although our results are line with these studies, suggestions towards the role rs57103849 or its proxy plays in context of T2D remain ambiguous until replicated in larger studies or supported by functional evidence.

In line with findings in other populations we also observed positive correlation between the major allele of rs7561317 near *TMEM18* and BMI (Thorleifsson et al., 2009; Willer et al., 2009). However, this variant had much weaker association with T2D risk compared to those from the *FTO* gene and it was lost after correction for BMI. SNP cluster in the *TMEM18* locus is less studied in context of T2D compared with variants found in *FTO*, but this relationship has also been reported to be most likely mediated by BMI (Renstrom et al., 2009; Sandholt et al., 2011), although contrary results exist (Thomsen et al., 2012).

Notably, those carrying protective allele of rs7561317 were diagnosed with the disease on average later than the mean age at T2D diagnosis of participants and this association persisted after adjustment for BMI and passed permutation test. One may speculate that polymorphisms near *TMEM18* may also contribute to the earlier onset of the disease through the mechanisms other than those leading to obesity on the genetic or environmental background already predisposing to T2D. However, more convincing evidence is needed to confirm the observed association.

TMEM18 is expressed in a wide range of tissues (Almen et al., 2010; Willer et al., 2009) and studies in murine models support its role in both regulation of higher cognitive functions as well as metabolism (Gutierrez-Aguilar et al., 2011; Rask-Andersen et al., 2011). Diet induced obesity in rats results in downregulation of TMEM18 expression in liver and skeletal muscle — tissues relevant in maintaining glucose homeostasis (Gutierrez-Aguilar et al., 2011). Recent studies provide evidence of its involvement in human adipogenesis (Bernhard et al., 2012). Nonetheless its functions in regard to metabolic disease are still under investigation.

We also examined the summary effects of risk allele number of variants representing *FTO* and *TMEM18* loci on studied traits. As expected rs62048402 and rs7561317 had in combination stronger impact on T2D risk than each SNP by itself, however the mean values for age at diagnosis remained within intervals provided by the strongest individual effect. Notwithstanding, subjects carrying all four risk alleles of rs57103849 and rs7561317 on average were more likely diagnosed with T2D at younger age than carriers of rs57103849 risk genotype thus showing that these SNPs may add to each other's effects (Table 3 and Fig. 2). Absence of substantial additive effects of rs62048402 and rs57103849 may further support their distinctive impact on the function of *FTO*, although this must be confirmed in a sample providing appropriate statistical power.

Yet our results are subject for discussion as the current study has several major limitations. Although we were able to replicate the association between variants in *FTO* and near *TMEM18* with BMI and T2D the sample size is too small to draw incontrovertible conclusions towards their impact on T2D. Larger sample including cases with information on age at diagnosis of T2D is also needed to confirm with certainty the observed impact of investigated variants on this trait. We used adjustment for BMI to evaluate obesity independent effects of SNPs representing two loci, yet this index is not the most reliable measure of body fat mass in a small study sample (Rothman, 2008). No information on BMI or age at the onset of the disease was available for diabetes patients in the case group. Nonetheless BMI in the patients with T2D may vary considerably due to antidiabetic treatment

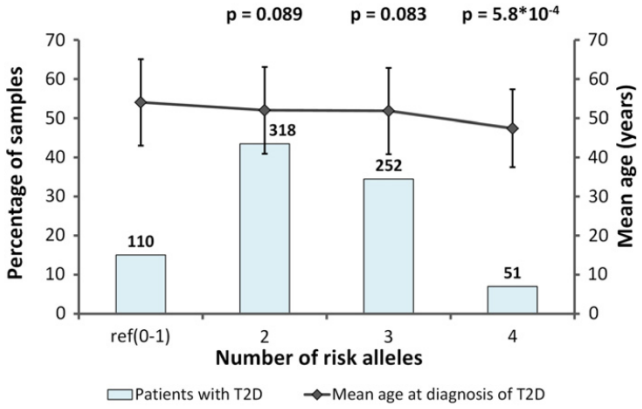

Fig. 2. Joint effects of rs57103849 in the fourth intron of *FTO* and rs7561317 near *TMEM18* on age at diagnosis of type 2 diabetes.

(Mitri and Hamdy, 2009), which may even further mask the real role of adiposity mediated effects. Besides, incorporation of data on age at onset of T2D may add information for estimation of impact by genetic variants on the earlier onset and severity of progress of the disease. One of the limitations of the study is lack of such metabolic traits like insulin resistance and glucose tolerance. Inclusion of larger number of healthy subjects with detailed metabolic profiles and more elaborate genetic analysis of the loci considering variants representing other independent association signals may clarify the impact on T2D of variants found within loci comprising *FTO* and *TMEM18* genes and possible molecular mechanisms underlying observed association.

In conclusion, this study supports that association of disease susceptibility variants in *FTO* and *TMEM18* loci with T2D may not only be a consequence of obesity, but may contribute to development of T2D through other pathways than those leading to increased BMI. Variants in the fourth intron of the *FTO* gene may represent alternative risk factors promoting earlier onset of T2D. However these findings have to be interpreted with caution before further confirmation.

Conflicts of interest

Authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.06.079.

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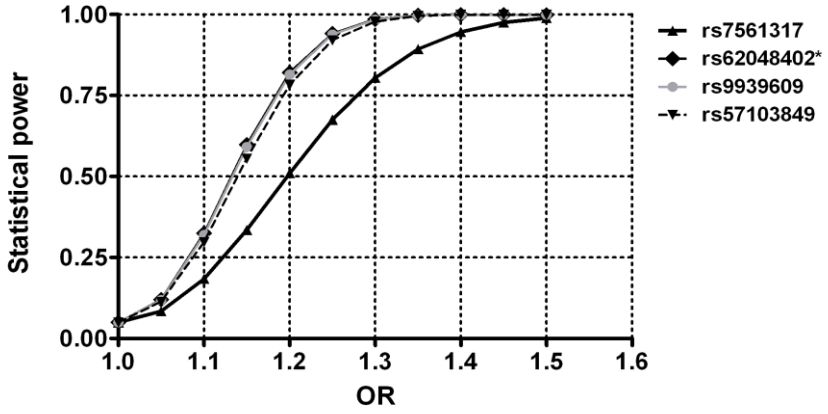
3.4 SUPPLEMENTARY DATA

Supplementary Figure 3.4.1 Graphical representation of statistical power to detect association between SNPs under investigation and type 2 diabetes.



*As rs62048402 and rs11642015 were in complete linkage only power in the case of rs62048402 is shown. In the power calculations we used allele frequencies derived from the 1000 genome project data on CEU population (Abecasis et al., 2010).

Supplementary Table 3.4.1 Differences between genotypes in continuous traits detectable with 80% power in the current study.

SNP	RAF	Mean age at diagnosis years ± SD	Difference in mean age at diagnosis	Mean logBMI ± SD	Difference in logBMI
rs7561317	0.85	52 ± 11.04 n = 738	≥ 2.25	1.46 ± 0.09 n = 2067	≥ 0.011
rs11642015	0.46		≥ 1.62		≥ 0.0079
rs62048402	0.46		≥ 1.62		≥ 0.0079
rs9939609	0.45		≥ 1.62		≥ 0.0079
rs57103849	0.38		≥ 1.66		≥ 0.0081

RAF – risk allele frequency; SD – standard deviation; logBMI – logarithmically transformed body mass index values; n – number of subjects used to calculate the mean values. In the power calculations allele frequencies derived from the 1000 genome project data on CEU population were used (Abecasis et al., 2010).

