

# MOLECULAR MECHANISMS IN OBESITY- ASSOCIATED METABOLIC DISEASE

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# ABSTRACT

Obesity is associated with increased morbidity and mortality. Subjects with obesity are at risk of developing several serious conditions such as type 2 diabetes, hypertension, coronary heart disease and stroke. This thesis aimed to identify genes that are implicated in the development of these obesity-associated metabolic diseases and to further increase our knowledge about these genes in relation to disease.

Adipose tissue, especially intra-abdominal adipose tissue, is tightly linked to metabolic disease. Identification of genes predominantly expressed in adipocytes can give new insights into adipocyte function and may thereby provide important information about genes involved in the development of obesity-associated metabolic disease. The acute-phase protein serum amyloid A (SAA) was unexpectedly found to be predominantly expressed in adipocytes during the nonacute-phase. Since SAA has been suggested to have multiple atherogenic effects, the production of SAA in adipose tissue may be a link between obesity and atherosclerosis.

Potential susceptibility genes for obesity-associated metabolic disease were identified based on their altered expression in adipose tissue from obese individuals with the metabolic syndrome compared with controls that persisted even when the disease phenotype was temporarily improved by a very low calorie diet treatment. Using this approach, S100 calcium binding protein A1 (S100A1), Zn-alpha2-glycoprotein (ZAG), and CCAAT/enhancer binding protein alpha (C/EBP $\alpha$ ) were identified as potential susceptibility genes. Subsequent genetic association study revealed a link between S100A1 and resting metabolic rate. A common ZAG genotype was associated with reduced ZAG gene expression, reduced serum levels of ZAG and low serum total cholesterol levels in humans. This genotype was also associated with coronary artery disease, which may be a result of decreased serum levels of adiponectin or HDL. Furthermore, data from studies in mice suggest that ZAG influences cholesterol synthesis. Thus, studies in both humans and ZAG-deficient mice showed a link between ZAG and cholesterol. Studies of the transcription factor C/EBP $\alpha$  showed that it is induced by insulin and in turn regulates multiple genes in the lipid and glucose metabolism including adiponectin, hexokinase 2, lipoprotein lipase, diacylglycerol O-acyltransferase 1 and 2.

In conclusion, SAA, S100A1, ZAG and C/EBP $\alpha$  were identified as potential susceptibility genes for obesity-associated metabolic disease using two different expression-based approaches. Our subsequent studies of these genes linked them to metabolic parameters known to influence or to be associated with metabolic disease e.g. resting metabolic rate, serum cholesterol levels and glucose and lipid metabolism.

# LIST OF PUBLICATIONS

This thesis is based on the following papers:

- I **Evaluation of Reference Genes for Studies of Gene Expression in Human Adipose Tissue**  
BG Gabriëlsson, LE Olofsson, A Sjögren, M Jernås, A Elander, M Lönn, M Rudemo, and LMS Carlsson  
*Obes Res. 2005 Apr;13(4):649-52*
  
- II **A Microarray Search for Genes Predominantly Expressed in Human Omental Adipocytes: Adipose Tissue as a Major Production Site of Serum Amyloid A**  
K Sjöholm, J Palming, LE Olofsson, A Gummesson, PA Svensson, TC Lystig, E Jennische, J Brandberg, JS Torgerson, B Carlsson, and LMS Carlsson  
*J Clin Endocrinol Metab. 2005 Apr;90(4):2233-9. Epub 2004 Dec 28*
  
- III **A link between *SI00A1* and human resting metabolic rate revealed by a strategy that identifies susceptibility genes for complex diseases**  
LE Olofsson, B Olsson, P Jacobson, L Pérusse, L Sjöström, C Bouchard, B Carlsson, and LMS Carlsson  
*Manuscript*
  
- IV **Zn-alpha2-glycoprotein is a susceptibility gene for metabolic disease influencing the cholesterol homeostasis**  
LE Olofsson, B Olsson, A Gummesson, P Jirholt, TC Lystig, K Sjöholm, P Jacobson, M Olsson, M Ståhlman, S Romeo, L Sjöström, P Eriksson, A Hamsten, LP Hale, DS Thelle, J Borén, B Carlsson, and LMS Carlsson  
*Manuscript*
  
- V **C/EBP $\alpha$  regulates genes in lipid and glucose metabolism and is dysregulated in subjects with the metabolic syndrome**  
LE Olofsson, L William-Olsson, K Sjöholm, B Carlsson, LMS Carlsson, and B Olsson  
*Manuscript*

