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Dorota Kamińska

Regulation of Alternative Splicing in Obesity and Weight Loss

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ABSTRACT

The prevalence of obesity and type 2 diabetes (T2DM) is increasing worldwide, making it one of the biggest health problems currently facing both developed and developing countries. The interactions between environmental signals and genes in the development of obesity and T2DM are poorly understood.

Alternative splicing can result in the generation of more than one protein from a single gene or in the complete switch off of the protein. Many different human diseases can be caused by impairment of alternative splicing or its regulation, resulting from genetic variations and environmental signals.

The main aim of the present study was to investigate interactions between alternative splicing of the genes and obesity. We aimed to investigate whether the expression profile of splice variants in human adipose tissue differs depending on fat depots and whether the splicing pattern significantly changes in response to weight loss.

The first study focused on the regulation of alternative splicing of candidate gene, *TCF7L2*, in obesity and weight loss. The distribution of *TCF7L2* splice variants was determined in subcutaneous and visceral adipose tissue and the livers of obese individuals. Furthermore, we demonstrated that weight loss regulates alternative splicing of *TCF7L2* in both subcutaneous adipose tissue and liver. We found that adipose tissue *TCF7L2* splicing was strongly associated with FFAs levels, suggesting an effect on insulin action in adipose tissue.

The second study investigated the relationship between splicing of insulin receptor gene (*ISNR*) and obesity or T2DM. Our results showed regulation of *INSR* splicing by weight loss in subcutaneous fat. Additionally, we found a strong correlation between splice variants and insulin levels in both fat depots.

In the third study we identified alternatively spliced genes in 11 obesity loci. We demonstrated that weight loss induced regulation of *MSH5* gene and that splicing of *BAT6* and *TRA2B* is fat depot dependent. Statistical analyses revealed that BMI is a main determinant of *TRA2B*, *BAG6* and *MSH5* splicing in subcutaneous fat.

The forth publication included in this thesis is a review of literature with respect to the role of alternative splicing in obesity and weight loss.

This thesis provides new information on the role of alternative splicing in pathogenesis of obesity and T2DM. These findings increase our understanding of splicing dysregulations and can improve the chances for a more targeted treatment and more accurate diagnostics.

National Library of Medicine Classification: QT 235, QU 58.7, QU 475, WD 210
Medical Subject Headings: Genes; Alternative Splicing; Gene Expression Regulation; Obesity; Overweight; Weight Loss; Adipose Tissue; Liver; Transcription Factor 7-Like 2 Protein; Receptor, Insulin; RNA-Binding Proteins; Insulin; Insulin Resistance; Diabetes Mellitus, Type 2; Glucose/metabolism; Fatty Acids/metabolism

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TIIVISTELMÄ

Lihavuuden ja tyypin 2 diabeteksen yleisyys lisääntyy maailmanlaajuisesti tehden niistä suurimman kansanterveysongelman, joka koskettaa sekä teollisuus- että kehitysmaita. Tyypin 2 diabetes ja lihavuus voivat molemmat kehittyä itsenäisesti johtuen geneettisten ja ympäristötekijöiden vaikutuksesta, mutta tästä yhteydestä on vain vähän tietoa.

Yhden geenin vaihtoehtoinen silmukointi voi johtaa useamman kuin yhden proteiinin muodostumiseen tai täydelliseen proteiinin toiminnan loppumiseen. Geenin silmukointiprofiili saattaa muuttua tapauksesta ja ympäristöstä riippuen. Ihmisillä monet sairaudet saattavat johtua vaihtoehtoisen silmukoinnin viasta tai sen säätelystä, geneettisistä variaatioista ja ympäristötekijöistä johtuen.

Tämän väitöskirjatyön kokonaistavoite oli tutkia geenien vaihtoehtoisen silmukoinnin ja lihavuuden vuorovaikutusta. Tavoitteenamme oli selvittää muuttuuko transkriptivarianttien ilmentymisprofiili ihmisen rasvakudoksessa riippuen rasvan sijaintikohdasta ja muuttuuko silmukointimalli laihdutuksen vaikutuksesta.

Ensimmäisessä osajulkaisussa keskityttiin kandidaattigeenin, *TCF7L2*, vaihtoehtoisen silmukoinnin säätelyyn lihavuudessa ja laihdutuksessa. *TCF7L2*-transkriptivarianttien jakautuminen määritettiin lihavien yksilöiden ihonalaisesta ja viskeraalisesta rasvakudoksesta ja maksasta. Tutkimuksessa osoitimme, että laihtuminen säätelee *TCF7L2*:n vaihtoehtoista silmukointia ihonalaisessa rasvakudoksessa ja maksassa. Totesimme, että rasvakudoksen *TCF7L2*:n silmukointi oli vahvasti yhteydessä vapaiden rasvahappojen pitoisuuksiin vihjaten vaikutuksesta insuliinin toimintaan rasvakudoksessa.

Toisessa osajulkaisussa tutkittiin insuliinireseptorigeenin, *ISNR,* vaihtoehtoisen silmukoinnin, lihavuuden ja tyypin 2 diabeteksen välistä yhteyttä. Tuloksemme osoittivat kuitenkin laihdutuksen säätelevän *ISNR*-geenin silmukointia ihonalaisessa rasvakudoksessa. Lisäksi havaitsimme vahvan korrelaation sekä ihonalaisen että viskeraalisen transkriptivarianttien ja insuliinipitoisuuksien välillä.

Kolmannessa osajulkaisussa tunnistimme vaihtoehtoisesti silmukoituja geenejä 11:ssä lihavuuslokuksessa. Osoitimme laihdutuksen säätelevän *MSH5*-geenin ilmentymistä ja *BAT6-* ja *TRA2B*-geenien vaihtoehtoisen silmukoinnin riippuvan rasvan sijaintikohdasta. Tilastolliset analyysit osoittivat, että painoindeksi on ihonalaisen rasvakudoksen *TRA2B*:n, *BAG6*:n ja *MSH5*:n silmukoinnin määräävä tekijä.

Väitöskirjatyöhön kuuluva neljäs osajulkaisu on kirjallisuuskatsaus vaihtoehtoisen silmukoinnin roolista lihavuudessa ja laihdutuksessa.

Tämä väitöskirjatyö tarjoaa uutta tietoa vaihtoehtoisen silmukoinnin roolista lihavuuden ja tyypin 2 diabeteksen patogeneesissa. Nämä tulokset lisäävät ymmärrystämme vaihtoehtoisen silmukoinnin säätelyn häiriöissä ja voivat edistää kohdennettujen hoitojen mahdollisuuksia ja tarkempaa sairauksien diagnosointia.

Luokitus: QT 235, QU 58.7, QU 475, WD 210
Yleinen Suomalainen asiasanasto: geenit; geenieksPressio; lihavuus; ylipaino; rasvakudokset; maksa; insuliini; insuliiniresistenssi; aikuistyypin diabetes; glukoosi; rasvahapot

To my beloved grandparents,
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Kuopio, April 2015

Dorota Kamińska

List of the original publications

This dissertation is based on the following original publications, referred to in the text by the Roman numerals I-IV:

I. Kaminska D, Kuulasmaa T, Venesmaa S, Käkelä P, Vaittinen M, Pulkkinen L, Pääkkönen M, Gylling H, Laakso M, Pihlajamäki J. Adipose tissue TCF7L2 splicing is regulated by weight loss and associates with glucose and fatty acid metabolism. *Diabetes 61(11):2807-2813, 2012.*

II. Kaminska D, Hämäläinen M, Cederberg H, Käkelä P, Venesmaa S, Miettinen P, Ilves I, Herzig KH, Kolehmainen M, Karhunen L, Kuusisto J, Gylling H, Laakso M, Pihlajamäki J. Adipose tissue INSR splicing in humans associates with fasting insulin level and is regulated by weight loss. *Diabetologia 57(2):347-351, 2014*.

III. Kaminska D, Käkelä P, Venesmaa S, Ilves I Herzig KH, Kolehmainen M, Karhunen L, Kuusisto J, Gylling H, Laakso M, Pihlajamäki J. Regulation of alternative splicing in human obesity loci. *Submitted*

IV. Kaminska D, Pihlajamäki J. Regulation of alternative splicing in obesity and weight loss. *Adipocyte 2(3):143-147, 2013.*

Contents

Abbreviations

ADA	American Diabetes Association
BAG6	BCL2-associated athanogene 6
BMI	Body mass index
EUGENE2	Functional Genomics of Type 2 Diabetes study
FFA	Free fatty acids
FPG	Fasting plasma glucose
GWAS	Genome wide association studies
HbA1c	Hemoglobin A1c
hnRNPs	Heterogeneous nuclear ribonucleoproteins
INSR	Insulin receptor
IVGTT	Intra venous glucose tolerance test
KOBS	Kuopio Obesity Surgery Study
LPIN1	Lipin 1
METSIM	Metabolic Syndrome In Men
mRNA	Messenger ribonucleic acid
MSH5	MutS protein homolog 5
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RYGB	Roux-en-Y gastric bypass
SNP	Single nucleotide polymorphism
snRNA	Small uridine rich ribonucleoproteins
SR	serine/arginine-rich proteins
T2DM	Type 2 diabetes mellitus
TCF7L2	Transcription factor 7-like 2
TRA2B	Transformer-2 protein homolog beta
VLCD	Very low calorie diet
Wnt	Wingless-type MMTV integration site family

1 Introduction

The prevalence of obesity is increasing worldwide, making it one of the biggest health problems currently facing both developed and developing countries. It has been long known that obesity is highly associated with insulin resistance and risk of type 2 diabetes (T2DM). However, obesity is not a homogeneous disorder and obese individuals differ in their body fat distribution. Individuals with excess visceral fat tend to be at an increased risk of developing T2DM (1).

The goal of obesity treatment is for an individual to reach and stay at a healthy weight. Because conventional treatments often fail to induce stable weight loss, bariatric surgery is currently the most effective approach that provides significant, sustained weight loss for patients who are severely obese (BMI> 40kg/m^2)(2). Surprisingly, bariatric surgery turned out to be the most effective therapy for T2DM (3). One of the most commonly performed obesity surgeries is Roux-en-Y gastric bypass, which involves creating a small stomach pouch and attaching it directly to the small intestine, bypassing a large part of the stomach and duodenum. Interestingly, visceral adipose tissue is more sensitive to weight reduction than subcutaneous tissue, which is a consequence of higher metabolic and lipolytic activity in the visceral region than in other fat depots (4). Moreover it was shown that all forms of weight loss affect visceral fat more than subcutaneous fat (percentage wise) (5).

Both T2DM and obesity are multifactorial complex diseases that are caused by a combination of genetic, environmental, and lifestyle factors. Obesity is a main risk factor for T2DM; however, these two conditions can also develop independently. The precise mechanisms linking the two conditions remain unclear as the interactions between environmental signals and genes in the development of obesity and T2DM are poorly understood.

Linkage and candidate-gene focused studies allowed for the identification of genes causing monogenic forms of T2DM (6) and obesity (7). However, these approaches were largely unsuccessful when applied to the common forms of the diseases (8, 9). Both obesity and T2DM are complex diseases, which do not show Mendelian inheritance pattern, and are associated with multiple genetic and environmental factors. Recent advances using genome wide association analyses have substantially increased the number of confirmed candidate genes for obesity and T2DM. However, the functional polymorphisms in these loci have not been identified. Many of the discovered polymorphisms are localized in non-coding sequences. Interestingly, at least 15% of all point mutations responsible for genetic diseases have been estimated to affect splicing (9, 10). The underlying mechanisms by which obesity and T2DM may or may not manifest together remain poorly understood. One possible explanation is that regulation of alternative splicing contributes to increased risk of obesity and T2DM.

Alternative splicing can result in the production of multiple mRNA products, and thus allows the creation of more than one protein from a single gene or the switching off the protein by including a stop codon in its mRNA. According to recent reports, almost all human multi-exon genes undergo alternative splicing, which would compensate for the relatively low number of genes present in human genome (10, 11). Depending on the situation and environment, one gene can be transcribed into suitable transcript (12). While the abundance of transcripts is mainly influenced by the promoter activity, the alternative splicing reflects in the structure of transcripts and their encoded proteins, subsequently influencing protein localization, activity, binding properties and post-translational modifications of numerous proteins (13).

Many human diseases result from defective splicing of crucial transcripts or abnormal splice variants in affected tissues (14). There are numerous, well-characterized genes in which disruption of alternative splicing is a cause of disease. Additionally, some genes associated with obesity and insulin resistance have been shown to be regulated by alternative splicing (15-19). Interestingly, rare SNPs in obese subjects have been shown to be enriched in splicing regulatory regions of introns (20) suggesting that genetic regulation of splicing may contribute to obesity. Taken into account the widespread occurrence of alternative splicing and its highly complex regulation, it is not surprising that dysregulation of normal splicing patterns can cause or modify human disease (21). A better understanding of these dysregulations can improve the chances for a more targeted treatment and more accurate diagnostics.

The key finding leading to studies described in this thesis was the report by Pihlajamäki *et al.* demonstrating that expression of several genes involved in RNA processing is decreased in both liver and skeletal muscle of obese humans (22), indicating that aberrant splicing in obesity might be a general phenomenon. The main aim of the present study was to determine the effect of obesity and weight loss on alternative splicing of candidate genes (*TCF7L2* and *INSR*) as well as to identify alternatively spliced genes in obesity loci in Kuopio Obesity Surgery (KOBS), very low calorie diet (VLCD), Metabolic Syndrome in Men (METSIM) and European Network on Functional Genomics of Type 2 Diabetes (EUGENE2) studies. We aimed to investigate whether the expression profile of splice variants in human adipocytes differs depending on fat depots and whether the splicing pattern significantly changes in response to weight loss.

2 Review of the Literature

2.1 OBESITY

2.1.1 Pathophysiology of obesity

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health (World Health Organization definition). Body mass index (BMI) has been used as a marker for overweight and obesity. BMI is calculated as person's weight (in kilograms) divided by the square of their height (in metres). An individual with a BMI equal to or more than 25 is considered overweight, a BMI of 30 or more indicates obesity, and BMI higher than 40 is considered severe obesity. Both overweight and obesity are well-recognized risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (23, 24). A major consequence of obesity is development of T2DM, characterized by insulin resistance and impaired insulin secretion (25). Obesity contributes to the development of insulin resistance via the release of free fatty acids, increased inflammation, cytokine production, adipokine changes and fat accumulation in the liver and muscle (26).

2.1.2 Adipose tissue

Adipose tissue is the largest energy reservoir in the body. Adipose tissue provides insulation and mechanical support, but the primary function of adipocytes is to store triglycerides in lipid droplets and release free fatty acids (FFAs). In periods of energy excess, energy is deposited efficiently in adipose tissue in the form of triglycerides (lipogenesis), whereas in the fasting state lipid reserves are released (lipolysis). The number of adipocytes is set in childhood and adolescence and stays relatively constant throughout the life (27). However, it was demonstrated that around 10% of adipose cells undergo annual replacement (27) and that subjects with hypertrophy generated 70% less adipocytes per year than individuals with hyperplasia (28). Hypertrophic obesity is associated with an inability to differentiate new adipose cells due to an inability to inhibit Wnt/β-catenin signalling (29). In obesity, adipose tissue is overloaded with triglycerides, resulting in decreased buffering capacity for lipid storage in adipocytes and exposure of other tissues to excessive influx of FFAs (30). Triglyceride accumulation in non-adipose tissues (e.g. skeletal muscle, pancreatic islets and the liver) may play a role in the development of insulin resistance and/or impaired insulin secretion in obese individuals (31). Importantly, adipose tissue serves as an endocrine organ that secretes hormones and adipokines which are involved in glucose and lipid metabolism and inflammation (32).