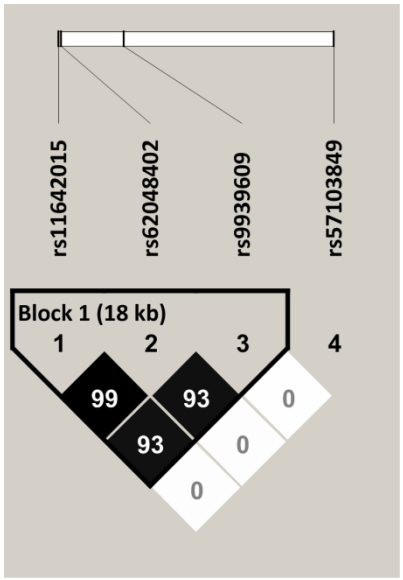
Supplementary Table 3.4.2 Description of genotyped polymorphisms.

SNP	Chr	Chr position[a]	Risk allele	Published RAF[a]	RAF	HWE test p-value	Genotyping success rate (%)
TMEM18 23kb downstream							
rs7561317	2	639894	G	0.85	0.84	0.22	99.08
FTO 1st intron							
rs11642015	16	39689324	T	0.46	0.48	0.20	99.71
rs62048402	16	39690053	T	0.46	0.48	0.24	99.95
rs9939609	16	39707357	A	0.45	0.47	0.18	99.13
FTO 4th intron							
rs57103849	16	39765365	G	0.38	0.33	0.34	98.60

Chr – chromosome; RAF – risk allele frequency; HWE – Hardy Weinberg equilibrium.

[a]SNP chromosomal location in base pairs and RAF according to the 1000 genome project data on pilot 1 CEU population low coverage panel (Abecasis et al., 2010).

Supplementary Figure 3.4.2 Linkage disequilibrium between SNPs within *FTO* gene. Each box contains pairwise r^2 coefficients.



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rs11642015	16	39689324	T	0.46	0.48	0.20	99.71
rs62048402	16	39690053	T	0.46	0.48	0.24	99.95
rs9939609	16	39707357	A	0.45	0.47	0.18	99.13
FTO 4th intron							
rs57103849	16	39765365	G	0.38	0.33	0.34	98.60

Chr – chromosome; RAF – risk allele frequency; HWE – Hardy Weinberg equilibrium.
[a]SNP chromosomal location in base pairs and RAF according to the 1000 genome project data on pilot 1 CEU population low coverage panel (Abecasis et al., 2010).

Supplementary Table 3.4.3 Association of polymorphisms near *TMEM18* and in *FTO* with body mass index.

SNP		Genotype freq n(%)	Mean BMI ± SD (kg/m^2)[a]	Mean logBMI ± SD [Mean BMI$^{b\text{-}transf}$]	Unadjusted values		Adjusted for gender, age	
					p-value	p- perm	p-value	p- perm
TMEM18 rs7561317	GG	1434 (70)	30.1 ± 6.53	1.47 ± 0.092 [29.4]	0.015	ns	0.0033	0.011
	AG	569 (27.8)	29.3 ± 6.80	1.46 ± 0.093 [28.6]				
	AA	45 (2.2)	29.5 ± 6.99	1.46 ± 0.097 [28.8]				
FTO 1st rs62048402	TT	495 (24.0)	30.5 ± 6.77	1.47 ± 0.094 [29.8]	0.0036	0.013	0.0053	0.017
	TC	1005 (48.6)	29.8 ± 6.69	1.46 ± 0.093 [29]				
	CC	566 (27.4)	29.3 ± 6.29	1.46 ± 0.09 [28.6]				
FTO 1st rs9939609	AA	464 (22.7)	30.5 ± 6.72	1.48 ± 0.094 [29.9]	0.0069	0.024	0.01	0.034
	AT	990 (48.3)	29.7 ± 6.79	1.46 ± 0.094 [29]				
	TT	595 (29.0)	29.4 ± 6.22	1.46 ± 0.089 [28.8]				
FTO 4th rs57103849	GG	209 (10.3)	29.7 ± 6.65	1.46 ± 0.095 [29]	0.46	ns	0.67	ns
	AG	918 (45.0)	29.7 ± 6.70	1.46 ± 0.093 [29]				
	AA	911 (44.7)	29. 9 ± 6.50	1.47 ± 0.091 [29.2]				

BMI – body mass index; logBMI - logarithmically transformed BMI values; freq - frequency; n- number; SD- standard deviation; p-perm – p - values derived performing permutation tests specifying 100000 permutations and corrected for multiple testing (EMP2); ns- non-significant, used for clarity of presentation of p-perm in cases were original p value was already above 0.05. Underlining indicates homozygous risk genotypes.
[a] mean BMI values before logarithmic transformation; [b-transf] BMI values back-transformed from the mean logBMI values. OR and p-values were calculated with linear assuming an additive model.

Supplementary Table 3.4.4 Combined effects of rs62048402 and rs57103849 on type 2 diabetes and age at diagnosis of type 2 diabetes.

Allelic score	Number	Type 2 diabetes			Age at diagnosis of T2D		
		Patients with T2D (%)	OR (CI95%)	p-values	Number	Mean age ± SD	p-value
Ref 0-1	954	428 (44.9)	-	-	318	53.1 ± 10.5	-
2	680	335 (49.3)	1.33 (1.082-1.63)	0.0067	263	51.5 ± 11.63	0.15
3	344	174 (50.6)	1.27 (0.98-1.65)	0.066	131	50.6 ± 10.95	0.038
4	59	29 (49.2)	1.35 (0.78-2.34)	0.28	21	48.9 ± 11.17	0.13

Ref 0-1 – reference group including subjects with none to one risk allele; T2D - type 2 diabetes; SD- standard deviation; OR – odds ratio; CI – confidence interval. OR and p-values were calculated with logistic or linear regression adjusted for age and/or gender.

4 DISCUSSION

The research carried out within the framework of the current thesis describes the contribution of genetic variation representing known susceptibility loci to the risk of major metabolic diseases like obesity and T2D in the population of Latvia. We have demonstrated a novel association between low frequency variant rs11575892 within the intronic region of *AGRP* gene with increased BMI and confirmed the link between higher risk of T2D and common variation within the *TCF7L2* gene and in or near obesity related genes *FTO* and *TMEM18*. On the contrary, common variants representing adiponectin gene locus showed no correlation with BMI or T2D, suggesting that variation within this locus probably has only marginal if any contribution to the variability of the traits under investigation.

The role of *AGRP* in the melanocortin system proposes it as a candidate gene implicated in the regulation of body weight (Rossi et al., 1998; Klovins et al., 2004; Stutz et al., 2005). However, so far correlation with phenotype has been described only for a few low frequency variants located within the *AGRP* gene locus (Mayfield et al., 2001; Argyropoulos et al., 2002; Marks et al., 2004; Bonilla et al., 2006; Loos et al., 2007). By performing direct sequencing of *AGRP* gene and its promoter region in 95 cases with severe obesity, we detected three polymorphisms from which rs5030980 in the third exon and intronic variant rs11575892 were selected for further investigation in a larger cohort. While we found significant association between BMI and intronic variant with previously unknown impact on phenotype rs11575892, no correlations shown in other populations were observed for rs5030980 (Argyropoulos et al., 2002; Marks et al., 2004; Li et al., 2013). According to data from HERITAGE family study the major G allele of the missense variant rs5030980 inducing change Ala67Thr was suggested to be a significant contributing factor to late onset obesity (Argyropoulos et al., 2002), whereas other studies have accented the role of minor allele in inheritance of leanness and *anorexia nervosa* (Vink et al., 2001; Marks et al., 2004). In contrast, in Dutch cohort directionally opposite although non-significant correlation was seen suggesting increased risk of being overweight for carriers of rs5030980 A(67Thr) allele , which is in agreement with our observations (van Rossum et al., 2006). Yet, due to the low frequency of the variant it was impossible to draw any valid conclusions in the moderate sample used in the current study. Similarly, despite the fact that association of rs11575892 with higher BMI retained its significance after correction for multiple testing and possible confounding factors, lack of homozygous carries of risk allele prevented evaluation of impact this polymorphism may exert on variation in BMI to full extent. Recently according to yet unpublished conference paper association of rs11575892 with BMI was described in individuals of west African descent, thereby supporting the link of rs11575892 with increased risk of being overweight (Shah, 2012). However, there was no further information available on direction of the association or possible effects in homozygous carriers, so it is hard to judge about the concordance between the two studies.