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## ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
Apo	apolipoprotein
BMI	body mass index
CEBP $\alpha$	CCAAT/enhancer binding protein alpha
CETP	cholesteryl ester transfer protein
DGAT	diacylglycerol O-acyltransferase
DNA	deoxyribonucleic acid
EL	endothelial lipase
ER	endoplasmic reticulum
HDL	high density lipoprotein
HL	hepatic lipase
HMG-CoA Reductase	3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase
IDL	intermediate density lipoprotein
IL	interleukin
Insig	insulin-induced gene
kDa	kilodalton
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LMF	lipid mobilizing factor
LPL	lipoprotein lipase
LRP10	low density lipoprotein receptor-related protein 10
NCEP	National Cholesterol Education Program
NEFA	non-esterified fatty acid
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase 1
PLTP	phospholipid transfer protein
PPAR $\gamma$	peroxisome proliferator-activated receptor-gamma
QFS	Quebec Family Study
QTL	quantitative trait locus
RNA	ribonucleic acid
RMR	resting metabolic rate
S100A1	S100 calcium binding protein A1
SAA	serum amyloid A
SCAP	SREBP cleavage-activating protein
SCARF	Stockholm Coronary Atherosclerosis Risk Factor
SNP	single nucleotide polymorphism
SOS	Swedish Obese Subjects
SREBP	sterol regulatory element-binding protein
TG	triglyceride
TNF $\alpha$	tumor necrosis factor alpha
TZD	thiazolidinediones
VLCD	very low calorie diet
VLDL	very low density lipoprotein
WHO	World Health Organization
WHR	waist-hip ratio
ZAG	Zn-alpha2-glycoprotein

## BACKGROUND

### ***Obesity and associated metabolic disease***

Overweight and obesity are defined as excessive fat accumulation that may impair health. Body mass index [BMI; BMI=weight (kg)/height<sup>2</sup> (m<sup>2</sup>)] is commonly used to classify overweight and obesity. Individuals are classified as overweight or obese if their BMI are  $\geq 25$  or  $\geq 30$  kg/m<sup>2</sup>, respectively. The International Obesity TaskForce has estimated that presently 1.1 billion adults worldwide are overweight including 312 million who are obese<sup>1</sup> and that this number will continue to rise in the early 21st century. Subjects with obesity are at risk of developing one or more serious medical conditions including type 2 diabetes, hypertension, coronary heart disease, stroke, as well as some types of cancers<sup>2,3</sup>. Obesity is thus associated with increased mortality<sup>4</sup>.

### ***Risk factors for cardiovascular disease***

Cardiovascular disease, including coronary artery disease and stroke, is rapidly increasing in prevalence in the wake of the obesity epidemic<sup>5</sup>. Several risk factors for cardiovascular disease have been identified<sup>6</sup>. These include:

- **Heredity** - Studies have estimated that the heredity of cardiovascular disease is approximately 40-60%<sup>7</sup>. For more details regarding the genetics of this disease see below.
- **Age** - Atherosclerosis begins at young age and increases in prevalence with age. Already at the age of 15, atherosclerotic lesions have formed<sup>8</sup>. However, according to the American Heart Association over 83% of the subjects dying from cardiovascular disease are 65 years or older.
- **Gender** - In middle-age individuals, coronary heart disease is 2 to 5 times more common in men than in women, which may partly be explained by sex differences in the major risk factors for coronary heart disease<sup>9</sup>.
- **Obesity/overweight** - Obesity, especially visceral obesity, is tightly linked to metabolic disease. Adipose tissue is known to be an endocrine organ, secreting factors that affect other organs in the body. The importance of the adipose tissue in metabolic disease is discussed in more detail below.
- **Smoking** - Smoking is a risk factor for myocardial infarction and stroke<sup>10,11</sup>. The risk of stroke decreased significantly two years after smoking cessation and returned to the level of nonsmokers after five years<sup>11</sup>.