In addition to adipocytes, adipose tissue contains the stromal vascular fraction of adipose tissue, which includes preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells.

Adipogenesis is the process by which precursor cells differentiate to adipocytes. Inhibition of Wnt signalling has been demonstrated to be essential for adipogenesis to proceed (33, 34). Activation of canonical Wnt signalling pathway leads to inhibition of GSK3β resulting in stabilization of β-catenin in the cytoplasm. Translocation of β-catenin to the nucleus and its further binding to TCF/LEF transcription factors, including *TCF7L2*, activates downstream targets and inhibits adipogenesis (35). Insulin signalling cascade is also crucial in adipogenesis. Insulin induced activation of AKT inhibits GSK3β (36), whereas insulin mediated activation of ERK1/2 pathway activation promotes adipogenesis (37) (Figure 1).

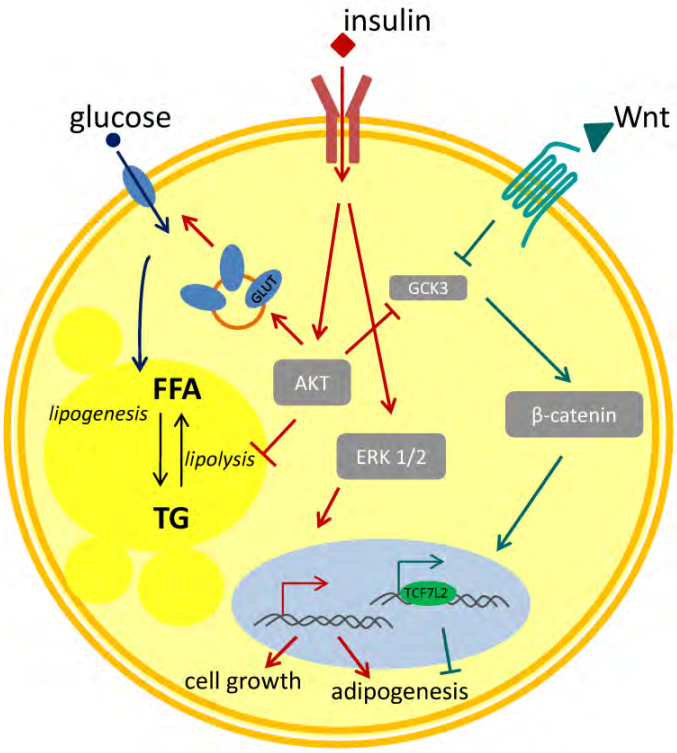

Figure 1. Wnt/β-catenin and insulin signalling pathways in adipocyte metabolism.

2.1.2.1 Subcutaneous and visceral fat depots

Body fat distribution varies between individuals. Adipose tissue localized around abdominal cavity, stored around internal organs such as the liver, pancreas and intestines, known as visceral fat, differs from adipose tissue localized under the skin (subcutaneous fat). Accumulation of visceral fat has been associated with hyperglycemia, hyperinsulinemia, hypertriglyceridemia, impaired glucose tolerance, and increased apolipoprotein B-rich lipoproteins, all of which characterize the insulin resistance syndrome (1). The risk of insulin resistance, the metabolic syndrome and cardiovascular diseases are more strongly associated with increased visceral fat than BMI alone (38). Individuals with excess visceral fat tend to be at an increased risk of developing diabetes (1). Furthermore, visceral adipose tissue is more sensitive to weight reduction than subcutaneous tissue, which is a consequence of higher metabolic and lipolytic activity in the visceral region than in other fat depots (4). Additionally, several studies reported elevated inflammation in visceral than subcutaneous fat (39, 40), which may result from higher expression of proinflammatory genes in visceral fat (41). Moreover, there are gender differences in adipose fat distribution. In general, women store more fat in the subcutaneous, whereas men store more fat in the visceral adipose tissue (42). Molecular differences between visceral and subcutaneous adipose tissue are not fully known. We investigated the differences in exon splicing profiles (explained in the paragraph 2.4) of candidate genes between visceral and subcutaneous fat.

2.1.3 Factors predisposing to obesity

The fundamental cause of obesity is an imbalance between energy intake and energy expenditure, which leads to increased storage of fatty acids in expanded adipose tissue.

Like many other diseases, obesity is a consequence of a complex interaction between an individual's genetic make-up and the environmental factors that an individual is exposed to. The study on all together 929 monozygotic twins, dizygotic twins and so called virtual twins (non-biological siblings of the same age who are raised together since infancy), revealed that 63.6% of the total variance of BMI was explained by a non-additive genetic

component, 25.7% by a common environmental component, and the remaining 10.7% by an unshared component (43) indicating that both genetics and environmental factors affect BMI.

2.1.3.1 Environmental factors

Longitudinal and cross sectional studies have indicated clear associations between environment and obesity risk. Obesity is caused by the changes in lifestyle over the past years, such as increase in the availability of energy-dense food, sedentary lifestyle and reduced physical activity, which create so called "obesogenic" environment (44). Additionally, environmental factors such as depression, sleep disorders and low socioeconomic status have been linked to obesity (45). The rapidity by which prevalence of obesity is increasing suggests that environmental factors, rather than slow changes in the human genome which occur in response to strong evolutionary pressures, have influenced the obesity epidemic.

2.1.3.2 Genetics of obesity

A significant body of evidence indicates that genetic factors play an important role in determining body weight (46). A study of twins separated from each other at birth and reared apart showed that twins were equally likely to be obese despite of the environment, such as what the parents and children ate, or how much they exercised (the intra-pair BMI correlation coefficients were 0.70 for men and 0.66 for women) (47). These and other results from twin studies suggest that genetic factors explain 50% to 90% of the variance in BMI (48). Genome wide association studies (GWAS) helped link genes to obesity risk. It was first expected that heritability of obesity would be explained by common genetic variations but each variant on its own has a very subtle effect on BMI. Recent GWAS analyses have identified 39 BMI associated loci (45), and each single variant raises body weight by approximately 180–1400 g (49). All the variants identified through GWAS explain only about 2–4% of the obesity risk (50). FTO (fat mass and obesity associated) gene was the first GWAS identified obesity locus, and it remains the one with the strongest effect, increasing BMI with a median per-allele change of 0.36 kg/m^2 (51). There are a small number of known monogenic forms of obesity, accounting only for less than 1% of the cases (52). The most common form of obesity is polygenic, which means that it results from interaction of several genes, each of which may have relatively small effects (53). Despite the large number of common risk variants identified for many complex diseases (such as obesity or T2DM), the proportion of heritability they explain remains small, leading to the important question where the "missing heritability" might be found (54). There are several possible sources of explanation. First, there are other forms of DNA variation (including copy number variants, insertions, deletions, inversions and translocations) which cannot be detected with commercial SNP genotyping arrays (55). Additionally, as demonstrated on mice model, complex phenotypes (such as T2DM and obesity) have a very complex genetic architecture due to the joint action of many loci of small effects contributing to the total variance (56). Another considered option to be a source of missing heritability is gene-gene interaction (G×G)(57, 58) as well as the gene-environment interaction (G×E) (54). Finally, epigenetic events, such as histone modifications, DNA methylation, and inherited expression of non-coding RNAs leave the genome intact, but modify the gene expression (59) and RNA splicing (60-63). Interestingly, it has been shown that some susceptibility variants for T2DM exhibit parental-origin specific associations. Moreover, rs2334499 was demonstrated to associate with T2DM when the risk allele was paternally inherited and was protective when maternally transmitted (64). Thus, to fully understand mechanisms behind the genetic predisposition, we need to relate them to the regulation of protein expression, which includes DNA sequence variations, joint action of several loci, chromatin remodelling, microRNAs and alternative splicing. Susceptibility to obesity is determined, to

a significant extent, by genetic predisposition, but an "obesogenic" environment is necessary for obesity to occur (65).

2.2 TYPE 2 DIABETES

2.2.1 Pathophysiology of type 2 diabetes

The main pathophysiological features of T2DM are impaired insulin secretion and increased insulin resistance which jointly contribute to the development of this disease. T2DM is a progressive disease which is characterized by decrease of functional pancreatic β-cell mass over time. T2DM is diagnosed based on elevated glucose levels or elevated hemoglobin A1c (HbA1c) levels. According to the American Diabetes Association (ADA) criteria, the diagnosis of T2DM is based on elevated HbA1c levels (≥6.5%) or fasting plasma glucose (FPG) levels (≥ 7.0 mmol/L) or elevated 2-hour plasma glucose (≥11.1 mmol/L) during an oral glucose tolerance test (OGTT) or, in patients with classic symptoms of hyperglycemia or hyperglycemic crisis, an elevated levels of random plasma glucose (≥11.1 mmol/L) (66).

2.2.1.1 Insulin secretion

Insulin is exclusively produced by pancreatic β-cells of the islets of Langerhans and lowers plasma glucose concentration. (67). The release of insulin from β-cells, in response to changes in blood glucose concentrations following food ingestion, is a highly dynamic process. The process is initiated by the transport of glucose into the β-cells by GLUT1 and GLUT3 transporters (67). In the β-cells glucose is metabolized in a process initiated by glucose phosphorylation by the glucokinase (GK). The increased ATP/ADP ratio leads to closure of ATP-sensitive K^+ channels (K_{ATP}) and depolarization of the membrane. Subsequently, activation of Ca^{2+} channels leads to increase of intracellular Ca^{2+} resulting in insulin release (68). Insulin release exhibits biphasic pattern. First phase is rapid and short lasting, followed by a second phase during which insulin release continues as long as the glucose concentration is elevated (69). In T2DM patients the first phase is absent and second phase is also lower than in normoglycemic subjects (70). Many of the T2DM associated risk variants identified to date regulate insulin secretion and not insulin action in insulin-sensitive tissues (71).

In healthy subjects, a postprandial insulin secretion is amplified by so called incretin effect. Incretins are a group of gastrointestinal hormones including the glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). In type 2 diabetic patients the incretin effect has been reported to be reduced (72). In particular, the impaired postprandial GLP-1 response has been reported in T2DM individuals, with obesity (72, 73), insulin resistance (74) and glucose intolerance (73) as a determinant of decreased GLP-1 secretion.

2.2.1.2 Insulin sensitivity

There are three major insulin-sensitive tissues: skeletal muscle (accounting for ~60-70% of the whole body glucose uptake), liver (~30%) and adipose tissue (~10%) (75). Insulin resistance is defined as an impaired ability of insulin to stimulate glucose uptake in insulin sensitive tissues. Obesity is the most common underlying cause of insulin resistance. Excess adipose tissue leads to an increased release of FFAs which directly affect insulin signalling, reduce glucose uptake, increase triglyceride synthesis and induce gluconeogenesis in the liver (76). Insulin resistance is a key pathophysiologic event in the development of T2DM. The pancreas compensates for impaired insulin action through increased insulin secretion, but after the period of compensated insulin resistance, T2DM usually develops due to impaired insulin secretion. Insulin acts through binding to insulin receptor, which results in

receptor autophosphorylation, activation of receptor tyrosine kinases, and followed by phosphorylation of insulin receptor substrates (IRS). Phosphorylation of IRS leads to activation of PI3K. Following steps include activation of Akt and PKC kinases which mediate translocation of GLUT4 to the plasma membrane (77).

2.2.1.2.1 Adipose tissue insulin sensitivity

Adipose tissue affects whole body glucose metabolism through regulation of FFAs levels, secreting adipokines and lipogenesis (78). Adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines (e.g., adiponectin, TGFβ, IL-10, IL-4, IL-13, IL-1Ra and apelin) while adipose tissue from obese subjects mainly releases proinflammatory cytokines (e.g., TNFα, IL-6, leptin, visfatin, resistin, angiotensin II and plasminogen activator inhibitor type 1) (79). It was demonstrated that the levels of FFAs and TNFα are significantly elevated, contributing to insulin resistance by affecting the insulin signalling cascade (80). On the other hand, adiponectin, a major adipocyte-secreted protein, is downregulated in obesity (81). Adiponectin increases insulin sensitivity by stimulating fatty acid oxidation and inhibition of hepatic gluconeogenesis (82).

2.2.2 Factors predisposing to type 2 diabetes

Similar to obesity, T2DM is caused by the combination of environmental signals and genetic factors to a varying extent. T2DM has been shown to aggregate in families. The risk of developing T2DM is approximately 40% in offspring of one diabetic parent and approximately 70% if both parents have the disease (83). However, family members share not only genes but also environment, such as dietary habits, socio-economical background and many other lifestyle aspects. Major risk factors for diabetes include obesity, with special emphasis on visceral obesity, aging, physical inactivity, drinking, smoking, sleep deprivation, low-socioeconomic status and ethnicity and impaired intrauterine environment (45, 84). Many of the T2DM associated risk variants regulate insulin secretion (71), suggesting that the deciding inherited component is related to insulin secretion, whereas the environmental component may relate to the energy excess, obesity and following insulin resistance (85).

2.2.2.1 Environmental factors

The epidemic of T2DM observed over the recent years indicates the importance of environmental factors in T2DM onset. Overweight and obesity are the most important determinant of T2DM (83). Even mild obesity increases the risk of T2DM; for example, it was observed that women with a BMI between 23 and 25 kg/m^2 have 3-times higher risk of developing diabetes compared with women with a BMI below 23 kg/m^2, whereas for women with BMI 35 the risk is 20 times higher (86). A number of lifestyle aspects are known to be important to the development of T2DM, including sedentary behaviour, diet choices and smoking (87). Lack of physical activity was shown to increase the T2DM risk, independent of obesity (88). Additionally, it was demonstrated that, independent of BMI, quality of diet plays an important role in the development of disease. Particularly, a low-fibre diet with a high glycemic load increases risk of T2DM (89), and higher intake of saturated fat and trans-fat (90) may modify insulin resistance and the risk of T2DM. Active smoking has been associated with increased risk of T2DM, which might be linked with antiestrogenic effect of smoking and abdominal obesity observed more often in smokers (91). The evidence from meta-analyses suggests an approximately 30% reduced risk of T2DM in moderate alcohol consumers (6-48 g/day) compared with heavier consumers or abstainers (92). Moderate alcohol consumption increases HDL cholesterol levels (93) and is associated with lower fasting insulin and triglyceride concentrations and enhanced insulin sensitivity (94). Sleep deficiency is associated with T2DM potentially by altering eating habits, inflammation, impairing glucose tolerance and insulin sensitivity (95).

2.2.2.2 Genetics of type 2 diabetes

Multiple lines of evidence support the view that T2DM is inherited. First, the genetic differences in T2DM disease predisposition has been observed in different ethnic groups (96). The T2DM prevalence varies from very low levels in tribes of Mapuche Indian, to very high levels in the Nauru and the Pima Indians (6, 97). Observation of full-blooded Nauruans revealed that the prevalence of T2DM is higher than those with admixture (98), and the prevalence of T2DM in the Pima Indians is inversely related to the extent of interbreeding with the European Americans (98, 99). Familial aggregation provides another evidence for a genetic contribution to disease. The heritability of T2DM in twin and adoption studies ranges from 45% to 85% (100-102). Only three genes, *PPARG*, *KCNJ11* and *TCF7L2* were identified as established genes associated with common forms of T2DM by linkage and candidate-gene studies (103). Advances using genome wide association analyses allowed the discovery of multiple risk variants. Importantly, the genes identified by linkage and candidate-gene approaches, *PPARG*, *KCNJ11* and *TCF7L2*, were confirmed as T2DM risk loci. The risk variant in the *TCF7L2* gene locus is the most influential common variant with allelic odds ratio of ~1.5 (104). *TCF7L2* is a key component of canonical Wnt signalling pathway. Carriers of the *TCF7L2* risk variant show impaired insulin secretion (105) and increased hepatic glucose production (106). The current count of firmly established T2DM risk loci is now 90 (45). However, these associated loci explain only 5–10% of the trait variance (107). As discussed previously, several options have been debated to be a source of missing heritability, such as structural variations (55), gene-gene (58), gene-environment interactions (54), and epigenetic modifications (59) to name a few.