Considering that several of phenotype linked variants found within locus harbouring *AGRP* gene show population specific distribution (Mayfield et al., 2001; Argyropoulos et al., 2002; Marks et al., 2004; Bonilla et al., 2006; Sozen et al., 2007), discrepancy in LD pattern could be one of the factors negatively affecting success of attempts to replicate findings for phenotype associated variants in *AGRP* and its promoter region (Vink et al., 2001; Marks et al., 2004; de Rijke et al., 2005; van Rossum et al., 2006). This is likely true in the case of rs5030980 in *AGRP* gene, since evidence obtained thus far investigating possible role of rs5030980 does not support this missense variant as being functional (de Rijke et al., 2005). On the other hand, *in silico* analysis performed in

the study conducted by us suggests that rs11575892 located within second intron of *AGRP* may yield more active form of peptide through alternative splicing or result in increased expression by disrupting binding site of inhibitory microRNA (Place et al., 2008). Still these hypotheses remain speculative until functionally proved and it cannot be excluded that rs11575892 serves as a proxy to the functional variants within regulatory elements of the *AGRP*. However, no other candidates were found by direct sequencing of the gene and its proximal regulatory region within the current study.

At the same time, we failed to observe a significant association between common variants representing the locus comprising another candidate gene, *ADIPOQ*, and BMI or the disease status within our study group. Only rs2241766 known from previous studies to correlate with lower adiponectin blood levels and T2D showed tendency towards association with increased risk of T2D (Xita et al., 2005; Li et al., 2007b; Melistas et al., 2009). Correlation of serum adiponectin with body adiposity, insulin resistance and T2D is well established and variation within the *ADIPOQ* gene and its regulatory region has been previously linked to these traits (Weyer et al., 2001; Yang et al., 2001; Vasseur et al., 2002; Matsuzawa et al., 2003; Spranger et al., 2003; Vasseur et al., 2006; Li et al., 2007b; Menzaghi et al., 2007; Melistas et al., 2009; Tabak et al., 2009). Yet, there is perceptible inconsistency among studies investigating impact of genetic variants within the *ADIPOQ* locus and phenotype with several studies similarly showing negative results (Mackevics et al., 2006; Potapov et al., 2008; Szopa et al., 2009). Besides, recent large scale Mendelian randomization study did not support causal link between genetically determined decrease in adiponectin level and impaired insulin sensitivity or T2D (Yaghootkar et al., 2013).

Adiponectin levels are regulated by the complex interactions of multiple environmental and genetic factors that may further complicate detection of effects of single variants and introduce some inconsistency in replication attempts across different populations (Tabak et al., 2009; AlSaleh et al., 2011; An et al., 2012). As adiponectin was not measured for the participants of the current study, it was impossible to tell whether there was any variation in adiponectin levels within the sample population that could have had an impact on investigated traits and modify observed association. Furthermore, adiponectin decreasing alleles in other loci also contribute to the variability in BMI, T2D risk and related metabolic traits (Dastani et al., 2012). In contrast to the variation within *ADIPOQ* locus playing the dominant role in the regulation of circulating adiponectin level, common variant in ADP-ribosylation factor-like 15 (*ARL15*) gene associated with the concentration of this hormone has been shown to correlate with measures of insulin resistance in GWA studies. However, it is possible that this variant may have pleiotropic effects and this relationship is not entirely explained by decrease in adiponectin (Richards et al., 2009; Scott et al., 2012). It would be valuable to take into account possible impact of these as well as established common susceptibility variants for T2D and BMI to estimate more precisely the role of polymorphisms in or near *ADIPOQ* on the investigated traits. Since the samples used in the study were not available for additional genotyping of common variants representing *TCF7L2*, *FTO* and *TMEM18*, it is impossible to tell, whether there are any side effects that could mask association of SNPs within the *ADIPOQ* locus and until further verification of data these remain only speculations. On the other hand, high proportion of patients with metabolic and cardiovascular diseases included in our study supposedly displaying altered levels of the circulating adiponectin could have masked possible correlations by artificially removing variability observed in population. Some studies have shown more potent association with markers of insulin resistance than the disease status (Menzaghi et al., 2007; Dastani et al., 2012), and therefore inclusion of fraction of patients with unknown disease etiology may have further diluted possible association between analysed SNPs and T2D.

Several risk genes initially identified as positional or biological candidate genes have been afterwards also picked up by GWA analysis. Such susceptibility loci usually contain variants displaying effects strong enough to be captured by studies involving relatively small study groups and with the incidence rate allowing for detection by GWA studies. This category of factors forming genetic background of common complex diseases includes *TCF7L2* gene. The fine mapping of strong T2D susceptibility locus suggested by linkage analysis in Mexican and Icelandic populations revealed a microsatellite marker DG10S478 within the well-defined LD block of 92.1 kb overlapping third and fourth introns of *TCF7L2*, which harbours five SNPs (rs12255372, rs7903146, rs7901695, rs11196205 and rs789540) highly correlated with this marker showing strong association with T2D. The strength of the association for minor alleles of rs12255372 and rs7903146 was reported to be comparable with that of DG10S478, but rs11196205 and rs789540 clearly displayed lower signal (Grant et al., 2006).

In agreement with the initial findings later confirmed in further studies in European populations and other ethnic groups, investigation of impact of common variants within the third and fourth intronic regions of *TCF7L2* gene on T2D risk estimated that rs12255372 and rs7903146 have the strongest association with T2D risk followed by rs7901695 (Groves et al., 2006; Saxena et al., 2006; Cauchi et al., 2007; Guo et al., 2007; Cauchi et al., 2008a; Sanghera et al., 2008). When analysed separately in non-obese (BMI under 30kg/m^2) and obese subjects (BMI above 30kg/m^2), the three variants showed significant association among non-obese subjects, whereas only the strongest variant rs12255372 retained significance within obese group. This is in concordance with the previous findings showing heterogeneous rs7903146 genotype distribution between obese and non-obese cases with substantially higher risk of T2D among carriers of rs7903146 risk allele with normal weight (Helgason et al., 2007; Cauchi et al., 2008a; Cauchi et al., 2008b). It is very likely that the *TCF7L2* variants are responsible for some proportion of T2D cases among overweighed part of population represented in our study, but due to moderate sample size we were able to observe the effects only for the strongest SNP among obese subjects. At the same time rs11196205 showed no significant correlation with the disease status in either analysis. Similarly, no association for this SNP was observed in another study performed in population from northern Sweden comprising comparable sample size (Mayans et al., 2007), whereas the significance threshold was reached in the larger replication study (Saxena et al., 2006). Together it supports the insufficient statistical power for the particular SNP as the most plausible cause of negative findings in the whole sample. LD pattern in our sample was compatible with that given by other authors and in HapMap database for Caucasians (Rel#28; www.hapmap.org) (Grant et al., 2006; Mayans et al., 2007; Altshuler et al., 2010). Since the weaker link between the rs11196205 and T2D coincides with lower pairwise LD with other variants and LD between three SNPs associated with T2D was rather high, it is likely that association of this SNP with T2D in other studies is based on linkage with true causal variation rather than having any functional impact *per se*. This assumption was also supported by the results of haplotype tests (data not shown), where the risk allele of rs11196205 was present in five out of six analysed haplotypes showing both lower and higher frequencies among patients with T2D compared to controls. However, only one haplotype comprising risk alleles of rs7903146 and rs12255372 reached significance level, while frequencies and/or effects of other haplotypes most likely were beyond threshold of sufficient power in our study, which prevented the proper analysis of their impact.