- **Blood lipids** - Epidemiological studies have shown a positive correlation between serum total cholesterol and mortality in cardiovascular disease. However, serum total cholesterol is not the best predictor of cardiovascular disease, since it is the sum of the cholesterol in the atherogenic very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) but also the atheroprotective high density lipoprotein (HDL). In a meta-analysis including 58 trials, a LDL cholesterol reduction of 1.0 mmol/L reduced the risk of ischemic heart events by 11% in the first year of treatment, 24% in the second year, 33% in years three to five, and by 36% thereafter<sup>12</sup>. Low HDL-C is also a strong and independent risk factor for coronary artery disease, commonly occurring in subjects with coronary artery disease<sup>13</sup>. In addition, the apolipoprotein B (apoB) to apoA-I ratio is positively related to fatal myocardial infarction<sup>14</sup>. Increased serum levels of triglycerides (TG) are also associated with increased coronary artery disease<sup>15</sup>.
- **Type 2 diabetes** - It has been estimated that type 2 diabetes results in a 2-4 fold increased risk of developing cardiovascular disease<sup>16</sup>. In addition, approximately 50-75% of the deaths of subjects with type 2 diabetes are related to cardiovascular disease<sup>17</sup>.
- **Physical inactivity** - Epidemiological studies suggest that physically active individuals have a 30-50% lower risk of developing type 2 diabetes and a similar risk reduction for coronary artery disease compared to sedentary individuals. The reduced risk is observed after as little as 30 minutes moderate-intensity exercise per day<sup>18</sup>.
- **Blood pressure** - Blood pressure is a strong and consistent predictor of development of cardiovascular disease<sup>19</sup>.
- **Low-grade inflammation** - Modestly increased levels of acute-phase proteins, including serum amyloid A (SAA) and C-reactive protein (CRP), are independent risk factors for coronary artery disease in both men and women<sup>20-22</sup>.
- **Stress** - Mental stress is considered a risk factor for cardiovascular disease<sup>23</sup>.

Some of these risk factors including dyslipidemia, type 2 diabetes, elevated blood pressure, smoking, obesity and physical inactivity can be treated or avoided to prevent cardiovascular disease.



## ***Definition of the metabolic syndrome***

It is well known that risk factors for coronary artery disease rarely occur alone. Instead, risk factors tend to cluster. This condition is referred to as the metabolic syndrome. Today, several different definitions of the metabolic syndrome exist. The World Health Organization (WHO) first published its definition in 1998<sup>24</sup>. Subsequently, other definitions were proposed including the definition from the National Cholesterol Education Program (NCEP) Expert Panel<sup>25</sup>. In an attempt to reach a consensus definition, the International Diabetes Federation modified the criteria for the metabolic syndrome in 2005<sup>26</sup>. For definitions, see Table 1. Alexander et al showed that metabolic syndrome as defined by NCEP is a predictor of prevalent coronary artery disease<sup>27</sup>. The NCEP-definition of metabolic syndrome was associated with a 2-fold increase in age-adjusted risk of fatal cardiovascular disease in men and non-fatal cardiovascular disease in women<sup>28</sup>. The definition by WHO resulted in slightly lower increase in risk. Metabolic syndrome defined by NCEP is also associated with increased mortality<sup>29</sup>. The prevalence of NCEP-defined metabolic syndrome is increasing and is now estimated to affect 21.8% of the adults in US<sup>30</sup>. The prevalence of the metabolic syndrome as defined by WHO was investigated in eight studies from seven different European countries. The overall prevalence of the metabolic syndrome was 14% in men and 4% in women under 40 years, 23% and 13% respectively for 40 to 55 years, and 41% and 26% respectively over 55 years of age<sup>31</sup>.

## ***Cardiovascular disease -Atherosclerosis***

Atherosclerosis is a progressive disease in which atherosclerotic lesions are formed in the vascular wall. These lesions may develop and cause coronary artery disease and stroke. The atherosclerotic lesions are thickenings of the innermost layer of the vascular wall and consist of lipids, fibrous connective tissue, cells, and debris<sup>32</sup>. The earliest stages of the atherosclerotic lesion are called fatty streaks. In this stage, the number of macrophages in the intima is increased and macrophages filled with lipids appear (foam cells)<sup>33</sup>. As the fatty streak develops, more macrophages are accumulated together with extracellular lipid pools and smooth muscle cells containing lipid droplets. A core of extracellular lipids is formed, as the atherosclerotic lesion evolves into an advanced one. In this stage, layers of fibrous connective tissues may also be formed. The advanced atherosclerotic lesions differ from one another. Some become calcified while others mainly consist of connective tissue<sup>32</sup>.