2.3 WEIGHT LOSS

Both obesity and T2DM are intimately linked, since obesity is an important contributor to the pathogenesis of insulin resistance (85). Treatment of obesity influences insulin resistance. Recommended treatments for both T2DM and obesity include dietary and behavioural modifications, bariatric surgery, or a combination of the above mentioned. All those approaches have their own advantages and shortcomings. Most of the programs modifying lifestyle change result in 5–10% weight loss and are effective for 6–12 months, after which weight regain is usually observed. Lifestyle changes and reduction of body weight improves glycemic control, mortality and morbidity. Weight loss induced by calorie restriction, exercise, life style changes or surgical interventions results in reduction of insulin resistance and glycemic control in patients with T2DM or a delay in the development of T2DM in pre-diabetic individuals (26). Successful lifestyle interventions include self-monitoring of weight, dietary intake, and physical activity (108). The usage of medications to assist in weight loss is limited by side effects. Most of the anti-obesity drugs that were approved and marketed over the years have been withdrawn due to severe adverse effects (109). The weight loss achieved with the medications is 2% to 8% higher compared to a placebo effect (108). To determine the role of alternative splicing in obesity and weight loss, we conducted experiments using tissue samples from before and after a very low calorie diet and the bariatric surgery.

2.3.1 Very low calorie diet

Weight reduction is a keystone of the modern treatment of overweight and obese individuals. Calorie-restriction strategies are the most common dietary approach, where calories are restricted every day, to a minimum of 40-50% of normal caloric intake (110). One of the calorie-restriction strategies for the treatment of obesity is very low calorie diet (VLCD) characterized by limiting energy intake to 800 kcal (3.35 MJ) per day. Weight loss on VLCD is on average approximately 1.5 to 2.5 kg/week with a total loss after 12 to 16

weeks of approximately 20 kg (111). The VLCD provides at least 50g of proteins and amino acids, essential fatty acids, and daily requirements of trace elements, vitamins and minerals (112). After weight reduction, the energy intake can be increased gradually to maintain a balance between calories consumed and calories expended. However, long-term weight loss maintenance is poor, with 50% of weight being regained within one year (113).

2.3.2 Roux-en-Y gastric bypass

Whereas many dietary interventions can lead to the short-term weight loss, most of them fail to result in a long-lasting effect. Bariatric surgery results in substantial and durable long-term weight loss and may be curative for T2DM. In recent years bariatric surgery has been used as a treatment for severe obesity and more recently as a treatment for T2DM (114). The Roux-en-Y gastric bypass (RYGB) leads to glycemia normalization in 84-98% patients (115). The gastric bypass surgery has become the most commonly used bariatric surgery procedure. The RYGB is a type of weight loss surgery that involves the division of the stomach into a small gastric pouch and gastric remnant. The gastric pouch is connected to the jejunum, the middle section of the small intestine, bypassing the remainder of the stomach and the duodenum (Figure 2). The surgery leads to the creation of two limbs, the alimentary and the biliopancreatic limb. The first one is the branch of the "Y" through which food passes, whereas the other transports secretions from the pancreas, liver, and gastric remnant (114). Interestingly, many T2DM patients who undergo RYGB are relieved of their hypoglycemic medications in a matter of days, before any significant weight loss takes place (115). The mechanisms of gastric bypass induced weight loss depends on many factors including gut hormones (increased GLP-1 and PYY and reduced ghrelin), changes in energy expenditure, reduction in food intake, changes in food choices (possibly due to changes in taste and food perception), changes to gut microbial and bile salts metabolism (26, 116, 117).

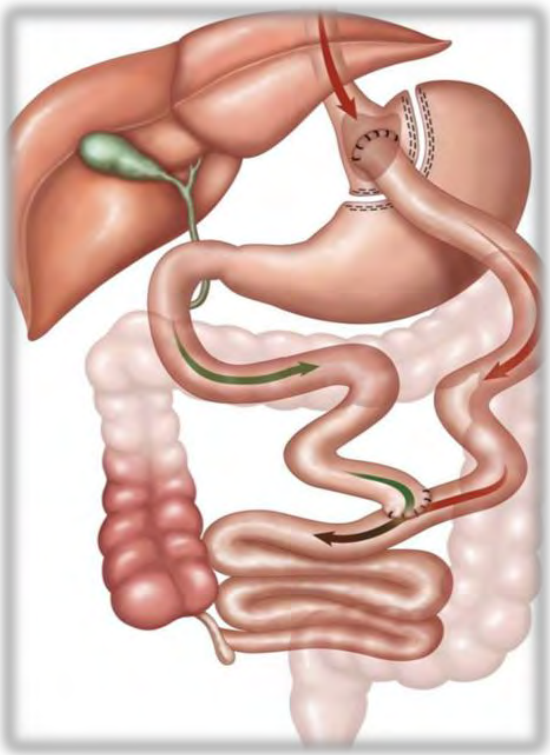

Figure 2. Roux-en-Y gastric bypass. Reproduced from Bariatric surgery: a best practice article, Neff KJ, le Roux CW, J Clin Pathol. 2013 Feb;66(2):90-8 with permission from BMJ Publishing Group Ltd.

2.4 ALTERNATIVE SPLICING

Despite the fact that obesity and T2DM are highly interrelated, both diseases can develop independently. There is no doubt that both T2DM and obesity are inherited, yet the overlap between loci for these traits is rather poor. Of 90 loci associated with T2DM only 5 loci is shared with 39 BMI associated loci (45). The mechanisms by which these diseases may or may not manifest together remain poorly understood. One possible explanation is that alternative splicing provides a mechanism for the modulation of risk factors associated with obesity and T2DM.

Genetic information flows from DNA to RNA and then to protein. DNA is transcribed in the nucleus into pre-mRNA which must undergo mRNA processing before being exported to the cytoplasm. mRNA processing is a complex cascade consisting of splicing (both constitutive and alternative), 5'capping, 3'polyadenylation and nuclear export of mature mRNA (118). Mature mRNA is then exported to the cytoplasm for translation into protein (Figure 3).

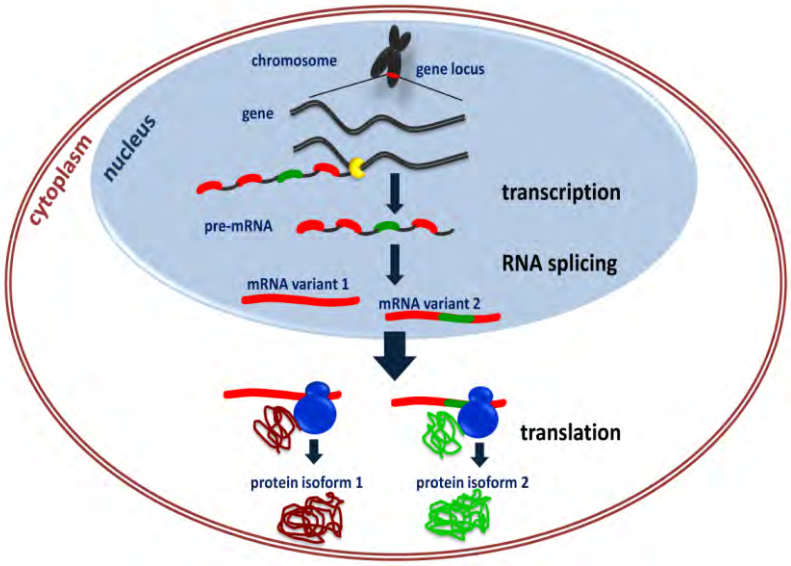

Figure 3. Flow of genetic information.

Alternative splicing was discovered in 1977 by two independent researchers, Richard J. Roberts and Phillip A. Sharp, who were jointly awarded the Nobel Prize in Physiology or Medicine in 1993 for discovery of "split genes" (119, 120). Recent estimates suggest that almost all human multi-exon genes are subject to alternative splicing (10, 11). The transcript abundance is regulated by promoter activity whereas the structure of transcripts – resulting in protein localization, activity, binding properties and post-translational modifications – is regulated by alternative splicing (13). Alternative splicing has drawn more attention after the completion of a map of the human genome in 2001, which revealed that our genome previously estimated to contain ~120,000 genes (121), contains only 20,000-25,000 protein-encoding genes (122). Alternative splicing of pre-mRNA is a common mechanism responsible for the generation of transcript and protein diversity. There are seven main types of common alternative splicing events: 1) alternative transcription start site, 2) alternative poly-A site, 3) exon skipping, 4) intron retention, 5) mutually exclusive exon usage, 6) 3' splice site selection and 7) 5' splice site selection (Figure 4). One gene can be subject to the combination of different types of alternative splicing, and the splicing events may differ between tissue types. Depending on the tissue type or the environment, one gene can be transcribed into suitable transcripts (12).

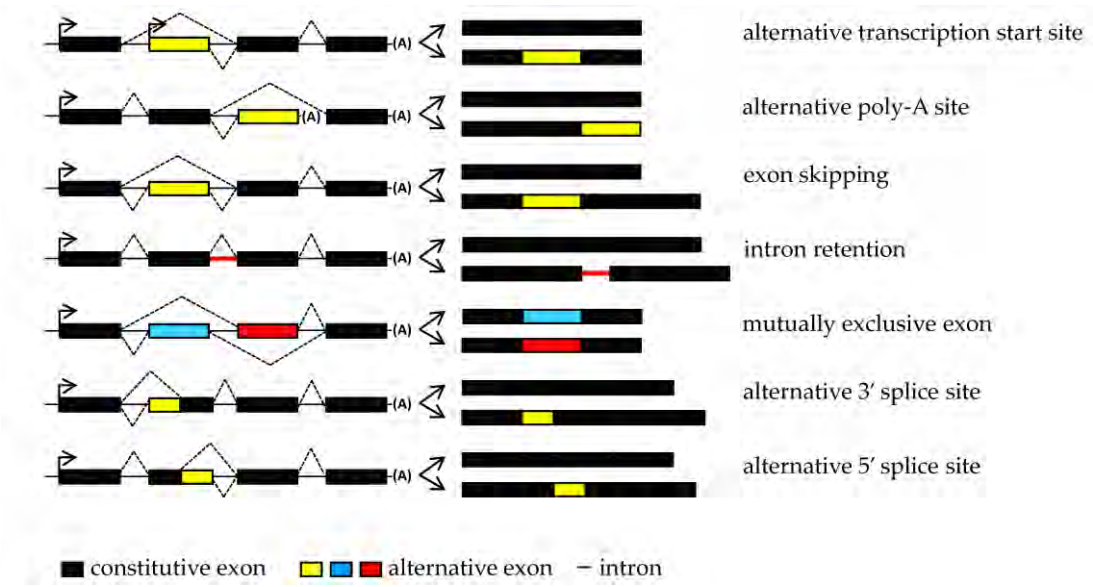

Figure 4. Common types of alternative splicing. Exons are represented by boxes, introns by solid lines indicate introns and splicing options are shown with dashed lines. Transcription start sites are indicated with arrows and polyadenylation sites with (A).

2.4.1 Regulation of alternative splicing

Regulation of alternative splicing is multi-factorial and depends on the regulatory sites on the pre-mRNA and corresponding protein activators. Additionally, alternative splicing is regulated by physiological signals, allowing an organism to adapt to the alterations in the environment (Figure 5).

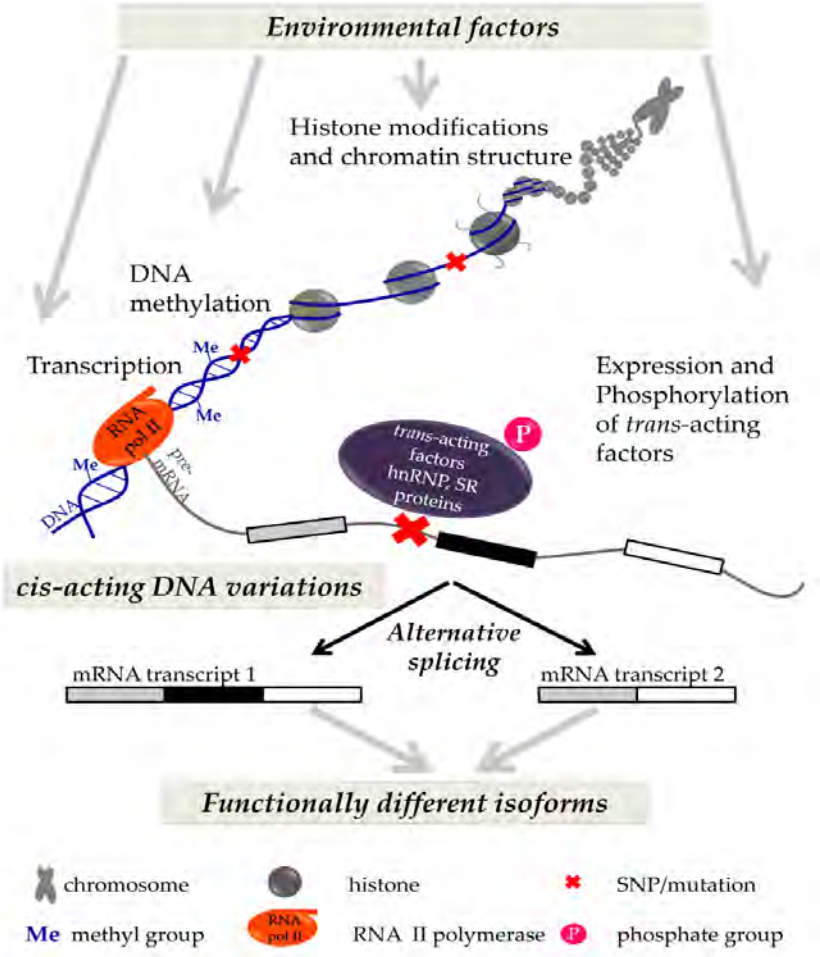

Figure 5. Different levels of regulation of alternative splicing

2.4.1.1 Basal splicing machinery

Splicing takes place in the nucleus and is catalyzed by a large ribonukleoprotein complex called spliceome. Spliceome includes five small uridine rich ribonucleoproteins (snRNAs: U1, U2, U4, U5, and U6) and at least 170 distinct proteins (123).

Alternative splicing is regulated by the *cis*-acting regulatory sequence elements and *trans*-acting RNA-binding proteins. There are four different types of *cis*-acting elements, which are short sequences within the pre-mRNA: 1) exonic splicing enhancers, 2) exonic splicing silencers, 3) intronic splicing enhancers and 4) intronic splicing silencers. One *cis*-acting element can serve both as an enhancer or silencer, depending on the *trans*-acting factor binding to it. The function of *cis*-acting elements is weak which is compensated by the clustering of many *cis*-acting elements. *Trans*-acting proteins include serine/arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNP proteins usually bind to splicing silencers preventing the binding of snRNPs or SR proteins whereas SR proteins usually bind to splicing enhancers (124).