Majority of loci identified in GWA studies are novel finds that have not been linked to the particular trait previously (Frayling et al., 2007; Scott et al., 2007; Voight et al., 2010; Morris et al., 2012; Scott et al., 2012). Among them the best association signals

provided by GWA analysis for BMI and risk of obesity are represented by clusters of common variants in high LD within the first intron of *FTO* gene and in close proximity to *TMEM18* gene (Thorleifsson et al., 2009; Willer et al., 2009). The association with increased BMI was replicated in our study group including patients with T2D for the most studied variants, rs7561317 near *TMEM18* and rs9939609 in *FTO*, as well as rs11642015 and rs62048402 recently proposed as representatives of potentially putative causative region tagged by SNPs from genome wide analysis (Almen et al., 2012).

Apart from the well established association with obesity and obesity related traits several studies have showed that BMI increasing alleles at the *FTO* locus also correlate with the higher risk of T2D (Frayling et al., 2007; Andreasen et al., 2008; Freathy et al., 2008; Meyre, 2012; Robiou-du-Pont et al., 2012). Yet, it is not clear to what extent observed correlation is attributable to secondary detrimental effects of obesity. Majority of the studies are in favour to consider these correlations to be secondary effects, since adjustment for BMI abolished observed associations (Frayling et al., 2007; Andreasen et al., 2008; Freathy et al., 2008; Renstrom et al., 2009; Meyre, 2012; Robiou-du-Pont et al., 2012), whereas findings reported by other authors in European and Asian populations suggest existence of BMI independent effects (Legry et al., 2009; Hertel et al., 2011; Takeuchi et al., 2011; Li et al., 2012). In the study described here the obesity related variants rs9939609, rs11642015 and rs62048402 were significantly associated with T2D. Although slightly attenuated, this association retained its significance after accounting for BMI in the regression analysis, thus providing further evidence that variation within this locus may contribute to development of T2D through mechanisms other than those mediated by increased adiposity. On the contrary, association of rs7561317 located near *TMEM18* gene with T2D disappeared after adjusting for BMI, which agrees with other reports supporting the BMI as the major mediator sustaining observed relationship (Renstrom et al., 2009; Sandholt et al., 2011). Still, there is contrary evidence suggesting that the impact of variation near *TMEM18* may be not completely attributable to confounding effect of increased BMI (Takeuchi et al., 2011; Thomsen et al., 2012). Probably variation near *TMEM18* displays weaker adiposity independent impact on T2D compared to variants found in *FTO*. Thus, it cannot be excluded that number of included subjects may have not provided enough power to detect BMI independent correlations. Since subjects from *TCF7L2* study partially overlapped with those from *FTO* and *TMEM18* study, it was possible to check for confounding effect of the strong susceptibility variants represented by rs12255372 on observed correlation with T2D. Inclusion of rs12255372 genotype among covariates in regression analysis introduced no significant changes in the association estimates for rs9939609, rs11642015, rs62048402 or rs7561317, indicating that observed correlation was not induced by random cosegregation with genotypes of rs12255372 (data not shown).

We further investigated possible impact of selected SNPs on the age of onset of T2D indirectly measured by age at diagnosis of the disease. In support of *FTO* and *TMEM18* being the risk genes for T2D, the rs57103849 located in the fourth intron and rs7561317 representing *TMEM18* were significantly associated with age at diagnosis of T2D and both associations persisted after correction for BMI. Of note, rs57103849 has not been linked to T2D, obesity or BMI within the current study or in other populations, although some tendency towards increased BMI has been previously observed among children (Jacobsson et al., 2008). Adjustment for genotype of rs12255372 had no effect on association results. Only non-significant tendency towards younger age at diagnosis of T2D was observed among the carriers of risk alleles of the three variants within the first intron of *FTO* gene. However, discrepancies in observed associations possibly would disappear, if larger number of subjects is included in the analysis.

The fact that rs57103849 may be related to younger onset of T2D independently of BMI was further supported in the smaller sample including 171 patients with T2D, where the SNP showed marginal association with younger age at the start of initial medical therapy. In contrast to the main sample, where the association was observed under recessive model, in the replication sample association between rs57103849 and the trait reached significance under additive model. Discordance in genetic models as well as the insufficient size of the replication sample should be taken into account when interpreting the data and before drawing any final conclusions these findings need further confirmation in an adequate study group (Table 4.1).

Carriers of minor non-risk allele of rs7561317 were diagnosed with the disease on average later than the mean age at T2D diagnosis of participants, suggesting possibly protective role against the development of T2D. The link between the rs7561317 and the trait was less persuasive compared to rs57103849, as the association failed to reach significance in the replication sample. Although the negative finding in this group most likely was connected with the small number of homozygous minor allele carriers and rs7561317 relationship with age at T2D diagnosis needs further investigation (Table 4.1).

Table 4.1 Relationship between polymorphisms near *TMEM18* and in *FTO* and age at initial medical therapy in the replication sample

Gene SNP	Mean age at initial medical therapy ± SD			Adjusted for gender	Adjusted for gender and BMI
				p-value [p-perm][a]	p-value [p-perm][a]
TMEM18 rs7561317	**GG (111)** 57.8 ± 10.83	**AG (54)** 60.2 ± 9.78	**AA (6)** 57.8 ± 15.68	0.25 [ns]	0.21 [ns]
FTO 1st rs62048402	**TT (51)** 57.5 ± 11.11	**TC (70)** 58.7 ± 10.93	**CC (50)** 59.4 ± 9.99	0.36 [ns]	0.61 [ns]
FTO 1st rs9939609	**AA (45)** 57.3 ± 11.5	**AT (70)** 58.3 ± 10.76	**TT (56)** 59.8 ± 9.94	0.28 [ns]	0.42 [ns]
FTO 4th rs57103849	**GG (20)** 55.9 ± 11.74	**AG (65)** 57.2 ± 12.26	**AA (86)** 60.2 ± 8.86	0.05 [ns]	0.043 [0.14]

[a] values derived performing permutation test specifying 100000 permutations (EMP2).Table shows values calculated with linear regression assuming an additive model.

Obesity status as an essential factor affecting risk of T2D development is well recognized variable modulating significance of association and possibly manifestation of disease susceptibility variants (Cauchi et al., 2008b; Voight et al., 2010; Manning et al., 2012; Scott et al., 2012). Thus, variants correlating with defects in beta cell functions and insulin secretion have been shown to account for larger proportion of non-obese T2D cases whereas variants acting on insulin resistance have appeared to be more important in obese population (Cauchi et al., 2008b; Voight et al., 2010; Manning et al., 2012; Scott et al., 2012). As an example of such segregation are SNPs found in *TCF7L2* locus displaying stronger impact on disease risk among normal weighted subjects compared to those with obesity. Transcription factor coded by the *TCF7L2* gene is involved in the regulation of beta cell turnover and insulin production (Lyssenko et al., 2007; Schinner et al., 2008; Shu et al., 2009). Furthermore, the risk allele of rs7903146 has been associated with decrease in beta cell mass and impaired insulin secretion (Damcott et al., 2006; Florez et al., 2006; Saxena et al., 2006; Lyssenko et al., 2007; Alibegovic et al., 2010; Le Bacquer et al., 2012; Faerch et al., 2013). The distribution of susceptibility variants according to their physiological role across the range of BMI is related to domination of different pathogenic mechanisms of disease development within non-obese and obese patients. In the non-obese

patients with T2D beta cell failure is the primary cause of the disease, since these patients usually are insulin sensitive (Alvarsson et al., 2005; Cauchi et al., 2008b). On the other hand, as pancreatic beta cells are highly adaptive, moderate deleterious effects of polymorphisms affecting insulin signalling such as SNP rs1801282 within *PPARg* in healthy subjects can be effectively compensated. In obese patients impact of such variants adds to the intense functional load caused by insulin resistance due to increased adiposity and triggers the disease (Del Prato et al., 2004; Jetton et al., 2005; Cauchi et al., 2008b).