**Table 1. Definitions of metabolic syndrome from WHO, NCEP and IDF.**

<b>Component</b>	<b>WHO diagnostic criteria (impaired glucose regulation<sup>a</sup> plus at least two of the following)</b>	<b>NCEP Expert Panel diagnostic criteria (at least three of the following)</b>	<b>IDF diagnostic criteria (central obesity plus at least two of the following)</b>
Abdominal/central obesity	Waist to hip ratio: >0.90 (men), >0.85 (women), and/or BMI >30 kg/m <sup>2</sup>	Waist circumference: >102 cm in men, >88 cm in women	Ethnic specific waist circumference <sup>26</sup> . If BMI>30 kg/m <sup>2</sup> , central obesity is assumed
Dyslipidemia	Plasma triglycerides ≥ 1.7 mmol/L and/or HDL cholesterol < 0.9 mmol/L for men, <1.0 mmol/L for women	Plasma triglycerides ≥ 1.7 mmol/L and/or HDL cholesterol <1.036 mmol/L for men, <1.295 mmol/L for women <sup>b</sup>	Plasma triglycerides > 1.7 mmol/L and/or HDL cholesterol <1.03 mmol/L for men, <1.29 mmol/L for women <sup>b</sup>
High blood pressure	SBP ≥ 160 mm Hg / DBP ≥ 90 mm Hg	SBP ≥ 130 mm Hg / DBP ≥ 85 mm Hg	SBP ≥ 130 mm Hg / DBP ≥ 85 mm Hg
Impaired glucose regulation	Impaired glucose tolerance, impaired fasting glucose, insulin resistance, or diabetes <sup>24</sup>	Fasting blood glucose ≥ 6.1 mmol/L	Fasting plasma glucose ≥ 5.6 mmol/L and/or previously diagnosed type 2 diabetes
Microalbuminuria	Urinary albumin to creatinine ratio: 20 mg/g, or albumin excretion rate: 20 µg/minute		

WHO = World Health Organization; NCEP = National Cholesterol Education Program; IDF, International Diabetes Federation; BMI = body mass index; HDL = high-density lipoprotein. <sup>a</sup>Impaired glucose regulation is defined as glucose intolerance, type 2 diabetes and/or insulin resistance. <sup>b</sup>Individuals with high serum triglycerides and low HDL-cholesterol fulfill two criteria.

When the lesion grows, it decreases the diameter of the artery lumen and causes stenosis. The blood flow and oxygen delivery to the tissue supplied by the artery are then reduced. However, the main cause of infarction is not the progressive narrowing of the arteries, but disruption of the lesion surface, hematoma or hemorrhage and thrombosis. Some lesions are more prone than others to disrupt and cause thrombosis. Several factors may decrease the stability of the lesion including presence of inflammatory cells (macrophages and lymphocytes), release of toxic substances and proteolytic enzymes as well as shear stress. Plaques that disrupt are often lipid-rich<sup>34</sup>, and it has been found that serum cholesterol, in particular elevated ratio of total cholesterol to high density lipoprotein cholesterol (HDL-C), predispose patients to rupture of vulnerable plaques<sup>35</sup>.

### ***Adipose tissue distribution and metabolic disease***

Adipose tissue can be divided into intra-abdominal and subcutaneous adipose tissue. The intra-abdominal adipose tissue is also called visceral adipose tissue and is located inside the peritoneal cavity. The subcutaneous adipose tissue is located beneath the skin. In addition to visceral and subcutaneous adipose tissue, muscle has been shown to contain a relevant amount of lipids<sup>36,37</sup>. Several anthropometric measurements are currently used to describe regional obesity including circumferences of waist and hip, ratio of waist-hip circumferences (WHR) and sagittal diameter<sup>38</sup>. The visceral depot is more closely associated to cardiovascular disease and type 2 diabetes compared with subcutaneous adipose tissue<sup>39</sup>. This divergence may be a result of depot differences in location in relation to other organs, function and response to signals. Unlike subcutaneous fat, visceral fat drains directly into the portal vein, which transports the blood to the liver. It is therefore believed that the visceral depot can affect hepatic lipid and glucose metabolism to a greater extent compared to the subcutaneous depot<sup>39</sup>. Visceral fat has also been reported to be resistant to insulin suppression of lipolysis<sup>40,41</sup> and the tissue is sensitive to  $\beta$ 3-adrenergic stimulation of lipolysis<sup>42,43</sup>. The resulting non-esterified fatty acid (NEFA) flux to the liver may lead to altered liver metabolism including increased hepatic glucose production<sup>44</sup>.