Regulation of alternative splicing depends on the abundance of *trans*-acting proteins, both hnRNPs and SR-proteins. The level of expression of *trans*-acting proteins may differ between different tissue types resulting in tissue-specific alternative splicing (125). Interestingly, the *trans*-acting factors can also be alternatively spliced and are involved in auto-regulation (126, 127).

2.4.1.2 Phosphorylation-mediated regulation

The components of the pre-mRNA splicing machinery are subject to phosphorylation and dephosphorylation during the splicing process. Both, phosphorylation and dephosphorylation, of SR proteins was shown to modulate splicing (128, 129).

2.4.1.3 Coupling between transcription and splicing

Regulation of alternative splicing involves also the coupling between transcriptions and splicing. Two models were proposed to explain the role of RNA polymerase II (RNA pol II) in alternative splicing: recruitment model and kinetic model. In the recruitment model, the binding of SR proteins to RNA pol II results in alternative exon skipping (130). The kinetic model proposes that alternative exon skipping is a result of the fast elongation rate, which can be influenced by the level of RNA pol II phosphorylation and chromatin structure (60).

2.4.1.4 Histone modifications and chromatin structure in splicing regulation

Recently, histone modifications such as methylation, acetylation and phosphorylation were reported to play a role in the regulation of alternative splicing (62). Additionally, siRNAs have been suggested to be involved in modulation of chromatin structure near alternatively spliced gene regions (131).

2.4.2 Alternative splicing in diseases

Misregulation of alternative splicing plays a significant role in diseases. Aberrant splicing can result from point mutations (SNPs), which have been compiled in the databases (132, 133). It is estimated that as much as one third of all disease-causing mutations modify mRNA splicing (134-136). Aberrant splicing leading to disease can be a consequence of polymorphism situated within *cis*-acting elements or misregulation of the splicing machinery by environmental stimuli. A large number of reviews describe the roles of *cis*-acting mutations in diseases (137, 138), and some of the mutations were linked to obesity and T2DM (Table 1).

Table 1. Splicing mutations in obesity and T2DM related phenotypes

Phenotype	Gene	Mutation	Manifestation		Ref.
			Clinical	Molecular	
Obesity	*PHF6*	NM_001015877.1:c.769A>G (rs104894919)	Mild Börjeson-Forssman-Lehmann syndrome	exon 3 skipping, impaired protein function	(139)
	GNB3	NM_002075.3:c.825C>T (rs5443)	Obesity	in frame deletion, truncated protein	(140)
	LEPR	NM_002303.5:c.2597+1G>A (CS982253)	Early-onset morbid obesity and pituitary dysfunction	exon 18 skipping, truncated protein	(141)
	LRRFIP1	NM_001137552.1:c.2069G>C (rs11680012)	Abdominal adiposity and inflammation	altered exonic splicing	(142)
	ALMS1	NM_015120.4 :c.11873-3T>G	Alström syndrome	exon 19 skipping, truncated protein	(143)
Hyper-glycemia	*INSR*	NM_000208.2:c.1124-2A>G (rs587776819)	Rabson-Mendenhall syndrome	exon 5 skipping, premature stop codon	(144)
	GCK	NM_000162.3:c.580-1G>A (CS052048)	MODY, gestational diabetes	in frame insertion	(145)
	HNF-1α	NM_000545.5:c.1623+1G>A (CS016023); NM_000545.5:C.956-2A>G (CS016022); NM_000545.5:c.1502-6G>A (CS024751)	MODY	exon 8 skipping; exon 5 skipping; exon 7 skipping; truncated proteins	(146)
	G6PC2	NM_021176.2:c.441-26T>C (rs560887); NM_021176.2:c.441-25G>A (rs2232321)	Elevated fasting plasma glucose	exon 4 inclusion	(147)
	GIPR	NM_000164.2:c.1152+820T>A (rs10423928)	Impaired insulin secretion, increased risk of T2DM	reduced expression of fully active receptor	(148)
	CDKAL1	NM_017774.3:c.371+11426A>C (rs10946398)	Increased risk of T2DM	reduced expression of CDKAL1	(149)

2.4.2.1 Regulation of alternative splicing by body weight and diet

There are few reports linking alternative splicing to obesity and weight loss. Reduced expression of several *trans*-acting factors in liver and skeletal muscle of obese humans was observed, suggesting common dysregulation of splicing in obesity. Importantly, reduced expression of RNA processing genes was also demonstrated in a mouse model of diet-induced obesity. Downregulation of trans-acting factor, TRA2B, led to a higher level of lipogenesis due to altered splicing of the LPIN1 and increase of β isoform (22). Alternative splicing of troponin T (TNNT3) is another example demonstrating the relation between body weight and alternative splicing. The ability to regulate skeletal muscle composition was partially impaired in obese rats due to aberrant splicing of *Tnnt3* in rats (150). Additionally, aberrant splicing of mTOR, caused by a knock-out of *trans*-acting factor Sam68, led to the generation of the truncated variant of *mTORi5* and lean phenotype in mice (151).

Gastric bypass surgery induced weight loss, but not diet, resulted in aberrant splicing of *Nnat* in mice, indicating different mechanisms of weight loss (152). Finally, high-fat diet was shown to affect splicing of tau in the brain of female mice (153). Mice fed a cafeteria diet demonstrated modified splicing pattern of insulin-degrading enzyme (*Ide*) in mouse liver (154).

2.4.3 Alternative splicing of *TCF7L2*

Common intronic variation within the gene encoding transcription factor 7-like 2 (*TCF7L2*) has been strongly associated with T2DM in all ethnic groups (104). Moreover, the GWAS analyses indicate the strongest association between T2DM and the *TCF7L2* locus (155), yet the molecular mechanisms on how variations in the gene lead to increased risk of developing T2DM have remained elusive. Alternative splicing of *TCF7L2* has been suggested to explain the association between *TCF7L2* SNPs and impaired *TCF7L2* function in T2DM. Several research groups reported tissue-dependent splicing of *TCF7L2* in human tissues including pancreas, pancreatic islets, colon, liver, monocytes, primary lymphocytes, skeletal muscle, subcutaneous and visceral adipose tissue, peripheral blood mononuclear cells and lymphoblastoid cell lines, but no association between splicing and genetic variants has been determined (156-160).

Human *TCF7L2* is composed of 18 exons and is characterized by a complex splicing pattern in different tissues. The 3′ terminus of *TCF7L2* gene is subject to extensive splicing, while the 5′ terminus is the stable region amongst different transcript variants. Alternative splicing of exons 12, 13, 13a and 13b results in proteins with short, medium or long reading frames (161). Exons 13, 13a and 13b are spliced in a mutually exclusive and specific manner, with exon 13 expressed in adipose tissue, both subcutaneous and visceral, exon 13a in liver tissue and exon 13b in pancreas (156, 160, 162, 163) (Figure 6).

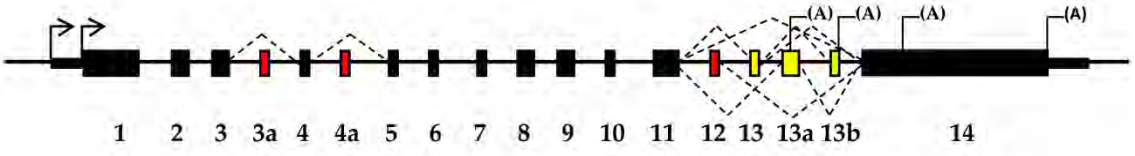

Figure 6. Alternative splicing of *TCF7L2* gene. Constitutive exons of *TCF7L2* are represented as black boxes and alternative exons as coloured boxes with yellow boxes indicating mutually exclusive exons.

Inclusion of full exon 14 introduces binding sites for C-terminal protein binding protein (CTBP), an inhibitor of Wnt/β-catenin signalling pathway (164-166), while only variants containing exon 13 encode a CRARF domain, required for potent activation of the Wnt signalling cascade (165, 167). Hence, changes in *TCF7L2* splicing could alter diabetes risk

through alterations in Wnt pathway. It has been reported that protein isoforms generated by alternative splicing of *TCF7L2* exhibit a different ability to activate Wnt signalling pathway. A reduced activation of Wnt/β-catenin target gene promoters was demonstrated for short *TCF7L2* transcript variant (exons 12, 13 and 13a deficient) (168). Furthermore, overexpression of the short mRNA transcript variant induced impaired insulin secretion and apoptosis in human islets while the overexpression of the full length *TCF7L2* mRNA transcript variant (including exons 12, 13 and 13a) exhibited the protective effect on β-cell function and survival (169). Moreover, it is well known that inhibition of Wnt/β-catenin signalling induces adipogenesis (170-172).

2.4.4 Alternative splicing of *INSR*

Human insulin receptor gene consists of 22 exons, with alternatively spliced exon 11 (Figure 7).

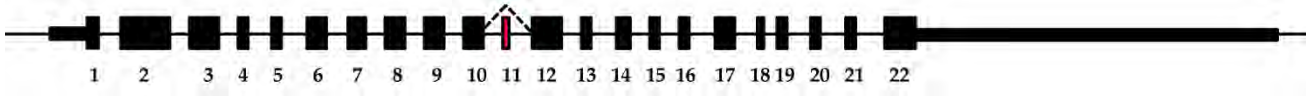

Figure 7. Alternative splicing of *INSR* gene. Constitutive exons are represented as black boxes and alternatively spliced exon is shown in red.

The insulin receptor (INSR) exists in two protein isoforms arising from inclusion (INSR-A) or skipping of exon 11 (INSR-B). Expression of the INSR protein isoforms varies between tissues and depends on tissue developmental stage. While the INSR-A protein isoform is predominantly expressed in fetal cells and is involved in fetal development, the INSR-B protein isoform is expressed in mature differentiated cells. These two protein isoforms exhibit different functions. INSR-A was shown to promote growth due to its ability to bind IGF-II and proinsulin in addition to insulin, while INSR-B is responsible for regulation of glucose homeostasis and is a highly specific receptor for insulin (173). The alternative splicing of *INSR* gene in T2DM has been studied mainly in skeletal muscle. In most, but not in all of the studies high expression of *INSR-B* transcript in skeletal muscle was associated with T2DM and insulin resistance (173). The increased expression of *INSR-B* was reported in adipocytes isolated from 10 T2DM patients compared with 11 normoglycemic individuals (17). Regulation of adipose tissue splicing of *INSR* in the context of obesity and weight loss has not been studied.

3 Aims of the Study

The main objective of this study was to investigate the role of the regulation of alternative splicing in obesity and T2DM (Studies I-III). Additionally we have published the review (IV) covering recent advances in understanding the role of alternative splicing in obesity and insulin resistance.

The specific aims were as follows:

1. To determine the splicing profile of the T2DM candidate gene, *TCF7L2*, in the subcutaneous and visceral adipose tissue and in the liver and its correlation with clinical parameters. To investigate whether the alternative splicing of *TCF7L2*, is regulated by weight loss (Study I).

2. To determine the splicing profile of *INSR* in the subcutaneous and visceral adipose tissue and its association with clinical parameters. To determine whether the alternative splicing of *INSR* is regulated by weight loss (Study II).

3. To investigate alternative splicing of genes in obesity loci, and to identify differences in the splicing profile between lean and obese individuals (Study III).

4 General Experimental Procedures

4.1 STUDY SUBJECTS

4.1.1 Kuopio Obesity Surgery study (Studies I, II & III)

All subjects undergoing Roux en-Y gastric bypass surgery at the Kuopio University Hospital were recruited into the ongoing Kuopio Obesity Surgery (KOBS) study (>90% participated) investigating metabolic consequences of bariatric surgery (174, 175). Study I included a total of 95 severely obese individuals, while Study II & III included 110 severely obese participants who underwent laparoscopic gastric bypass surgery with alimentary limb of 100 cm in Kuopio University Hospital. There were three criteria for surgery (1) BMI≥40 kg/m^2 or BMI≥ 35 kg/m^2 with significant comorbidity (T2DM), (2) failure of dietary and drug treatments and (3) no other contraindication for operation. Each participant had a one-day visit in hospital for screening eligibility to bariatric surgery, which included an interview on disease history, current medication status and an assessment of glucose tolerance and cardiovascular risk factors. Fasting blood samples were drawn after 12 hours of fasting. Subcutaneous and visceral adipose tissue biopsies were collected during the operation. Subcutaneous fat biopsies were taken a year after surgery. Clinical parameters were assessed prior to the surgery (at baseline) and at 1-year postsurgery (follow-up) (Table 2). In Study I, 95 liver biopsies were analyzed at baseline and 11 liver biopsies at follow-up (Table 1 in (163)).

The study was approved by the Ethics Committee of the University of Eastern Finland and Kuopio University Hospital, and it was in accordance with the Helsinki Declaration.

Table 2. Characteristics of the KOBS study subjects.

	Study I			Study II & III		
	Baseline n=54	Follow-up n=46	p value	Baseline n=108	Follow-up n=81	p value
Sex male/female	15/39	14/32		34/74	26/55	
Age years	45.4±8.0	46.6±8.1		47.1±9.1	47.6±9.0	
BMI kg/m^2	45.0±5.9	34.6±6.1	6×10^{-21}	45.0±6.3	34.6±5.8	2×10^{-37}
Fasting glucose mmol/l	6.7±1.8	5.5±0.8	3×10^{-6}	6.7±1.7	5.6±0.9	2×10^{-8}
Fasting insulin pmol/l	123.0±68.4	64.8±70.8	1×10^{-5}	135.7±135.5	71.4±67.4	3×10^{-10}

4.1.2 Metabolic Syndrome in Men study (Studies I, II & III)

A total of 49 men were included to a Study I & II and 46 participants were included in Study III from the population-based Metabolic Syndrome in Men (METSIM) study (176).

The male subjects, aged from 45 to 70 years, were randomly selected from the population register of the town of Kuopio. Each participant had a one-day visit to the Clinical Research Unit at the University of Eastern Finland, which included an interview on disease history, current medication status and an assessment of glucose tolerance and cardiovascular risk factors. Fasting blood samples were drawn after 12 hours of fasting followed by an oral glucose tolerance test (OGTT). The characteristics of the subjects are given in Table 3.

The study was approved by the Ethics Committee of the University of Eastern Finland and Kuopio University Hospital, and it was in accordance with the Helsinki Declaration.

Table 3. Characteristics of the METSIM study subjects.

	Study I, II			Study III		
	NGT n= 28	**T2DM n= 21**	***p* value**	**NGT n= 26**	**T2DM n= 20**	**p value**
Sex male/female	28/0	21/0		26/0	20/0	
Age years	56.7±5.3	56.3±5.5	0.803	56.5 ± 5.5	56.7 ± 5.3	0.902
BMI kg/m^2	23.2±1.4	26.0±3.9	0.004	23.1 ± 1.4	26.1 ± 3.9	0.003
Fasting glucose mmol/l	5.5±0.3	7.0±1.1	3×10^{-6}	5.5 ± 0.3	7.1 ± 1.0	1×10^{-6}
Fasting insulin pmol/l	27.6±8.4	65.4±47.4	0.002	27.6 ± 8.8	62.9 ± 47.3	0.004

4.1.3 Functional Genomics of Type 2 Diabetes study (Study I)

Functional Genomics of Type 2 Diabetes (EUGENE2) study included healthy non-diabetic individuals but who were the offspring of patients with T2DM. One of the parents had to have T2DM while the other a normal glucose tolerance or a lack of history of T2DM in first-degree relatives. The probands were randomly selected among T2DM subjects living in the town of Kuopio. T2DM among the probands was defined according to the WHO criteria. All participants underwent physical examination, routine blood tests, OGTT, an IVGTT on a separate occasion, followed by a hyperinsulinemic-euglycemic clamp (177). A total of 113 individuals were included to the Study I (Table 4). The study was approved by the Ethics Committee of the University of Eastern Finland and Kuopio University Hospital, and it was in accordance with the Helsinki Declaration.