Majority of known T2D susceptibility genes are related to beta cell functions and insulin secretion, that raises the question about the nature of factors forming the genetic component of insulin resistance (Stancakova et al., 2009; Dupuis et al., 2010; Voight et al., 2010; Watanabe, 2010; Morris et al., 2012; Scott et al., 2012). Given the role of obesity in the development of insulin resistance, it is tempting to speculate that some proportion of variability of this trait may be associated with variants within risk loci for obesity and obesity related traits. Combined analysis of several common obesity related variants showed positive association between these variants and increased predisposition to T2D, but these effects appeared to be solely mediated by the impact of these variants on the BMI (Li et al., 2011). Moreover, several loci correlating with insulin resistance, including those representing *GRB14*, *PPARG*, *IRS1* and *LYPLAL1* genes, also have been linked to BMI, measures related to fat distribution like WHR or detrimental changes in lipid profile (Beamer et al., 1998; Voight et al., 2010; Kilpelainen et al., 2011; Manning et al., 2012; Morris et al., 2012). Although these findings support the role of adiposity related variants in development of insulin resistance, as it could be expected due to tight physiological link between insulin resistance and obesity, this relationship is not necessarily as straightforward as initially thought. Thus, PPARg improves insulin sensitivity by stimulating expression of genes involved in transduction of insulin signal as well as indirectly via improving functions of adipocytes (He et al., 2003; Sugii et al., 2009; Monsalve et al., 2013), while variation within regulatory region of *IRS1* affecting its expression, apart from interfering with direct role of IRS1 in signal transduction downstream of insulin receptor, was also associated with higher circulating levels of triglycerides and decreased subcutaneous fat deposition that may promote ectopic fat accumulation and add to insulin resistance (Kilpelainen et al., 2011).

Similarly, FTO and TMEM18 may be engaged in more complex interplay with phenotype involving distinct mechanisms. While the contribution of FTO and TMEM18 to obesity has been linked to their role in the central regulation of metabolism including its cognitive aspects (Cecil et al., 2008; Fredriksson et al., 2008; Speakman et al., 2008; Gutierrez-Aguilar et al., 2011; Rask-Andersen et al., 2011), altered functions of the two genes in peripheral tissues may propose valid mechanisms explaining BMI independent effects on T2D. Overexpression of *FTO* has been shown to potentiate GSIS in pancreatic beta cell line (Russell and Morgan, 2011), however there is more evidence towards its modifying effects on insulin action rather than secretion. Increased expression of *FTO* as well as presence of risk genotype of rs9939609 have been linked to altered metabolism in skeletal muscle, hepatic insulin resistance and increased fasting blood glucose independently of body fatness (Grunnet et al., 2009; Bravard et al., 2010). Besides, the link between intronic variation within *FTO* gene and insulin action has also been suggested in GWA studies (Voight et al., 2010; Morris et al., 2012). Interestingly that, as already mentioned, rs57103849 located within the fourth intron of *FTO* gene also has been associated with fasting insulin levels and insulin resistance (Jacobsson et al., 2008).

Similarly to FTO, there is evidence towards possible functions of the TMEM18 in relation to insulin action in peripheral tissues relevant to maintaining glucose homeostasis. Thus, diet induced obesity in rats resulted in downregulation of *TMEM18* expression in

liver and skeletal muscle (Gutierrez-Aguilar et al., 2011), while recently evidence was provided towards its involvement in human adipogenesis (Bernhard et al., 2012). However, effects of variants representing *TMEM18* locus are less clear and in contrast to *FTO* locus none of variants near *TMEM18* have been detected among association signals for insulin related traits (Voight et al., 2010; Morris et al., 2012; Scott et al., 2012). It is possible that investigation in larger study groups than used to date will bring some consistency towards extent of BMI impact on association between T2D and *TMEM18* locus.

In addition, meta-analysis involving 34,195 cases and 89,178 controls of European and Asian descent recently confirmed BMI independent association of common obesity risk variants with T2D for represented by rs17782313 near *MC4R* (Xi et al., 2012). Like variants within *FTO*, SNPs near *MC4R* gene have been shown to correlate with impaired insulin action (Chambers et al., 2008; Morris et al., 2012). Interesting that rs5030981 (-38C>T) linked with higher promoter activity of the *AGRP* gene, coding for antagonist of MC4R, has been associated with T2D in Africans, though impact of BMI on the association was not evaluated (Mayfield et al., 2001). Theoretically, if presence of the risk allele at the *MC4R* gene locus causes aberrations in signal transduction mediated by the receptor, then variants interfering with functions of AGRP could possibly lead to T2D through the same pathway downstream of MC4R. However, association of SNPs representing *AGRP* locus with T2D was not considered in our study due to low number of included patients with T2D.

Together these findings contribute to increasing evidence indicating that obesity and T2D being linked on the physiological level may also have shared genetic component, particularly regarding genetic background of insulin resistance (Voight et al., 2010; Morris et al., 2012). Yet, majority of studies investigating the role of obesity related variants in the development of T2D use BMI as a measure of adiposity. Considering that BMI does not distinguish between lean and fat mass its usage may introduce bias inflating independency of effects from adiposity status. Furthermore, not always data on weight measures at onset or diagnosis of T2D are available and BMI included in the statistical analysis is calculated including weight measures at the time of recruitment like in our study aiming to investigate effects of variation in *FTO* and *TMEM18* loci. Since treatment of T2D is often linked with changes in BMI, using of post-onset or post-diagnosis measures could further contribute to erroneous estimation of impact of increased adiposity. Studies with moderate sample size are particularly prone to such bias. Thus, considering the major deleterious effects of obesity on insulin sensitivity along with drawbacks of methods used to evaluate body fatness, before drawing any conclusions this possible connection still needs further functional approval.

No doubt, GWA studies and investigation of information derived from such studies in the context of more specific phenotypes have immensely improved the knowledge about genetic background of complex diseases. In contrary to candidate gene studies, which have repeatedly demonstrated the link between variants representing *AGRP* locus and variability in BMI, no signals within this gene or its promoter have been detected by GWA studies for *anorexia nervosa* or obesity related traits (Nakabayashi et al., 2009; Wang et al., 2010). Another such example is the locus comprising nicotinamide phosphoribosyltransferase or visfatin (*NAMPT*) gene. Investigation of this locus using tagSNPs in French population revealed rare intronic variant rs10487818 with marked protective effect against obesity, which also does not appear among loci described by GWA studies (Blakemore et al., 2009; Thorleifsson et al., 2009; Willer et al., 2009; Wheeler et al., 2013).