### ***Storage of triglycerides in adipose tissue***

Most of the body's energy is stored as triglycerides and glycogen. Adipose tissue is the main energy reservoir and stores mainly triglycerides. Triglycerides contain three fatty acid molecules esterified to one glycerol molecule. The synthesis of triglycerides has previously been extensively reviewed<sup>45</sup>. Triglycerides can be synthesized from glucose, which during glycolysis forms

glycerol-3-phosphate. Glycerol-3-phosphate is then further metabolised through a series of reactions, catalyzed by mitochondrial glycerol-3-phosphate acyltransferase (GPAM), glycerol-3-phosphate O-acyltransferase (GPAT3), phosphatidic acid phosphohydrolase (PAP2) and diacylglycerol O-acyltransferase 1 and 2 (DGAT1 and 2), to form triglycerides. In addition, triglycerides can be formed from components of the plasma membrane i.e. phosphatidylinositol and phosphatidylcholine. This synthesis is catalyzed by phospholipase C and DGAT 1 and 2. The transcription factors sterol regulatory element-binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), and liver X receptor are important regulators of triglyceride synthesis.

### ***Lipolysis in adipose tissue***

The breakdown of triglycerides stored in the adipose tissue is known as lipolysis. In this reaction, triglycerides are hydrolyzed into free fatty acids and glycerol. This process is important during starvation to supply other organs with fuel and to provide the liver with substrates for the gluconeogenesis and lipoprotein synthesis<sup>46,47</sup>. It was recently discovered that during lipolysis the first ester bond in triglycerides is predominantly catalysed by adipose triglyceride lipase. The resulting diacylglycerols are mainly hydrolysed by hormone sensitive lipase (HSL) and the hydrolysis of monoacylglycerol is performed by monoglyceride lipase<sup>48</sup>. In addition to these lipases, several proteins known as PAT-proteins including perilipin, adipocyte differentiation-related protein, and tail interacting protein TIP-47 are important in lipolysis<sup>49</sup>. The mobilization of triglycerides is tightly regulated. The insulin-mediated inhibition and catecholamine-mediated stimulation of lipolysis are well characterized. The catecholamine stimulation is mediated by binding to the  $\beta$ 3 adrenergic-receptor leading to phosphorylation of HSL and perilipin by protein kinase A. Phosphorylation of HSL activates this enzyme. In addition, phosphorylation of perilipin is essential for translocation of HSL to the surface of the lipid droplets<sup>49</sup>. In addition to insulin and catecholamines, other hormones and adipokines regulate lipolysis including growth hormone, glucocorticoids, atrial natriuretic peptide, leptin, resistin, TNF- $\alpha$ , IL-6, and adiponectin<sup>48</sup>. However, the mechanisms involved in the regulation by these factors are unknown or not fully understood.

### ***Adipose tissue as an endocrine organ***

Adipose tissue secretes factors, so called adipokines, that affect other organs in the body (Figure 1). These include adipokines that effects energy regulation,

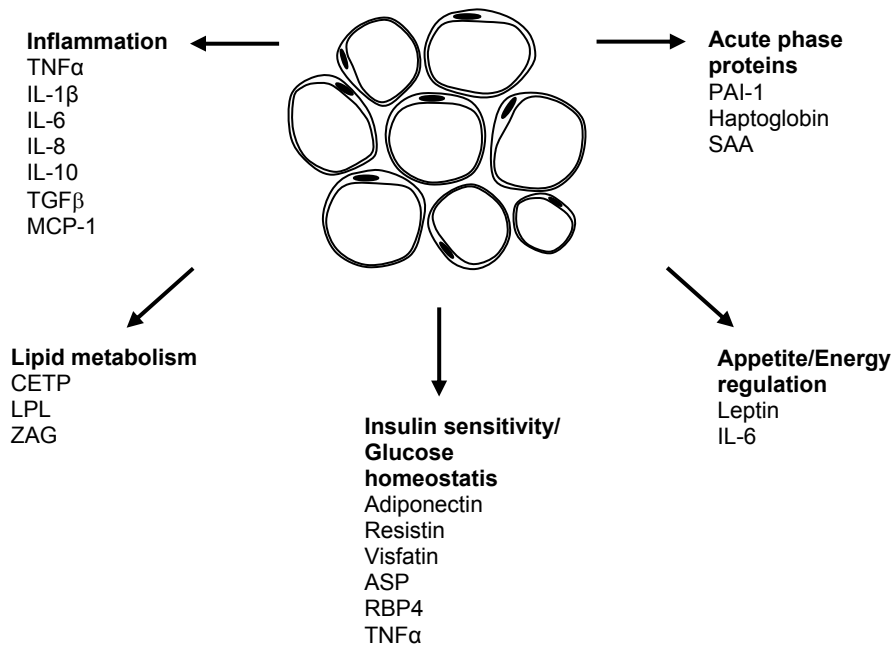
insulin sensitivity, lipid metabolism, and inflammation. Leptin and adiponectin are two of the most studied adipokines.