Table 4. Characteristics of the EUGENE2 study subjects.

	Study I
	NGT n=113
Sex male/female	49/64
Age years	40.4± 5.9
BMI kg/m^2	26.8 ± 5.1
Fasting glucose mmol/l	5.3 ± 0.5
Fasting insulin pmol/l	63.6 ± 33.6

4.1.4 Very Low Calorie Diet study (Studies II & III)

A study group of 35 obese, non-diabetic subjects with a BMI between 30 and 45 kg/m^2 were recruited to the very low calorie study (VLCD). Participants followed very low calorie diet (VLCD) of 2,510 kJ/day (600 kcal/day) for 7 weeks (Nutrilett; Leiras Co., Finland). They supplemented their diet with low calorie vegetables as desired. The weight loss period was followed by a 24-week weight-maintenance period (WM). Clinical parameters and subcutaneous tissue biopsies were collected at all visits (baseline, 7 weeks and 24 weeks) (178). A total of 32 participants were included to the Study II & III (Table 5).
The study was approved by the Ethics Committee of the University of Eastern Finland and Kuopio University Hospital, and it was in accordance with the Helsinki Declaration.

Table 5. Characteristics of the VLCD study subjects.

	Study II & III				
	Baseline n=32	**7 weeks VLCD n=32**	**24 weeks WM n=32**	***p* value (VLCD)**	***p* value (WM)**
Sex male/female	9/23	9/23	9/23		
Age years	48.8±8.7	49.0±8.8	49.5±8.8		
BMI kg/m^2	34.7±2.7	29.9±2.4	30.3±2.8	3×10^{-24}	2×10^{-16}
Fasting glucose mmol/l	6.1±0.8	5.6±0.6	5.7±0.6	1×10^{-4}	6×10^{-5}
Fasting insulin pmol/l	82.8±46.2	40.8±19.2	51.0±27.0	3×10^{-6}	4×10^{-6}

4.2 MATERIALS AND METHODS

4.2.1 Analytical methods used in human studies

Plasma glucose, insulin, and serum lipids and lipoproteins (total cholesterol, HDL cholesterol, and triglycerides) and FFAs were measured from fasting venous blood samples. Plasma glucose was measured by enzymatic hexokinase photometric assay (Konelab Systems Reagents; Thermo Fischer Scientific, Vantaa, Finland). Plasma insulin was determined by immunoassay (ADVIA Centaur Insulin IRI, number 02230141; Siemens Medical Solutions Diagnostics, Tarrytown, NY). An oral glucose tolerance test (OGTT) was carried after a 12-h fasting period. Serum FFAs were assayed with an enzymatic colorimetric method (Wako NEFA C test kit; Wako Chemicals, Neuss, Germany). Glucose, insulin, and FFA levels were determined at 0, 30, and 120 min in an OGTT. Intravenous glucose tolerance test (IVGTT) and hyperinsulinemic-euglycemic clamp were performed after a 12-h fast. In order to determine the first-phase insulin release, an IVGTT was performed. A bolus of glucose (300 mg/kg in a 50% solution) was given within 30 s into the antecubital vein. Samples for the measurement of blood glucose and plasma insulin were drawn at −5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 min. Whole-body insulin sensitivity was assessed with the hyperinsulinemic-euglycemic clamp technique (insulin infusion rate of 40 mU $\times min^{-1} \times m^{-2}$ body surface area), as described (179). Blood glucose was clamped at 5.0 mmol/L for the next 120 min by infusion of 20% glucose at various rates according to blood glucose measurements made every 5 min. The mean amount of glucose infused during the last hour was used to calculate the rates of whole-body glucose uptake. Adipose tissue insulin sensitivity was estimated from the levels of serum FFAs during the clamp (163).

4.2.2 Adipose tissue biopsies

Adipose tissue samples of the KOBS study were taken as open biopsies from subcutaneous and visceral adipose tissue during Roux-en-Y gastric bypass surgery (at baseline) and at 1-year postsurgery (follow-up) open biopsies from subcutaneous adipose tissue were taken under local anaesthesia. Adipose tissue samples of the METSIM and EUGENE2 studies were taken by a needle biopsy from subcutaneous adipose tissue under local anaesthesia. Adipose tissue samples of the VLCD study were taken at all visits (baseline, 7 weeks and 24 weeks) as an open biopsy from subcutaneous adipose tissue under local anaesthesia. Collected adipose tissue samples were stored in -80°C until used for RNA extraction.

4.2.3 Cell-culture experiments

Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes, which are characterized by a high capacity for adipogenic differentiation (180), were morphologically differentiated into mature adipocytes. Preadipocytes were cultured in Dulbecco's modified Eagle's

medium/F12 Nut mix supplemented with 33 μM biotin, 17 μM pantothenate, 1% penicillin/streptomycin, and 10% FBS until reaching confluence. To induce differentiation, preadipocytes at day 0 were washed three times with PBS and cultured in serum-free 3FC medium (Dulbecco's modified Eagle's medium/F12 nut mix, 33 μM biotin, 17 μM pantothenate, 1% penicillin/streptomycin, 0.01 mg/mL transferrin, 20 nmol/L insulin, 0.1 μM hydrocortisone, and 0.2 nmol/L T3) with 25 nmol/L dexamethasone, 0.5 μM isobutylmethylxanthine, and 2 μM rosiglitazone. At day 4 of postdifferentiation, the medium was changed to 3FC medium with 25 nmol/L dexamethasone and 0.5 μM isobutylmethylxanthine. At day 7 of postdifferentiation, the medium was changed to 3FC medium, and after that, the medium was replenished twice a week with 3FC medium (163).

4.2.4 RNA isolation and reverse transcription

Total RNA was extracted and purified using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was converted to cDNA with random primers using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. (163).

4.2.5 Analysis of alternative splicing and sequencing

Relative ratio of splice variants of analyzed genes were analyzed with the PCR-capillary electrophoresis method. Our method, based on a single PCR, was optimized to give reliable information on the isoform distribution and on changes of the isoform distribution due to alternative splicing. The cDNA equivalent of 2 ng total RNA was amplified by PCR in a thermocycler (ABI PRISM 2720; Applied Biosystems). PCR reaction was performed using DyNAzyme II Hot Start DNA Polymerase (Finnzymes, Espoo, Finland) and a suitable primer pair flanking an alternatively spliced region resulting in PCR products that vary in size depending on splicing. The reverse primers were fluorescein (FAM)-labelled. The primers used in this study are shown in Table 6. Amplicon reactions were run on the ABI 3100 the ABI 3100 DNA Genetic Analyzer (Applied Biosystems) using POP6 polymer (Applied Biosystems). The results were analyzed using Peak Scanner Software v1.0 (Applied Biosystems). Peak areas were used to calculate the relative quantities of PCR products. PCR products were initially gel-purified and sequenced in order to determine exon incorporation and ensure the specificity of amplification using BigDye Terminator v1.1 (Applied Biosystems) (163).

Table 6. Primer pairs used for detection of splice variants

PCR primer	Primer forward (5′ – 3′)	Primer reverse (5′ – 3′)	Study
TCF7L2 exon 3 - 5	ATCCCCGACCTGACGAGCCC	GAGGTGGGTTTCCCGGCGTG	I
TCF7L2 exon 11-14	CAGCCGGGAGAGACCAATG	TGGGTCTGCTCAGTCTGTGACT	I
INSR	ACCAGAGTGAGTATGAGGATTCGG	TCCGGACTCGTGGGCACGCTGGTC	II
RPGRIP1L	GCACCAAGACCTAAACCAAGACAACG	TGCCAAGCTTTGTTCTGCAAGCTG	III
MSH5	TGGACCCACATGCCCCCACA	TGATGGGTTTTCCACTGCGGATCA	III
CKAP5	CCTGAACCCAAGATCCGGGCTG	CCTCCCGGGCAAGGCTCTCT	III
SMARCD1	CTGCTGTCCACTGCCAGCCA	CGGCACACAGCCTCCTGAGC	III
SPNS1	GTAGTGCGGGAGCCGCCAAG	CCCAGGACTCCGGTCAGGCA	III
SH3YL1	TACACCGCGGCCTGCTCAAG	TCCCCAGGCTGCTGTCCTTCA	III
ACP1	TGTCGATCACCCATTGCAGAAGC	TGCAGCACCTGACACACTGC	III
BAG6	GTTCCGAGTGCTCCCACTGGC	CGCCCTGCAGTGTCCCAGAG	III
TRA2B	GTCATGAGCGACAGCGGCGA	ATGGCTGTGGCTGTGCCGTC	III
LAT	TGGTGCCAACAGTGTGGCGAG	AGACGCTTCTGCGCTCTCCC	III

4.2.6 Total gene expression analysis

Quantitative RT-PCRs were conducted in a 7500 Real-Time PCR System (Applied Biosystems) using cDNA as template (equivalent of 3 ng total RNA) and gene-specific primers that placed on constitutive exons flanking a region which was not subject to alternative splicing. PCR products were detected using SYBR Green (KAPA SYBR FAST qPCR Kit; Kapa Biosystems, Woburn, MA). Gene expression was normalized to RPLP0 (ribosomal protein, large, P0). Primers for SYBR Green PCR were designed using Primer3 v. 0.4.0. (163). Primer information is presented in Table 7. Additionally, expression of HNRNPA1, *SF3A1*, *SFRS7* and *TRA2B* was determined by the TaqMan Gene Expression Assays (Applied Biosystems) (Hs01656228_s1, Hs01066327_m1 and Hs01032694_g1 respectively) according to the protocol and their expression was normalized to *RPLP0* expression (Hs99999902_m1).

Table 7. Primer pairs used for detection of total gene expression

PCR primer	Primer forward (5′ – 3′)	Primer reverse (5′ – 3′)	Study
TCF7L2 total	TGTCTCTAACAAAGTGCCAGTG	GGTAAGTGTGGAGGTGGGTTT	I
INSR total	ACCCCAATGGCAACATCACCCAC	TGGACAGGAGCAGCATTCGCC	II
RPLP0	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC	I, II

4.2.7 Genotyping

In study I, genomic DNA was isolated from whole blood, and used as a template for genotyping of *TCF7L2* rs7903146 with the TaqMan Allelic Discrimination Assay (Applied Biosystems).

4.2.8 Statistical analysis

Data analyses were conducted with the SPSS/Win programs (SPSS Inc., Chicago, IL). Data from the same individual at the different time points (KOBS and VLCD studies) were compared with paired t tests, whereas data between study groups with unpaired two-tailed t tests. Correlations were assessed with nonparametric Spearman correlations. A P value <0.05 was considered statistically significant. Logarithmic transformation was used to obtain normal distribution whenever needed (indicated in the tables and figures). Descriptive statistics are presented as mean ± SDs (163).

5 Results

5.1 ADIPOSE TISSUE *TCF7L2* SPLICING IS REGULATED BY WEIGHT LOSS AND IS ASSOCIATED WITH GLUCOSE AND FATTY ACID METABOLISM

In Study I (also reviewed in R IV) we investigated alternative splicing of *TCF7L2* in the context of obesity surgery induced weight loss and the association of *TCF7L2* splicing with the levels of plasma glucose and serum free fatty acids (FFAs) in three independent study cohorts (n=216 combined: KOBS n=54, EUGENE2 n=113 and METSIM n=49). Furthermore, we investigated the possible association between *TCF7L2* splicing and rs7903146 polymorphism associated with T2DM.

We have demonstrated reduced expression of the short mRNA transcript variant (lacking exons 12, 13, 13a and 13b) after weight loss both in subcutaneous fat (n=46) and also in the liver (n=11) (Figure 8).

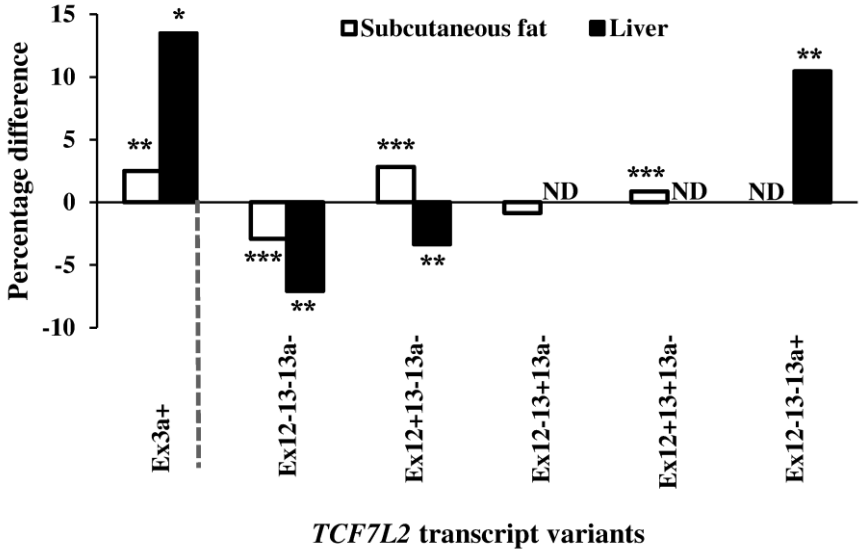

Figure 8. Change in expression level of *TCF7L2* splice variants during the year after gastric bypass surgery in subcutaneous fat and liver. *p<0.05; **p< 0.01; ***p< 0.001, paired t-test. ND- not detected (163).

In addition, observed expression of the short mRNA transcript variant was higher among individuals with T2DM than in those with normal glucose tolerance in both KOBS and METSIM studies (Figure 9A). The short mRNA transcript variant positively correlated with the level of fasting glucose in individuals of all three studies (Figure 9B). Finally, short mRNA transcript variant exhibited positive correlation with FFAs in both subcutaneous and visceral adipose tissue depots (Figure 9C). We observed the association between short mRNA transcript variant and high levels of serum FFAs during hyperinsulinemia suggesting impaired insulin action in adipose tissue (Figure 9D). We did not observe any association with insulin secretion (Figure 9E) or insulin-stimulated whole body glucose uptake (Figure 9F) in the EUGENE2 study. We did not find an association between splicing and common risk SNP, which may be due to a relatively small sample size. Finally, we observed no change in total *TCF7L2* expression suggesting that effect on splicing is independent of transcription regulation.