GWA studies are based on the genotyping a large number of common SNPs selected as best markers being in LD with as large as possible number of variants across extent LD regions (Cleveland and Deeb, 2012). Identification of low frequency variants in

GWA study is possible if there is a strong LD between the variant and common representative SNP or effect of the functional variant is large enough to remain detectable even when artificially diluted by decrease in LD and increased difference between frequencies with the rare variant (Wray et al., 2011). If particular locus does not corresponds mentioned criteria and lacks suitable SNP markers, like *AGRP* harbouring low frequency variants with moderate impact, it is prone to be skipped by existing GWA studies due to insufficient statistical power. To gain more power constant increase of study samples is needed. Taking into account that recent meta-analysis already comprised more than 120,000 participants (Speliotes et al., 2010), these requirements could not always be fulfilled. It is possible that some association signals from low frequency variants can be still captured, but are dropped as they do not pass stringent significance thresholds set for genome wide analysis due to the high rate of multiple comparisons (Yang et al., 2011; Morris et al., 2012; Panagiotou and Ioannidis, 2012). These drawbacks are less stressing using candidate gene association analysis making this approach a more powerful tool for identification of weak associations (Wilkening et al., 2009; Holliday et al., 2013). Although having advantage in terms of statistical power favouring detection of signals missed by GWA studies, candidate gene studies are liable to type one error and it has been considered as the main source of discrepancy in risk loci yielded by the two approaches as well as replication problems in general. Candidate gene studies tend to include moderate number of subjects, which increases risk of errors due to sampling bias like unequal distribution of ancestral subgroups or samples collected following different protocols among cases and controls, confounding factors specific to particular population and genotyping errors. Besides, preference for positive finds and underreporting of negative ones can result in artificial loosening of correction for multiple testing in a study (Holliday et al., 2013). However, given that the replication requirements are fulfilled, positive findings within the current study further support candidate gene studies as a valid approach to reveal some proportion of the so called "missing heritability" especially when low frequency variants are involved.

In general, markers showing the strongest association within GWA studies have not been assumed as causal variants themselves, which leaves the question about the structure and properties of variation captured by these markers. One of the suggestions is that common variants selected as markers for genome wide association analysis reflect effects of rare causal variants (Dickson et al., 2010). Yet, as just discussed, common variants tend to be weak proxies to low frequency variants because differences in LD and frequencies would be expected to dissolve the observed statistical correlation with the trait for the high frequency variant. To explain association signals captured by common markers the causal low frequency or rare variant should have a strong individual effect or there should be strong enough combined impact from multiple rare variants, which would make such locus already detectable by linkage studies (Wray et al., 2011). However, majority of top signals from GWA studies represent novel loci and only the strongest region detected by GWA for T2D representing *TCF7L2* was initially described by linkage study (Grant et al., 2006).

Since population specific variation in LD and variant frequencies are likely to induce variability in observed effects (Wray et al., 2011), rare causal variants as an explanation of association of leading SNPs from GWA studies also seems to contradict with permanence of observed effects of marker polymorphisms across distinct populations (Waters et al., 2010; Marigorta and Navarro, 2013). This is also true for common variants within the regions of *FTO*, *TMEM18* and *TCF7L2* genes displaying comparable effects in populations of European, African and Asian descent (Cauchi et al., 2007; Renstrom et al., 2009; Palmer et al., 2010; Waters et al., 2010; Hertel et al., 2011; Takeuchi et al., 2011; Li et al., 2012; Meyre, 2012). In particular it applies to risk SNPs representing *TCF7L2*.

Despite the much lower frequency of risk allele of rs7903146 within *TCF7L2* among Asians the magnitude of detected effects was close to that observed in Europeans (Miyake et al., 2008; Takeuchi et al., 2009; Dou et al., 2013). So far no rare functional variants linked to common forms of obesity or T2D have been found by sequencing coding parts of *TCF7L2*, *FTO* and *TMEM18* or regions covering whole genes (Meyre et al., 2010; Palmer et al., 2010; Almen et al., 2012; Deliard et al., 2013). However, it cannot be excluded that in some cases of extensive LD the low frequency causal variant may be located outside the screened region (Dickson et al., 2010). For example, two association signals for height rs1814175 and rs5017948 located approximately 1,76Mb initially regarded as independent later appeared to be proxies in high LD (Yang et al., 2013). The listed evidence argues that associations with phenotype exceeding stringent thresholds set for statistical significance in GWA studies are predominantly induced by lead polymorphisms or tagged variants falling within the same frequency range.

This hypothesis is supported by data recently described by The Encyclopedia of DNA elements (ENCODE) project. ENCODE data revealed remarkable overlap between regions harbouring lead SNPs highlighted by GWA analysis and non-coding functional elements such as enhancers and sites with promoter like features. Besides, the approach used to annotate non-coding functional elements indicates that if the lead SNPs are not functional themselves causal variants should be located in a very close proximity (The ENCODE Project Consortium, 2012). Similarly to intronic variation within *TCF7L2* (Gaulton et al., 2010; Savic et al., 2012), susceptibility variants of obesity and T2D found within the first intron of *FTO* gene and near *TMEM18* gene have been suggested to affect gene expression (Bell et al., 2010; Berulava and Horsthemke, 2010; Almen et al., 2012; Gong et al., 2013). The rs7561317 unlikely represents functional variant in European populations, nevertheless this variant is almost in complete LD with the lead SNPs with similar frequency from association conducted in population of African descent. The novel leading variant lies within enhancer probably interacting with the promoter of *TMEM18* gene (Gong et al., 2013). The rs9930609 has been linked to changes in methylation rate (Bell et al., 2010), whereas rs62048402 also locates to the enhancer binding site (Almen et al., 2012). Although allele specific transcription of *FTO* has been actually shown for rs9930609 in blood cells, another possibility is that these variants may exert regulatory effects on other distant genes like *IRX3*, which is involved in pancreatic function (Ragvin et al., 2010). ENCODE project has described tissue specific long range interactions spanning over distances up to several megabases as a relatively common phenomenon involving hundreds of genes (The ENCODE Project Consortium, 2012). Tissue specificity of such interaction at least to some degree could explain independent impact of variation within *FTO* and *TMEM18* regions on obesity and T2D.

In summary, it seems that rare variants are more likely to be responsible for separate associations observed for common variants rather than form the major class of genetic risk factors explaining associations detected using high frequency markers.

The fine mapping of risk loci and deciphering the real causal variant among numerous candidates in high LD in majority of cases has proved thus far to be a challenge. Moreover, an assumption that among highly correlated variants within associated loci link with the trait is provided by single causal variant is not generally applicable. More detailed conditional analysis of associated loci has revealed several susceptibility regions containing correlated variants representing independent impact that merges under single association signal (Browning and Browning, 2008; Voight et al., 2010; Yang et al., 2013). For T2D probably the best example is *CDKN2A/B* locus, within which existence of secondary signal is supported by haplotype analysis yielding stronger association than that observed for single markers (Browning and Browning, 2008; Voight et al., 2010).

Both *TCF7L2* variants underlying best association signals rs12255372 and rs7903146 were in strong LD, therefore complicating recognition of potential causal variant or its closest proxy solely based on the data obtained in our study. However, more detailed dissection of the risk locus overlapping *TCF7L2* gene has failed to bring forward other potentially functional variants and these studies have led to the conclusion that rs7903146 is the most plausible functional variant underlying association signal (Grant et al., 2006; Helgason et al., 2007; Ren et al., 2008; Palmer et al., 2010). Evidence towards rs7903146 being a causal variant is further supported by functional studies, where the risk allele T was shown to alter enhancer activity within the *TCF7L2* locus harbouring common risk variants (Cauchi et al., 2008b; Gaulton et al., 2010). The situation is more complicated with SNPs forming haploblock covering part of the first intron of *FTO*, hence there is evidence, although not incontestable, offering functional explanation of association with phenotype for both traditionally studied rs9930609 and rs62048402 as novel representative of the particular putative causal site (Bell et al., 2010; Almen et al., 2012). Recently performed fine mapping of the region harbouring *FTO* gene in American Africans discarded rs9930609 as a causal candidate, but concurrently brought forward novel variant rs56137030 within the same susceptibility cluster as the strongest association signal (Gong et al., 2013; Peters et al., 2013). The rs56137030 was found to be in strong LD with several important candidates for causal variants in particular rs1421085, which locates to conservative binding site of transcription factor cut-like homeobox 1 (*CUX1*) (Peters et al., 2013). Conditional analysis conducted within the same study failed to nominate a single causal variant (Peters et al., 2013). Thus, despite the fact that in accordance with data from our study group haplotype analysis did not yield stronger results than observed for individual SNPs and this region was not among those defined as harbouring multiple association signals (Browning and Browning, 2008; Voight et al., 2010; Yang et al., 2013), the risk locus within the first intron of *FTO* gene may harbour several functional variants influencing investigated traits.