Leptin was originally cloned in 1994 and lack of this hormone causes obesity in ob/ob-mice<sup>50</sup>. Leptin is secreted by the adipose tissue and regulates appetite and food intake by signalling to the central nervous system<sup>51,52</sup>. Serum leptin concentrations correlate with percent body fat and decrease during weight loss<sup>53</sup>. Leptin-deficiency in humans is rare and results in obesity, hyperinsulinemia, dyslipidemia and immune dysfunction<sup>54,55</sup>. These disturbances are improved by leptin-treatment<sup>55</sup>.

Adiponectin is a 244 amino acid protein of approximately 28 kDa that occurs in the circulation as low molecular weight oligomeres and high molecular weight multimeres<sup>56</sup>. Adiponectin is secreted from adipose tissue and appears to have multiple beneficial and protective effects including anti-inflammatory, vasculoprotective and anti-diabetic effects<sup>56</sup>. Studies of adiponectin-deficient mice show that these mice have impaired insulin sensitivity<sup>57,58</sup>. In contrast to leptin, mRNA and serum levels of adiponectin decrease with increasing adipose tissue mass<sup>59,60</sup>. These adipokines illustrates the importance of adipose tissue as an endocrine organ.

### ***Insulin resistance and adipose tissue***

Insulin resistance is a condition where normal levels of insulin fail to evoke normal response in target tissues e.g. liver, skeletal muscle, and adipose tissue. Insulin stimulates glucose up-take in skeletal muscle and adipose tissue and suppresses the endogenous glucose production in the liver<sup>61</sup>. These effects result in decreased blood glucose concentration in response to insulin. In individuals with insulin resistance, the response to insulin is limited. To compensate for the loss of action,  $\beta$ -cells in pancreas secrete more insulin leading to hyperinsulinemia<sup>62</sup>. This compensatory hypersecretion of insulin is a result of both expansion of  $\beta$ -cell mass and alterations in  $\beta$ -cell metabolism<sup>62</sup>. In subjects with insulin resistance, hyperinsulinemia can keep the glucose levels within a normal range for some time. However, glucose levels are mildly increased in these subjects, which may be toxic to the  $\beta$ -cells and cause  $\beta$ -cells dysfunction<sup>63,64</sup> and cell death. Some studies have found a reduced number of  $\beta$ -cells in individuals with type 2 diabetes<sup>65</sup>.



**Figure 1. Adipose tissue is an endocrine organ secreting adipokines that affect energy regulation, insulin sensitivity, lipid metabolism, and inflammation.** TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; IL, interleukin; TGF $\beta$ , transforming growth factor  $\beta$ ; MCP-1, monocyte chemotactic protein 1; CETP, cholesteryl ester transfer protein; LPL, lipoprotein lipase; ZAG, Zn-alpha2-glycoprotein; C3, complement component 3; RBP4, retinol-binding protein 4; PAI-1, plasminogen activator inhibitor 1; SAA, serum amyloid A.

Insulin resistance is strongly associated with obesity<sup>64</sup> and studies indicate several mechanisms by which adipose tissue is implicated in the pathology. Hormones, cytokines and NEFA, secreted from the adipose tissue, can affect insulin signalling. Both obesity and type 2 diabetes are associated with increased serum levels of NEFA<sup>66,67</sup>. The release of NEFA is important in modulating insulin sensitivity<sup>68</sup>. It inhibits both insulin-stimulated glucose up-take in skeletal muscle and stimulates gluconeogenesis in the liver. Furthermore, NEFA may be harmful for  $\beta$ -cells and thus contribute to its abnormal function during development of type 2 diabetes<sup>69</sup>. In addition to the effects mediated by NEFA, adipose tissue secretes hormones, including adiponectin, retinol-binding protein 4, visfatin and resistin, that can affect insulin sensitivity. The important role of adipose tissue in the pathology of insulin resistance is also demonstrated by the fact that the class of anti-diabetic drugs called Thiazolidinediones (TZD) act via the transcription factor peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), mainly expressed in adipose tissue<sup>70</sup>. TZD decrease insulin resistance partly by modulating the expression of adipokines including adiponectin, retinol binding protein-4 and leptin<sup>71,72</sup>. In addition, DGAT1 mRNA are positively