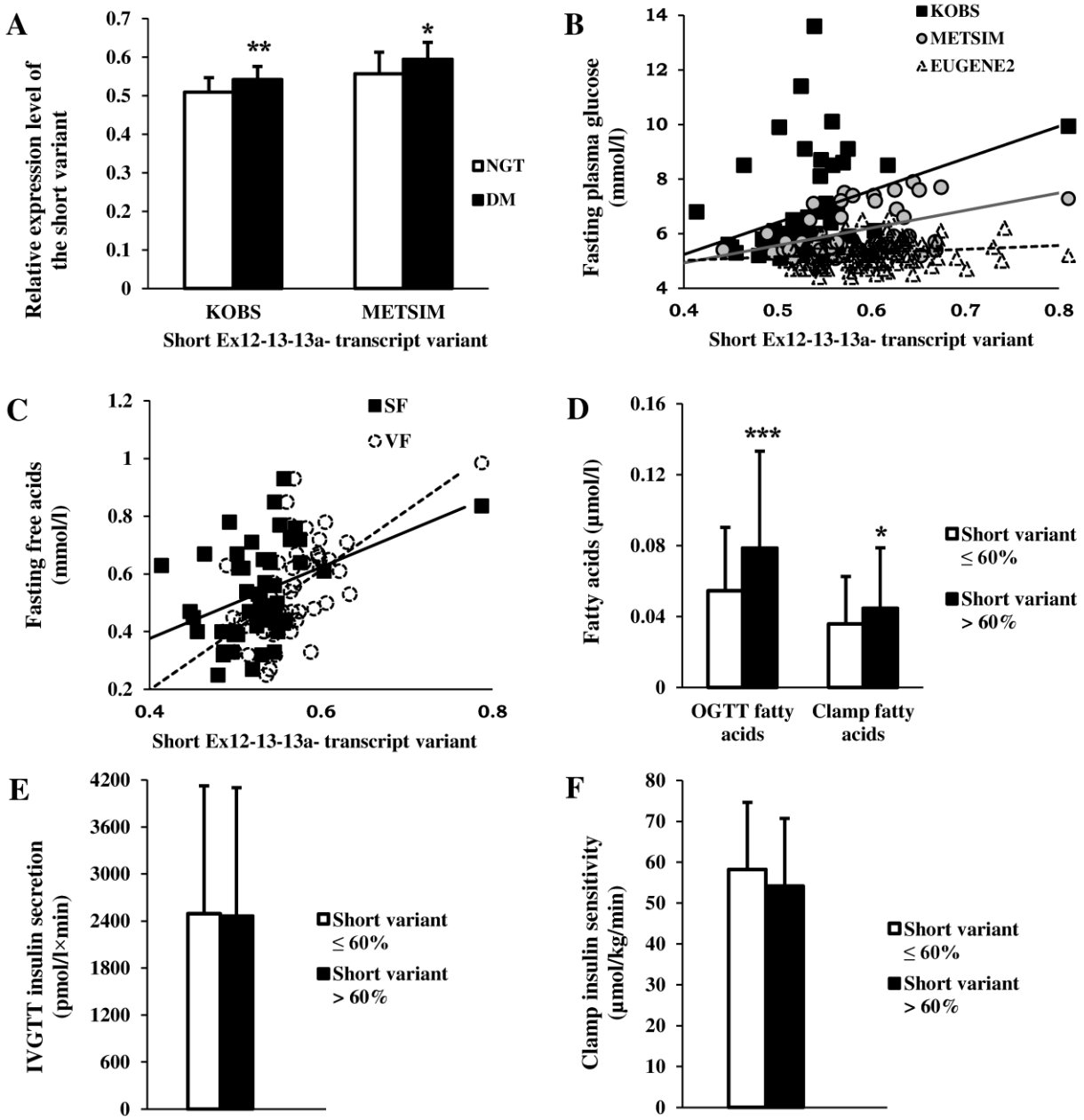

Figure 9. **A.** Prevalence of the short *TCF7L2* transcript variant in subcutaneous fat in individuals with and without T2DM in the KOBS and METSIM studies. Mean±SD shown. *p<0.05; **p<0.01. **B.** Scatter plot demonstrating correlation between the short *TCF7L2* transcript variant expression and fasting plasma glucose in the KOBS, EUGENE2 and METSIM studies in subcutaneous fat (KOBS, r=0.335, p=0,016; METSIM r=0.382, p=0.011; EUGENE2, r=0.218, p=0.026). **C.** Scatter plot demonstrating correlation between the expression of the short *TCF7L2* transcript variant in subcutaneous fat (SF, r=0.350, p=0.014) and visceral fat (VF, r=0.581, $p=1.8\times10^{-5}$) and fasting plasma glucose and serum free fatty acids (FFAs) in the KOBS study. **D.** Insulin action in adipose tissue measured as serum FFA levels at 120min after an oral glucose load (OGTT) (n=216, all studies combined) or as serum FFA levels at the end of 40mU euglycemic clamp (n=113, EUGENE2 study) in individuals with low or high prevalence of the *TCF7L2* short transcript variant (divided by the median value). Mean ± SD shown. *p<0.05; ***p< 0.001 **E.** Insulin secretion measured during intravenous glucose tolerance test (IVGTT) and **F.** insulin sensitivity measured as a whole-body glucose uptake during the hyperinsulinemic-euglycemic clamp in the EUGENE2 study in individuals with low or high prevalence of the *TCF7L2* short transcript variant (divided by the median value) (163).

In addition, we observed an increased proportion of short *TCF7L2* transcript variant, with reduced capacity to activate Wnt/β-catenin signalling, during adipocyte differentiation. Importantly, inhibition of Wnt/β-catenin signalling is well known to activate adipogenesis (170-172).

To conclude, we demonstrated that the short *TCF7L2* mRNA transcript variant, characterized by lack of exons 12,13,13a and 13b, in subcutaneous adipose tissue is regulated by weight loss and associated with hyperglycemia and impaired insulin action in adipose tissue.

5.2 ADIPOSE TISSUE *INSR* SPLICING IN HUMANS IS ASSOCIATED WITH FASTING INSULIN LEVEL AND IS REGULATED BY WEIGHT LOSS

In Study II we investigated alternative splicing of *INSR* in response to weight loss, induced either by obesity surgery or dietary intervention. Based on our earlier findings, that the expression of several RNA processing genes is reduced in obesity, we hypothesized that *INSR* splicing in adipose tissue is modified by weight loss due to changes in expression of *trans*-acting factors regulating alternative splicing. We investigated the association of *INSR* splicing with the expression of *trans*-acting factors and metabolic traits in three independent studies (n=189 combined: KOBS n=108, VLCD n=32 and METSIM n=49).

In Study II we reported for the first time that alternative splicing of *INSR* in adipose tissue is associated with BMI (Figure 10A). Furthermore, we demonstrated that alternative splicing of *INSR* in adipose tissue is regulated by weight loss induced both by gastric bypass surgery (Figure 10B) and dietary intervention (Figure 10C). The proportion of *INSR-B* mRNA variant was decreased in adipose tissue of T2DM subjects in METSIM study and similar trend was observed in KOBS study (Figure 10D). We observed strong negative correlation between insulin levels and *INSR-B* splice variant in subcutaneous adipose tissue in all three studies (Figure 10E).

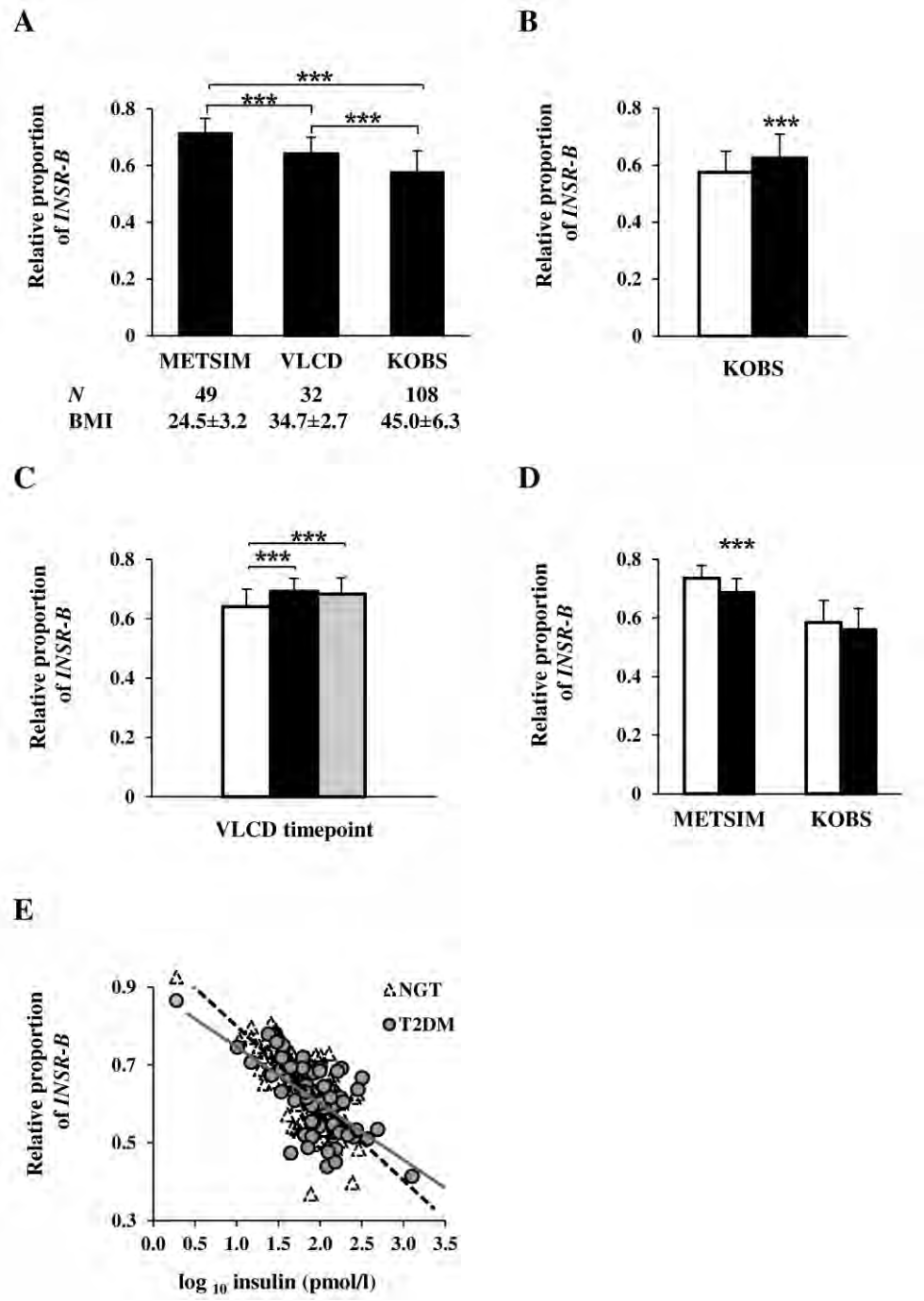

Figure 10. The relative proportion of *INSR-B* in subcutaneous fat (SF) (**A**) in the METSIM, VLCD and KOBS studies at baseline and in response to (**B**) obesity surgery in the KOBS (white bar, baseline; black bar, 1 year postsurgery) and (**C**) to a 7 week very low calorie diet (VLCD) followed by a 24 week weight-maintenance (WM) period (white bar, baseline; black bar, 7 weeks VLCD; grey bar, 24 weeks WM). (**D**) Relative proportion of the *INSR-B* variant in individuals with and without type 2 diabetes in the METSIM and KOBS studies. White bars, non-diabetic subjects; black bars, subjects with type 2 diabetes (T2DM) (**E**) Scatter plot demonstrating the correlation of *INSR-B* with logarithmically transformed fasting insulin levels in pooled samples from the KOBS, VLCD and METSIM studies (r=-0.649, p=3×10^{-22}). White triangles, non-diabetic subjects (ND, n=123, r=-0.676, p=4×10^{-16}); grey circles, subjects with type 2 diabetes (T2DM n=66, r=-0.601, 3×10^{-7}). Mean±SD shown. ***p<0.001 (A,B) vs. baseline of same study (C) and vs. non-diabetic subjects of same study (181).

We did not detect any change in total expression of *INSR* gene, indicating that effect on splicing was independent on transcription. Finally, *INSR* splicing was correlated with expression of *HNRNPA1,* a known regulator of *INSR* splicing (182).

To conclude, we demonstrated that *INSR* splicing is strongly associated with fasting insulin levels and is regulated by weight loss, possibly through the alterations in the *trans*-acting factors expression.

5.3 REGULATION OF ALTERNATIVE SPLICING IN HUMAN OBESITY LOCI

Recent GWAS analyses increased the number of loci associated with obesity risk. Yet, the mechanisms of action by which these loci increase susceptibility to obesity remain unclear. Most of the functional polymorphisms are non-coding, i.e., they are situated in intronic or intergenic regions. However, non-coding polymorphisms can influence either the regulation of transcription or act at the level of regulation of mRNA processing leading to change in protein expression or protein sequence. Reduced expression of several *trans*-acting factors in the liver and muscle of obese individuals suggests an important role of alternative splicing in human obesity (22). The role of genetic regulation of splicing in obesity was suggested based on enrichment of rare SNPs in intronic splicing regulatory regions of obese subjects (20).

We postulate that alternative splicing of genes in obesity loci may contribute to obesity. To this aim we selected 136 genes in 11 obesity loci based on analysis of approximately 40000 individuals (38, 183) and we screened 25 genes, coding for different protein isoforms (Figure 11).

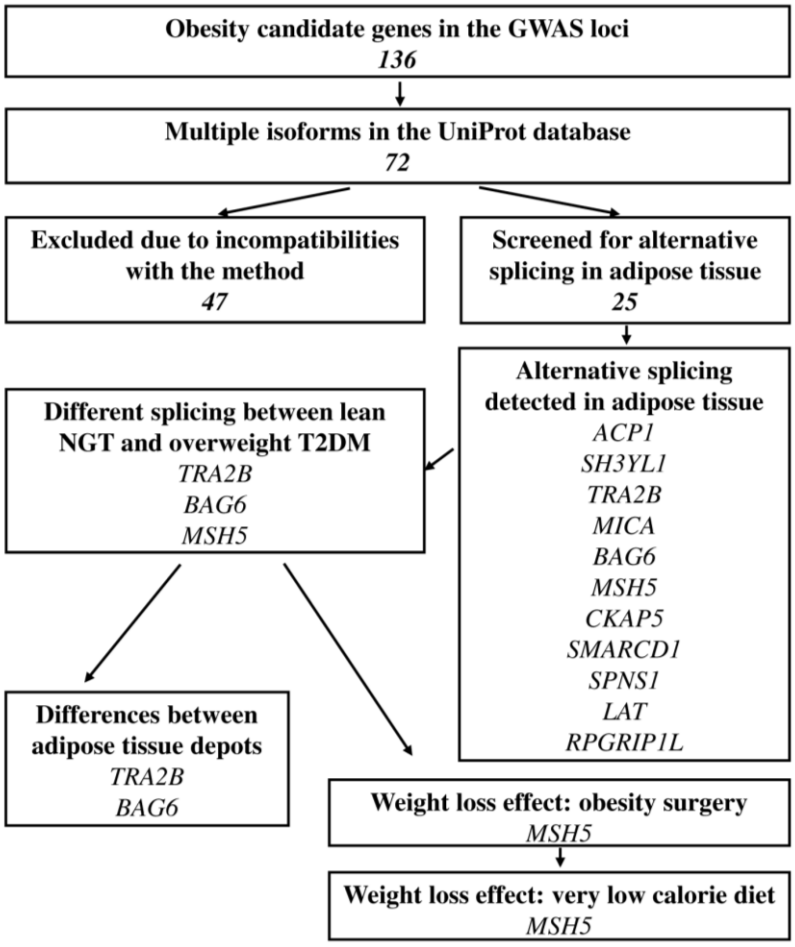

Figure 11. Analysis pipeline to identify alternatively spliced variants in the obesity loci.

We detected alternative splicing of 10 genes in obesity loci, three of which (*TRA2B, BAG6, MSH5*) were differently spliced between lean normoglycemic and overweight T2DM subjects (Figure 12).