To even further complicate deciphering of the genetic background underlying multifactorial diseases such as obesity and T2D, several candidate genes revealed by GWA studies have been suggested to contain secondary association signals distinct from major susceptibility loci (Voight et al., 2010; Morris et al., 2012; Yang et al., 2013). Such variants may be missed by large scale studies, which traditionally are concentrated on the strongest signals from particular region. The rs57103849 within the fourth intron of *FTO* associated with the age of diagnosis of T2D according to our data may represent independent risk factor for the development of the disease. The variant was not in LD with the risk locus within the first intron of *FTO* and it showed different association pattern. Additional association signals independent from locus tagged by rs9939609 for BMI have been described within the first intron and the third intron of the gene in Old Order Amish and population of Sorbs in Germany (Rampersaud et al., 2008; Tonjes et al., 2009). Effect of the variant described in Old Order Amish was strongly augmented in presence of low physical activity (Rampersaud et al., 2008). Further support towards the significance of lifestyle factors in modulation of associations observed for variation in *FTO* comes from studies showing interaction of rs9939609 with diet and physical activity (Andreasen et al., 2008; Sonestedt et al., 2009). Considering that rs57103849 was not associated with disease status it is possible that its impact on phenotype becomes apparent in presence of specific genetic or lifestyle factors. Prospective study where it could be possible to detect first pathogenic changes, to fix the age at onset and to collect long term data on variability in weight and lifestyle could be more informative for evaluation of impact of this SNP on pathogenesis of T2D and mechanisms underlying phenotypic manifestation of the SNP. However, associations falling within this category are not always successfully replicated.

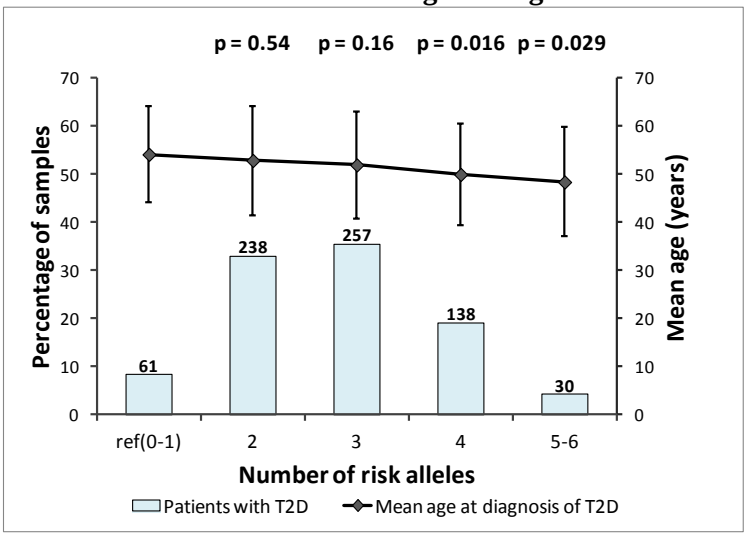
Given that errors in study design are maximally avoided, replication failures may be explained by population specific effects, low power of studies to detect weaker secondary signals or presence of specific additional factors necessary for manifestation of effects of the variant. These may not always been accounted in genome wide association scans including thousands of participants (Wilkening et al., 2009; Yang et al., 2013). Current findings clearly show that further studies of candidate genes pointed out by GWA studies within the well characterized cohorts may significantly contribute to the dissection of genetic background of common traits. Yet, some of these associations still may be false positive findings and without further confirmation must be interpreted with caution.

Although it has been demonstrated that higher genetic risk score is associated with increased susceptibility to T2D, in clinical practice models including conventional risk factors such as gender, age, adiposity and lifestyle factors have much higher predictive value of developing the disease (Weedon et al., 2006; Talmud et al., 2009). Low predictive value of genetic score based on common variants may be at least partly linked to incompleteness of the set of genetic factors included in the analysis due to limited information on genetic background of the disease and large proportion of carriers of medium number of risk alleles in the population (Weedon et al., 2006; Talmud et al., 2009). Nevertheless, combined SNP analysis may give new insights about interrelation among different genetic factors.

Subsequently besides the single SNP analysis we examined joint effects of investigated variants. As expected, correlation with T2D became stronger among participants carrying increasing numbers of risk alleles in an additive manner. Thus subjects carrying four risk alleles, considering rs62048402 in *FTO* and rs7561317 near *TMEM18*, displayed OR 2.25 (95% CI 1.97 – 3.3), whereas, after inclusion of rs12255372 representing *TCF7L2* gene, OR reached 3.49 (95% CI 2.07 – 5.86) among carriers of five to six risk alleles. Increase of susceptibility to T2D among carriers of risk alleles of variants in *TCF7L2, FTO* and *TMEM18* could have been anticipated since these variants have been suggested to influence both of the major pathogenic mechanisms underlying the development of T2D. Risk alleles of two SNPs rs57103849 and rs7561317 in combination lowered age at diagnosis beyond mean values observed, when analysing each SNP separately. Association with younger age at diagnosis of T2D has been previously described for minor alleles at well established risk locus within intronic region of *TCF7L2* (Silbernagel et al., 2011; Tangjittipokin et al., 2012). Although there was some tendency towards correlation between presence of risk allele at rs12222372 and earlier diagnosis of the disease, among participants included in our study this association did not reach significance (data not shown). Addition of rs12255372 in calculation of allelic score along with rs57103849 and rs7561317 had no further impact on the trait (mean age at diagnosis among carriers of five to six risk alleles was 48.4 (SD ± 11.38)) (Chapter 3.4 Figure 2; Figure 4.1). However, it cannot be excluded that significance of summary effects of the risk alleles would have been more pronounced in a larger sample.

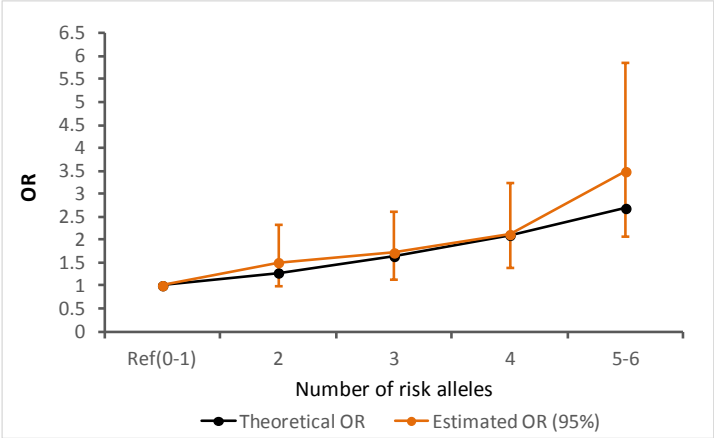
Although the current study had low power for evaluation of possible interactions between the investigated risk loci, these findings along with no substantial effects on estimations after adjustment for rs12255372 incline to support existence of joint additive effects rather than interaction (p = 0,125; Figure 4.2). Suggestion towards variants representing loci overlapping the first intron of *FTO*, *TCF7L2*, and *TMEM18* as being independent risk factors for development of T2D is in concordance with impact on regulation of different physiological mechanisms underlying the disease suggested for genes overlapping with regions harbouring risk variants. Thus *TCF7L2* is involved in the regulation of beta cell functions while *FTO* and *TMEM18* more likely interfere with insulin signalling (Lyssenko et al., 2007; Grunnet et al., 2009).