correlated with insulin sensitivity and increase in response to TZD. These data suggest that lipid storage in adipose tissue can prevent peripheral lipotoxicity<sup>73</sup>.

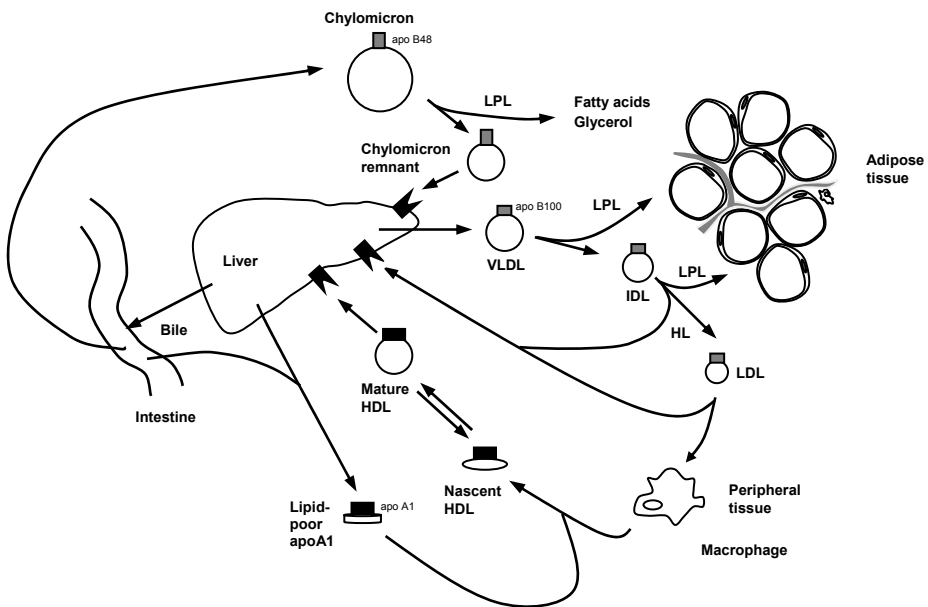
### ***Lipoproteins –synthesis and metabolism of apoB-containing lipoproteins***

Lipids are transported in the blood in complex with apolipoproteins forming so-called lipoprotein particles. These particles have a neutral core consisting of cholesterol esters and TG. The surface of the particles is a monolayer of the partly hydrophilic molecules; phospholipids and free cholesterol. The apolipoproteins are embedded in this surface layer. The lipoproteins are classified based on their density and include chylomicrons, VLDL, IDL, LDL, and HDL. Multiple apolipoproteins have been discovered and are constituents of the lipoproteins<sup>74</sup>. ApoB100 and apoB48 are encoded by the same gene. Post-transcriptional deamination of cytidine to a uridine in apoB48 results in a stop-codon instead of the glutamine codon present in apoB100. Hence, apoB48 is identical to the N-terminal part of the apoB100. ApoB100 is mainly synthesised in human liver, where VLDL is assembled and secreted into the bloodstream. The production rate of VLDL is primarily controlled by the availability of neutral lipids i.e. triglycerides and cholesteryl esters<sup>47</sup>. The apoB100 production is also important. However, apoB is produced in excess and will therefore not regulate the VLDL production under most physiological conditions. In contrast to apoB100, apoB48 is mainly produced in the intestine and is a constituent of chylomicrons. VLDL and chylomicrons are metabolized by lipoprotein lipase (LPL), hepatic lipase (HL), and cholesteryl ester transfer protein (CETP) in the circulation. LPL and HL hydrolyze triglycerides and phospholipids present in circulating plasma lipoproteins<sup>75,76</sup>. CETP promotes redistribution of lipids among lipoproteins with a net transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins (VLDL and chylomicrons), and LDL and of triglycerides from triglyceride-rich lipoproteins to LDL and HDL<sup>77</sup>. During the processing of VLDL and chylomicrons, the metabolites IDL, LDL and chylomicron remnants are formed (Figure 2). Removal of these remnants from the bloodstream occurs mostly by uptake mediated by the LDL-receptor, the LDL receptor-related protein and the macrophage scavenger receptor (MSR1)<sup>78-82</sup>.