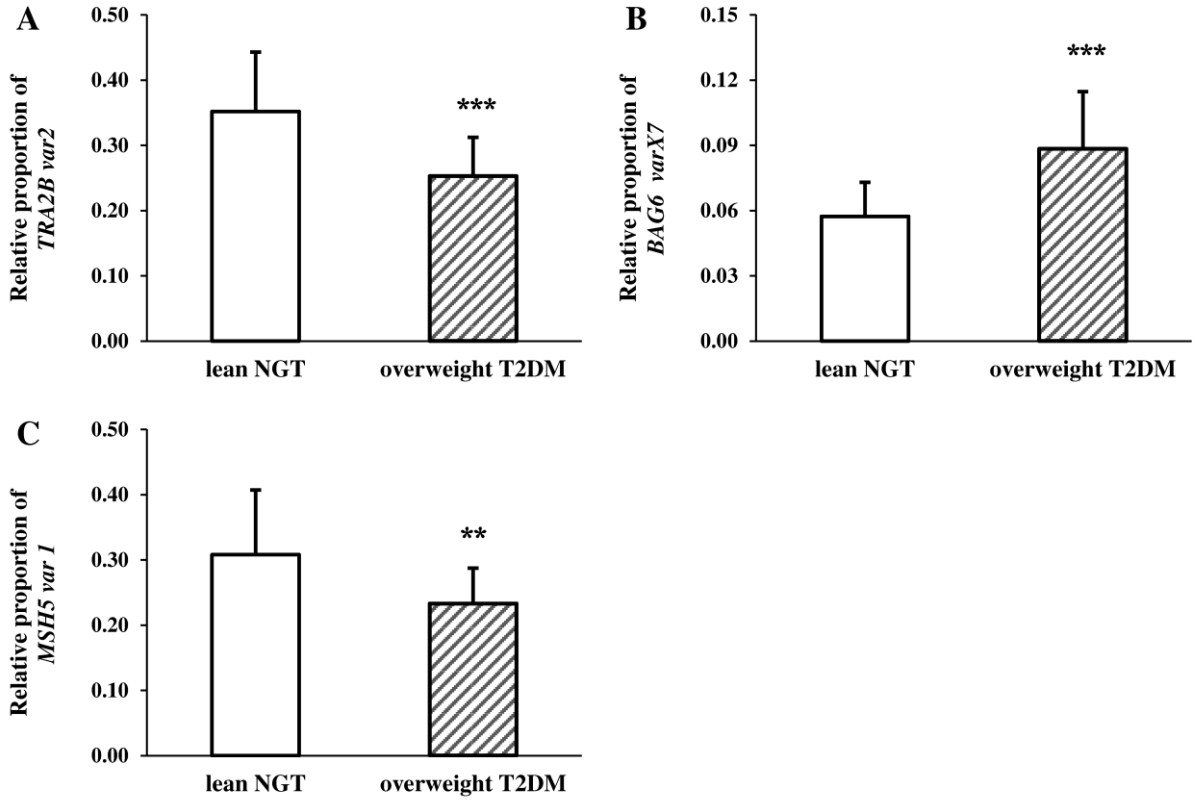

Figure 12. The relative proportion of (a) *TRA2B var2* (b) *BAG6 varX7* and (c) *MSH5 var1* in the subcutaneous fat of the lean normoglycaemic individuals (NGT) and overweight type 2 diabetic individuals in the METSIM study. Mean±SD shown. Comparison made using t- test, **p< 0.01, ***p<0.001.

In addition, we detected different splicing pattern of *BAG6 varX7* and *TRA2B var2* transcript variants between subcutaneous and visceral fat depots (Figure 13).

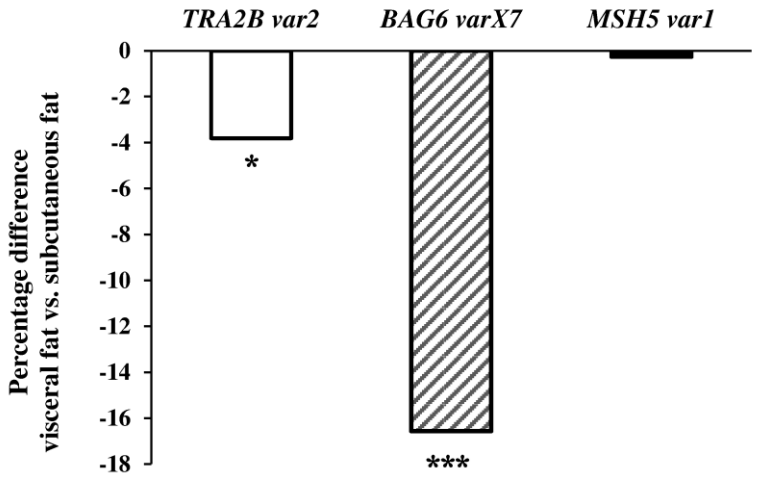

Figure 13. Difference in expression level of *TRA2B*, *BAG6* and *MSH5* splice variants between subcutaneous fat and visceral fat in the KOBS study. Mean±SD shown. Comparison made using paired t- test, *p< 0.05, ***p<0.001.

Moreover, our analysis revealed that *MSH5* splicing was regulated by weight loss in two independent intervention (KOBS and VLCD) studies (Figure 14).

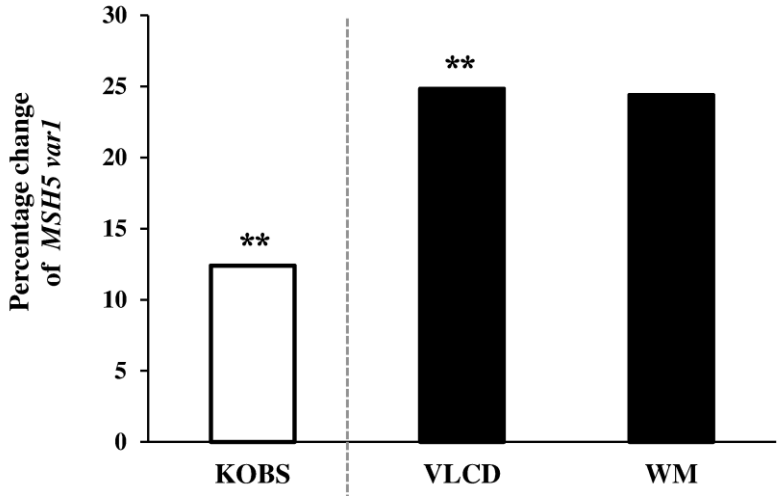

Figure 14. Change in expression level of *MSH5 var1* during the year after gastric bypass surgery (white bar, KOBS study) and during a 7 week very low calorie diet (VLCD) followed by a 24 week weight-maintenance (WM) period (black bar, VLCD study) in subcutaneous fat. Comparison made using paired t- test, **p< 0.01.

Finally the forward stepwise multivariate analysis indicated that BMI was an independent determinant of *TRA2B, BAG6* and *MSH5* splicing profile in pooled data from the METSIM and KOBS studies. Age was the only determinant of *TRA2B* splicing in visceral fat.

To conclude, our study identifies alternative splicing in obesity loci. These findings imply that the obesity-associated SNPs might act through regulation of splicing which in turn might underlie the pathogenesis of obesity in individuals carrying the risk SNPs.

6 Discussion

6.1 REGULATION OF *TCF7L2* BY OBESITY AND WEIGHT LOSS (STUDY I)

TCF7L2 gene, coding for transcription factor 7-like 2, is the most important candidate gene for T2DM, confirmed in many populations. Since its discovery in 2006 (184), several research groups have reported associations of *TCF7L2* rs7903146 polymorphism with impaired insulin secretion, linking the polymorphism function with the pancreatic β-cell (105, 185). TCF7L2 is known to play a role at the last stage of the canonical Wnt/β-catenin signalling pathway, regulating the expression of a set of target genes. Despite the obvious association between T2DM risk and *TCF7L2*, the mechanism through which *TCF7L2* has effects on T2DM remains very unclear (186).

In the Study I we demonstrated, that weight loss regulates alternative splicing of candidate gene, *TCF7L2,* in subcutaneous fat and liver (Figure 8). Subcutaneous adipose tissue splicing of *TCF7L2* is associated with obesity, levels of fasting plasma glucose and FFAs. Importantly, we observed an association between *TCF7L2* splicing and fasting FFAs in both subcutaneous and visceral fat. Additionally, we observed the association between *TCF7L2* splicing pattern and insulin action in adipose tissue (Figure 9D). The observed correlations are highly statistically significant but they are relatively weak which results from both, relatively small sample size and human genetic diversity. We did not observe any change in total gene expression following weight reduction. Our results are in agreement with the earlier reports with respect to distribution of transcript variants in adipose tissue and liver (159, 160). We did not find the association between rs7903146 genotype of *TCF7L2* and transcript variants, but we cannot exclude the possibility of an association with our relatively small sample size for genetic study.

TCF7L2 splicing can affect cell metabolism through modifying Wnt/β-catenin signalling, as it was shown that transcript variant lacking exons 12,13, 13a and 13b has impaired ability to induce Wnt/β-catenin signalling (168, 169). Exclusion of exons 13 and 13a results in lack of CRARF and CRALF motifs (165, 167) required for the activation of Wnt/β-catenin target promoters (168). The exact pathways responsible for the change in *TCF7L2* splicing after obesity surgery should be investigated in experimental studies. Aberrant splicing of *TCF7L2* in obesity could be related to insulin resistance. Insulin has been shown to regulate *trans*-acting factors (22, 187) and their reduced expression was demonstrated in obesity (22).

To summarize, in Study I we demonstrated that the short *TCF7L2* transcript variant characterized by lack of exons 12, 13, 13a and 13b in subcutaneous adipose tissue is regulated by weight loss and is associated with high levels of plasma glucose and impaired insulin action in adipose tissue. Findings of the Study I could be explained by impaired ability of the short *TCF7L2* transcript variant to activate Wnt/β-catenin signalling in adipose tissue. These results suggest that the regulation of *TCF7L2* splicing in adipose tissue may contribute to increased diabetes risk (Figure 15).

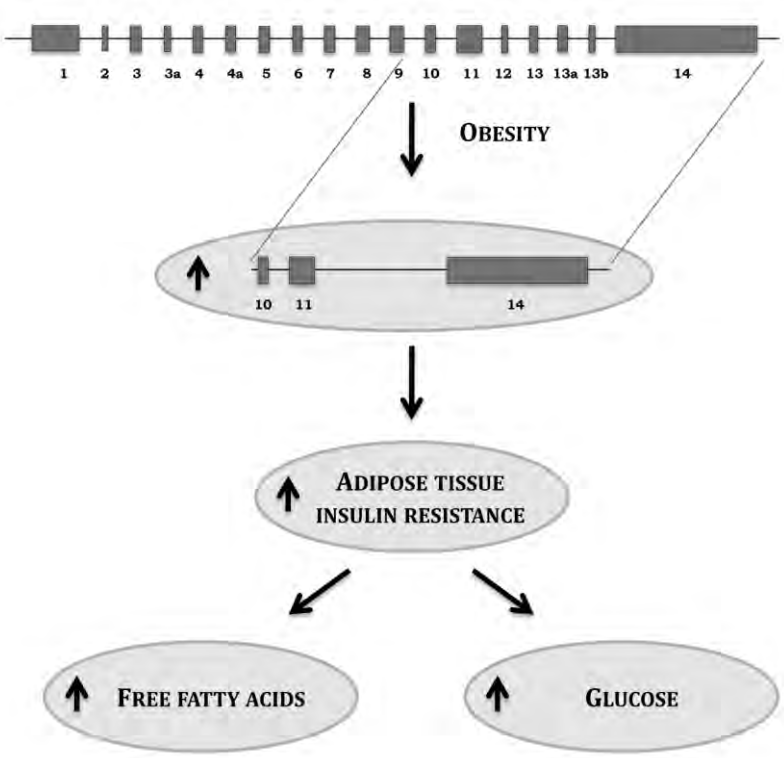

Figure 15. Alternative splicing of exons 12-13b of *TCF7L2* can lead to a short less functional isoform. This isoform associates with adipose tissue insulin resistance and with elevated levels of serum free fatty acids and plasma glucose. This suggested model is based on our study published in Diabetes (2012, vol. 61, (11), pages 2807-2813) (188).

6.2 REGULATION OF *INSR* BY OBESITY AND WEIGHT LOSS (STUDY II)

The insulin receptor, coded by the *INSR* gene, exists in two isoforms, which are result of alternative splicing of 36 nucleotide-long exon 11. Both *INSR* variants were shown to be expressed in developmentally and tissue-specific manner. The *INSR-A* (lacking exon 11) variant is predominantly expressed in fetal cells and plays a role in fetal development, whilst the *INSR-B* (including exon 11) variant is expressed in differentiated cells. INSR-A binds insulin, IGF-II and proinsulin, while INSR-B binds highly specifically insulin. As a result INSR-A predominantly promotes growth and INSR-B regulates glucose homeostasis (173). Almost thirty years after discovery of the two insulin receptor splice variants, the role of INSR isoforms in T2DM remains unclear since data reported by different groups are contradictory (173).

In Study II we demonstrated that weight loss regulates alternative splicing of *INSR* in adipose tissue, regardless of the weight reduction approach. The *INSR* splicing pattern depends on BMI, with reduced expression of *INSR-B* transcript variant in obese subjects. The proportion of *INSR-B* mRNA variant, the more active isoform in insulin signalling (189), was increased in response to weight loss induced by both obesity surgery and VLCD (Figure 10A-C). We observed higher expression of *INSR-B* was higher in normoglycaemic individuals than T2DM subjects in METSIM study. But the strongest detected correlation in all examined studies was with insulin levels (Figure 10E). Our analysis revealed that fasting insulin is the main determinant of the *INSR* splicing in subcutaneous adipose tissue, which is in line with a previous study in monkeys, suggesting a link between hyperinsulinemia and *INSR* splicing (190, 191). Importantly, insulin levels were strongly negatively correlated

with *INSR-B* proportion both in subcutaneous and visceral fat indicating common insulin action regulation.

Aberrant splicing of *INSR* in obesity could be related to insulin levels, as it is known that insulin regulates *trans*-acting factors activity (22, 187, 192) and their reduced expression was demonstrated in obesity (22). Moreover, we observed a correlation between splicing pattern of *INSR* and expression of *HNRNPA1,* previously reported to inhibit exon 11 inclusion in HepG2 and HEK293 cells (182). We acknowledge that other regulators of *INSR* splicing exist (182), and they may also be modified by weight loss. The main limitation of this study is that INSR protein isoforms created by alternative splicing could not be detected because the difference between the protein isoforms is only 1 kDa.

To summarize, in Study II we demonstrated that the adipose tissue *INSR-B* transcript variant, characterized by inclusion of exon 11, is regulated by weight loss and is strongly associated with fasting insulin. Findings of the Study II could be explained by changes in splicing factor activity through phosphorylation (192). We propose that changes in adipose tissue *INSR* splicing in response to obesity could be mediated by changes in expression of splicing factors (Figure 16).