Figure 4.1 Joint effects of rs12255372 in *TCF7L2*, rs57103849 in the fourth intron of *FTO* and rs7561317 near *TMEM18* on age at diagnosis of T2D



Crosslink between pathways involving proteins coded by *FTO*, *TMEM18* and *TCF7L2* cannot be excluded as many aspects of their functions still remain obscure. Moreover, considering the low LD between rs57103849 and rs62048402, absence of substantial deviation of summary impact of the two SNPs on age at diagnosis of T2D from stronger effects estimated for rs57103849 as well as no significant changes observed after adjustment of analysis for rs62048402 (p = 0.0059 obtained from linear regression analysis adjusted for gender, BMI and rs60248402; Chapter 3.4, Table 3) may further indicate towards distinct role of variants representing different haploblocks within the region of *FTO* gene. However, these findings need further confirmation in a sample with larger statistical power for performance of this kind of analysis.

Figure 4.2 Comparison of theoretically estimated OR and OR calculated within the study sample supporting additive joint effects on T2D of variants representing *TCF7L2*, *FTO* and *TMEM18*.



In summary, studies included in the doctoral thesis are in line with general conclusions that may be drawn from genetic research of complex traits. Although common variants with moderate effects predominantly on gene expression may be responsible for the largest proportion of genetic component of traits like obesity and T2D, impact of low frequency variants like those described within *AGRP* gene likely displaying stronger impact on protein functions must not be underestimated. Furthermore, susceptibility loci discovered by GWA studies like those within proximity of *TMEM18* and in *FTO* gene may

contain variants with pleiotropic effects that may provide genetic link between T2D, particularly, insulin resistance and obesity. Thus it is clear that detailed analysis of biological candidate genes as well as those represented by susceptibility loci discovered in GWA studies may be a valuable approach to reveal some proportion of „missing heritability" and describe new aspects towards the mechanisms linking genetic variation affecting involved genes with development of particular phenotypes.

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CONCLUSIONS

1. *AGRP* gene intronic variant rs11575892 in heterozygous state is associated with increased body mass index and this association may be explained by its functional impact on alternative splicing and expression of the gene.
2. Polymorphisms found in the adiponectin gene do not significantly correlate with higher risk of type 2 *diabetes mellitus* or increased body mass index in the study population.
3. Minor allele of rs182052 within the adiponectin gene may protect against myocardial infarction, while haplotype determined by C allele of rs1501299, A allele of rs3774261 and common alleles of other polymorphisms associates with atrial fibrillation.
4. Common variants in the third intron of the *TCF7L2* show significant association with type 2 *diabetes mellitus* with stronger effects observed among subjects with body mass index under 30kg/m^2.
5. Obesity risk polymorphisms representing *FTO* and *TMEM18* loci are associated with higher body mass index, risk of type 2 *diabetes mellitus* and younger age at the time of diagnosis of type 2 *diabetes mellitus* in the population of Latvia. Observed impact on type 2 *diabetes mellitus* and age at diagnosis of the disease appears to be at least partly independent from obesity mediated effects.
6. Molecular mechanisms explaining the link between variation within the fourth intron of the *FTO* gene and development of type 2 *diabetes mellitus* may differ from those by which polymorphisms in the first intron of the same gene increase the risk of the disease.

MAIN THESIS FOR DEFENCE

1. Variation in *AGRP* gene contributes to human obesity.
2. Genotype at the adiponectin locus is unlikely to be significant determinant of type 2 *diabetes mellitus* risk or body mass index, but is modifying susceptibility to coronary heart disease in the study population.
3. *TCF7L2* is an important susceptibility gene for type 2 *diabetes mellitus* among non-obese subjects.
4. Body mass index is not the major factor mediating observed impact of obesity risk variants found in *FTO* and *TMEM18* loci on type 2 *diabetes mellitus*.

LIST OF ORIGINAL PUBLICATIONS

1. **Kalnina I**, Kapa I, Pirags V, Ignatovica V, Schioth HB & Klovins J. Association between a rare SNP in the second intron of human Agouti related protein gene and increased BMI. BMC Medical Genetics, 2009; 10:63.
2. **Kalniņa I**, Latkovskis G, Ņikitina-Zaķe L, Mackevičs V, Pečulis R, Kapa I, Fridmanis D, Ērglis A, Pīrags V, Klovins J, Analysis of Polymorphisms at the Adiponectin Gene Locus in Association with Type 2 Diabetes, Body Mass Index and Cardiovascular Traits in Latvian Population. Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences, 2009; 63, No 4-5, pp 174-179.
3. **Kalnina I**, Geldnere K, Tarasova L, Nikitina-Zake L, Peculis R, Fridmanis D, Pirags V, Klovins J. Stronger Association of Common Variants in TCF7L2 Gene with Nonobese Type 2 Diabetes in the Latvian Population. Exp Clin Endocrinol Diabetes 2012; 120(8):466-8.
4. **Kalnina I**, Tarasova L, Vaivade I, Ignatovica V, Nikitina-Zake L, Peculis R, Fridmanis D, Geldnere K, Jacobsson JA, Almen MS, Pirags V, Schiöth HB, Klovins J. Polymorphisms in *FTO* and near *TMEM18* associate with type two diabetes and predispose to younger age at diagnosis of diabetes. Gene, 2013:462-468.

APPROBATION OF THE RESEARCH, PUBLISHED THESIS

1. **I.Kalnina,** L. Tarasova, I. Vaivade, V. Ignatovica, J. A. Jacobsson, M. S. Almen, H. B. Schiöth, V. Pirags, J. Klovins; Association of polymorphisms representing obesity-related loci comprising FTO and TMEM18 with type 2 diabetes and age at diagnosis of the disease in the population of Latvia. European Human Genetics Conference Nürnberg, Germany, June 23-26, 2012. Eur J Hum Genet Vol 20, Supp 1, 2012 June 1, p247, P09.136.
2. **I.Kalnina**, L.Tarasova, I.Vaivade, V.Pirags, J.A. Jacobsson, M.S. Almen, H.B. Schiöth, J.Klovins. Common Variants in FTO and TMEM18 Loci Associate with Type 2 Diabetes and BMI in the Population of Latvia. International Congress on Personalized Medicine: Up Close and Personalized, Florence, Italy, February 2-5, 2012. Abstract book, 2012, p83.
3. J.Klovins, **I.Kalnina**, L.Tarasova, K.Geltnere, V.Pirags. Replication study of polymorphisms associated with type 2 diabetes in Latvian population. Genomic Disorders, Wellcome Trust Conference, Hinxton, UK, March 24-27, 2010. Abstract book 2010, P55
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5. **I.Kalniņa**, L.Tarasova, V.Pīrāgs, J.Kloviņš. Characterisation of type two diabetes susceptibility variants in the population of Latvia. 68th Scientific Conference of the University of Latvia, Riga, 2010, January-May.

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2001 - 2007 Latvian Biomedical Research and Study Centre - laboratory technician;

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2009 - 2013 Latvian Scientific council project No 10.0010 „Genetic investigation of disease aetiology, pathogenesis and ageing processes in the Latvian population", Type 2 diabetes research project No 10.0010.04.

2013-till now Latvian Scientific council project No. 343 „Identification of various pathogenic mechanisms of type 2 diabetes development using patient specific cell models".

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