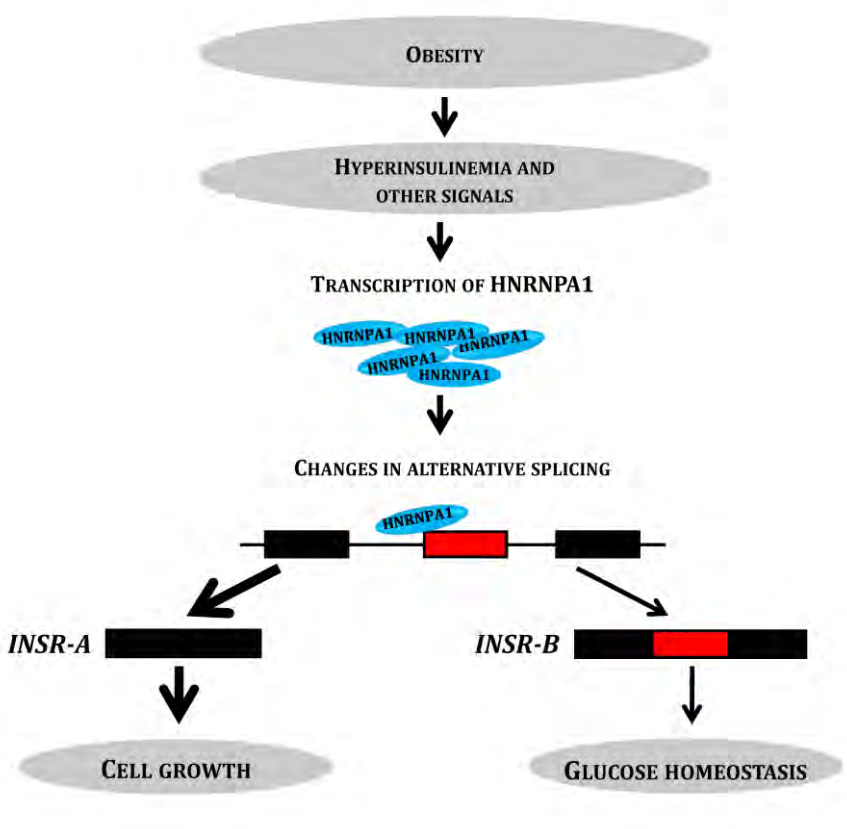

Figure 16. Proposed mechanisms for changes in alternative splicing of *INSR* gene in obesity. Obesity induced changes in transcription of *trans*-acting factors, e.g. *HNRNPA1*, result in altered splicing pattern of *INSR* gene favouring INSR-A variant involved in cell growth.

6.3 IDENTIFICATION OF ALTERNATIVE SPLICING OF GENES IN OBESITY LOCI (STUDY III)

During past years GWAS analyses revealed several loci associated with obesity, yet the causal gene and mechanism in most cases remains elusive. The attempts to resolve the functional significance of these loci are challenging since majority of them are located in

intronic or intergenic regions. One possibility is that SNPs in the obesity loci could affect splicing of genes.

In Study III we applied bioinformatics approach and conducted PCR capillary electrophoresis to investigate the importance of alternative splicing of genes in obesity loci. We demonstrated the evidence that 10 out of 136 genes residing in obesity loci are alternatively spliced in human adipose tissue. We identified altered splicing pattern between T2DM and non-diabetic individuals in 3 out of 11 genes (MSH5, BAG6 and TRA2B). Furthermore, we detected different splicing patterns of *BAG6* and *TRA2B* genes between subcutaneous and visceral fat depots. Moreover, we have identified lower expression of *MSH5var1* in overweight T2DM subjects and increase of this variant following weight loss, regardless of the weight reduction approach used.

We demonstrated that BMI was an independent determinant of *TRA2B* splicing in subcutaneous fat. *TRA2B* splicing has a known autoregulatory function, with low expression levels of TRA2B inducing the generation of variant missing exon 2 (193). Additionally, expression of *TRA2B* was demonstrated to be reduced in skeletal muscle and liver of obese individuals (22). Thus, our observations support the role of *TRA2B* in human obesity (22).

We also detected lower proportion of *BAG6 varX7* in visceral compared to subcutaneous adipose tissue. BAG6 is involved in apoptosis, gene regulation and is a regulator of protein synthesis and degradation (194). A mechanism on how the aberrant splicing of *BAG6* contributes to obesity remains to be determined.

MSH5 protein plays a role in development and meiosis (195) and in non-meiotic tissues in DNA damage repair (196). The function of MSH5 in adipose tissue metabolism remains unknown. The *MSH5* gene is subject to alternative splicing and exists in three protein isoforms. Functional properties of these protein isoforms are unknown. Polymorphisms within *MSH5* gene have been associated with many human diseases such as cancer, immune diseases and reproductive disorders (197). Additionally, we investigated the effect of inflammatory factors on the *MSH5* splicing pattern in SGBS cell strain, but we observed, if any, the opposite trend than expected suggesting that reduced inflammation associated with weight reduction is not responsible for observed changes in three independent studies. Statistical analyses revealed that BMI is a main determinant of *TRA2B, BAG6* and *MSH5* splicing in subcutaneous fat.

To summarize, in Study III we identified alternative splicing in obesity loci. We identified altered splicing pattern between obese T2DM and lean non-diabetic individuals suggesting the importance of alternative splicing in pathogenesis of obesity and/or T2DM. We showed the fat depot dependent regulation of alternative splicing of *BAG6* and *TRA2B*. The significance of these variants needs to be further investigated. Finally, we have observed reduced expression of *MSH5var1* in obese T2DM subjects and weight loss induced increase of this variant in two independent weight reduction studies. The function of alternative splicing of *MSH5* in adipose tissue and its possible implications to obesity requires further examination.

6.4 EVALUATION OF THE METHODS

6.4.1 Study subjects

This work was based on the results from four study cohorts (KOBS, METSIM, EUGENE2 and VLCD) all of them well characterized and the subjects were phenotyped in detail. Subjects for weight intervention studies were carefully selected; subjects were obese (VLCD) and severely obese (KOBS). All subjects of this study had a one day visit to the hospital or Clinical Research Unit at the University of Eastern Finland, including an

interview on their history of previous diseases, current drug treatments and cardiovascular risk factors. All study subjects were of Finnish descent.

6.4.2 Alternative splicing analysis

We implemented PCR capillary electrophoresis for sensitive detection of subtle changes in the splicing pattern. The benefit of this method is that many transcript variants can be analyzed at the same time using single PCR. Internal size standard allows for determination of the analyzed fragment size. These factors make PCR capillary electrophoresis a great choice of method for candidate gene study. Relative abundance of each transcript variant was determined by dividing the area under the peak by the total of all peak areas. The advantage of this method is that there is no need to use endogenous control. Additionally, the change in relative ratio between existing splice variants is not affected by a small fluctuations in the total transcript level. The method provides exceptional sensitivity and transcript size accuracy which in turn allows the identification of the transcript variants and the quantification of the ratio of all transcript variants examined. The PCR capillary electrophoresis method do not allow for absolute quantification of the variants. There are however few limitations of the method to be mentioned. The PCR capillary electrophoresis method is suitable for analysis of single gene, not a high-throughput analysis which is the main limitation of the methods. Additionally, primers spanning the regions around alternatively spliced regions allow for detection of cassette exons but PCR based method is not suitable for detection of alternative transcription start site and alternative poly (A) site events. These limitations can be overcome by implementation of the high-throughput transcriptome assessment technologies. Development of the high-throughput techniques, such as RNA-sequencing (RNA-seq), enables the investigation of alternative splicing patterns on a genome wide scale. RNA-seq provides millions of short sequence reads from one- or both-ends of the sequenced fragment, which results in large scale and high sensitivity transcript data. However, analysis of RNA-seq data, especially in the presence of genes with large numbers of alternative transcripts has turned this into a challenging task due to the complexity of the information.

6.4.3 Total gene expression

Quantitative RT-PCR method was implemented in order to determine the total gene expression level. The method is sensitive and allows quantification of total gene expression. The data obtained with qPCR are relative and require normalization using the reference gene, which is the main limitation of the method.

6.5 CONCLUDING REMARKS (REVIEW IV) AND FUTURE PLANS

The overall function of alternative splicing is to increase transcriptome variability and coding capacity of the genome. Aberrant splicing leading to disease can result from point mutation within *cis*-acting elements (137, 138) or misregulation of the splicing machinery by environmental stimuli. There are numerous obesity related genes regulated by alternative splicing. The finding, that expression of several RNA processing genes is reduced in liver and skeletal muscle of obese humans, suggests that aberrant splicing in obesity might be a common phenomenon. It is important to notice that although *trans*-acting factors and alternative splicing events can be modulated in obesity they can also contribute to the metabolic status, as demonstrated for trans-acting factor, TRA2B, and its target-exon 6 of *LPIN1* gene (22). The aberrant splicing in obesity might be related to insulin levels since activity of *trans*-acting factors, SR (192) and HNRNP proteins (198), were shown to be regulated by insulin (22, 187, 192). Therefore, both, insulin resistance observed in obese

individuals and improved insulin signalling accompanying weight loss may influence splicing (Figure 17).

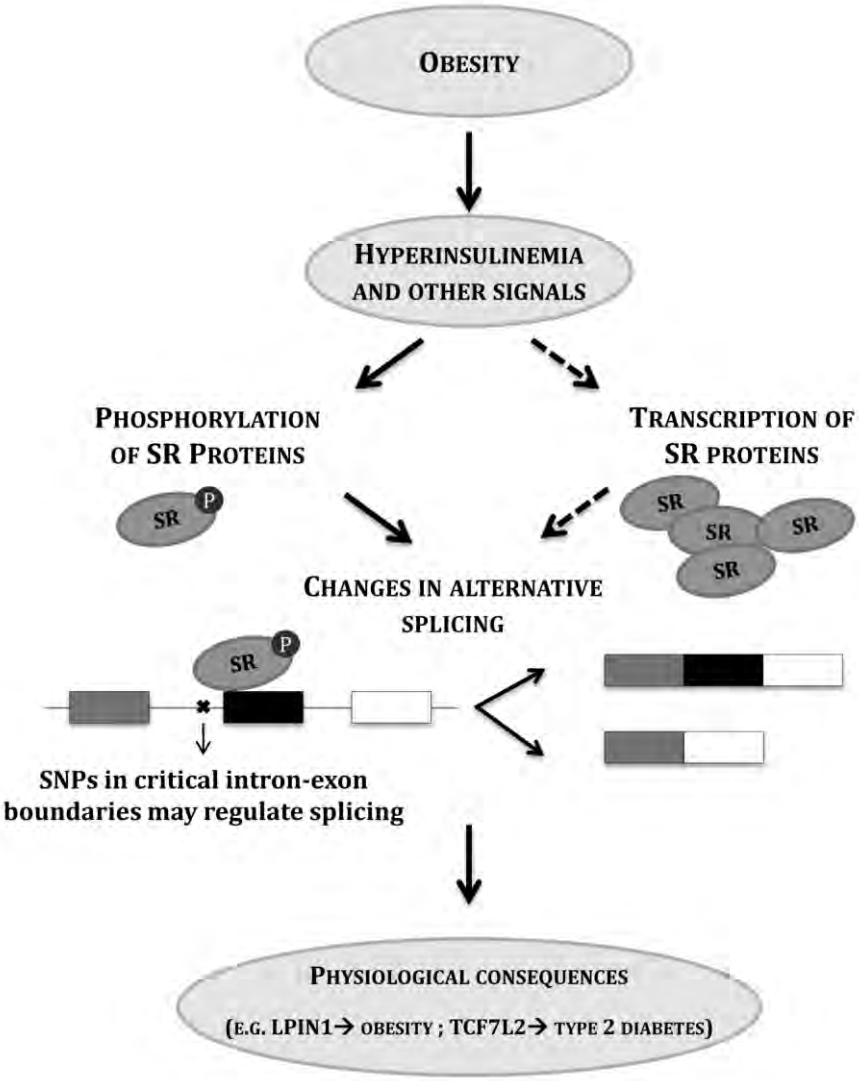

Figure 17. Proposed mechanisms for changes in alternative splicing in obesity. Changes in phosphorylation (P in the figure) and transcription of SR proteins may alter splicing leading to protein isoforms with different function. These effects may interact with single nucleotide polymorphisms (SNPs) in critical intron-exon boundaries. Dashed arrows refer to our own recently published data in Cell Metabolism (2011, vol. 14, (2), pages 208-2018)(188).

There is undisputed evidence that both obesity and T2DM are hereditary conditions. In spite of the fact that GWAS analyses have identified several genetic risk variants for obesity and T2DM, the SNPs identified so far only explain a fraction of the obesity (50) and T2DM (107) risk. The missing heritability may result from rare variants (55), copy number variants (55), interaction effects between different SNPs (199), epigenetic modifications (59-63), gene-gene (G×G)(57, 58) and gene environment (G×E) (54) interactions that are not detected in GWAS. It is important to point out that regulation of alternative splicing might be affected at several levels, as shown in Figure 5, such as transcription, epigenetic modifications or environmental stimuli, which provides a possible link between missing heritability and alternative splicing. Studies described here, providing the evidence that alternative splicing of candidate genes is affected by weight loss, indicate an important role of alternative splicing in mediating the gene-environment interaction.

Study III, describing a systematic evaluation for alternative splicing in GWAS loci, revealed the need of utilizing the whole transcriptome RNA sequencing to detect all splicing events in a large sample size with a longitudinal setting allowing for investigation of intra-individual regulation. Our future plans include a genome wide analysis of alternative splicing in adipose tissue and its relation to risk gene variants in the METSIM and KOBS studies. Additionally, we aim to determine the association between alternative splicing and weight loss in KOBS study. Genotyping and RNA-seq analysis on samples extracted from the KOBS and METSIM studies will be performed in collaboration with Prof. Päivi Pajukanta at the University of California, Los Angeles (UCLA). This systematic study will provide a better understanding of the relationship between alternative splicing, genotypes and obesity and/or T2DM on genome wide scale.

There is certainly much more to discover about alternative splicing function in metabolic pathways and pathophysiology of metabolic diseases. The number of genes affected as well as *trans*-acting factors affecting alternative splicing in metabolic disorders is very likely to expand. Future studies, implementing genome wide analyses, should help in identifying the role of alternative splicing in metabolism, but also provide opportunities for new diagnostic and possible personalized therapeutic approaches.

7 Summary

The main findings of Studies I-III were as follows.

Study I: Alternative splicing of *TCF7L2* is regulated by weight loss and is associated with high levels of plasma glucose and serum FFAs. These findings could be explained by different ability of *TCF7L2* splice variants to activate Wnt/β-catenin signalling in adipose tissue.

Study II: Alternative splicing of *INSR* is regulated by weight loss and is strongly associated with fasting insulin. These findings could be explained by changes in splicing factor activity

Study III: Genes in obesity loci are regulated by alternative splicing. These findings imply that the obesity-associated SNPs might act through regulation of splicing which in turn might underlie the pathogenesis of obesity in individuals carrying the risk SNPs. Adipose tissue depot dependent splicing of *TRA2B* and *BAG6* and weight loss induced regulation of *MSH5* were demonstrated. Additionally, BMI was shown to be a main predictor of *TRA2B*, *BAG6* and *MSH5* splicing.

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ORIGINAL PUBLICATIONS (I-IV)

Dorota Kamińska

Regulation of Alternative Splicing in Obesity and Weight Loss

Many human diseases can be caused by impairment of alternative splicing or its regulation. The main aim of the present study was to investigate interactions between alternative splicing of the genes and obesity. This thesis provides new information on the role of alternative splicing in pathogenesis of obesity and type 2 diabetes. These findings increase our understanding of splicing dysregulations and can improve the chances for a more targeted treatment and more accurate diagnostics.

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