### ***Reverse cholesterol transport***

HDL is important for reverse cholesterol transport i.e. removal of cholesterol from peripheral tissue and transporting it to the liver (Figure 3). The major apolipoprotein of HDL is apoA-I<sup>74</sup>. Both the liver and the intestine are able to

synthesize and secrete apoA-I. Subsequent lipidation of apoA-I is mediated by the lipid transporting protein ATP-binding cassette transporter A1 (ABCA1), which promotes efflux of unesterified cholesterol and phospholipids. Studies of mice specifically lacking hepatic or intestinal ABCA1 together with liver-specific partial gene knockdown of ABCA1 have shown that ABCA1 in liver and intestine are important in this lipidation process<sup>83-85</sup>. Although liver and intestine ABCA1 may be the most critical for lipidating newly synthesized lipid-free apoA-I, substantial cholesterol efflux to HDL occurs from other tissues.

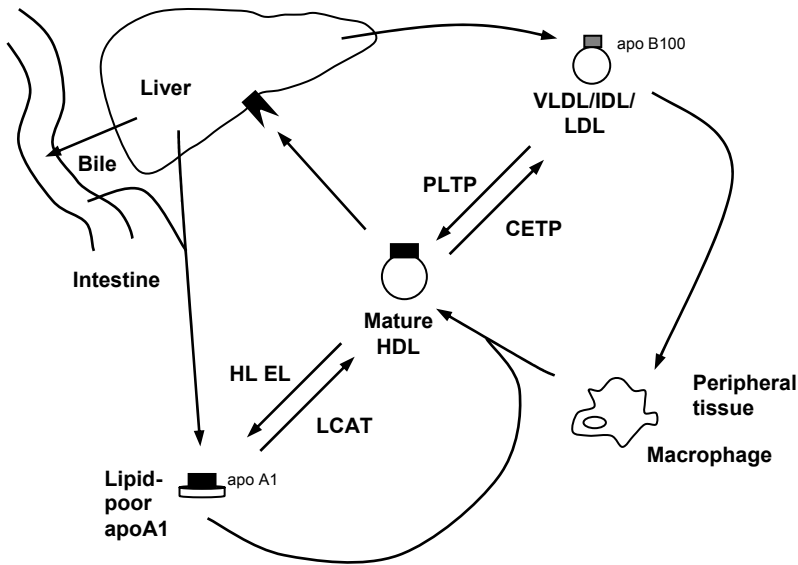


**Figure 2. Lipoprotein metabolism.** Chylomicrons and very low density lipoproteins (VLDL) are secreted from the intestine and liver, respectively. These lipoproteins are metabolized by lipoprotein lipase (LPL), hepatic lipase (HL), and cholesteryl ester transfer protein (CETP) in the circulation, forming the metabolites chylomicron remnants, intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). These metabolites are removed from the bloodstream by receptor-mediated uptake. ApoA-I is secreted from the intestine and liver, and lipidated in the blood to form mature high density lipoproteins (HDL). Apo, apolipoprotein.

In addition to ABCA1, ABCG1 also mediates cholesterol efflux from cells. However, ABCG1 only promotes cholesterol efflux from cells to HDL and other lipoprotein particles and not to lipid-free apoA-I<sup>86</sup>. Studies in mice suggest that approximately 90 mg cholesterol per kg body weight is effluxed from peripheral tissues every 24h<sup>87</sup>. In the circulation, HDL is modified by multiple proteins including lecithin:cholesterol acyltransferase (LCAT), HL, endothelial lipase



(EL), phospholipid transfer protein (PLTP), and CETP. LCAT converts cholesterol and phosphatidylcholines (lecithins) to cholesteryl esters and lysophosphatidylcholines on the surface of HDL<sup>88</sup>. PLTP is also important in remodeling of the HDL particle and has for example been found to transfer surface lipids from triglyceride-rich lipoproteins to HDL<sup>89</sup>. Scavenger receptor type B class I binds HDL with high affinity and mediates selective uptake of cholesterol<sup>82</sup>.



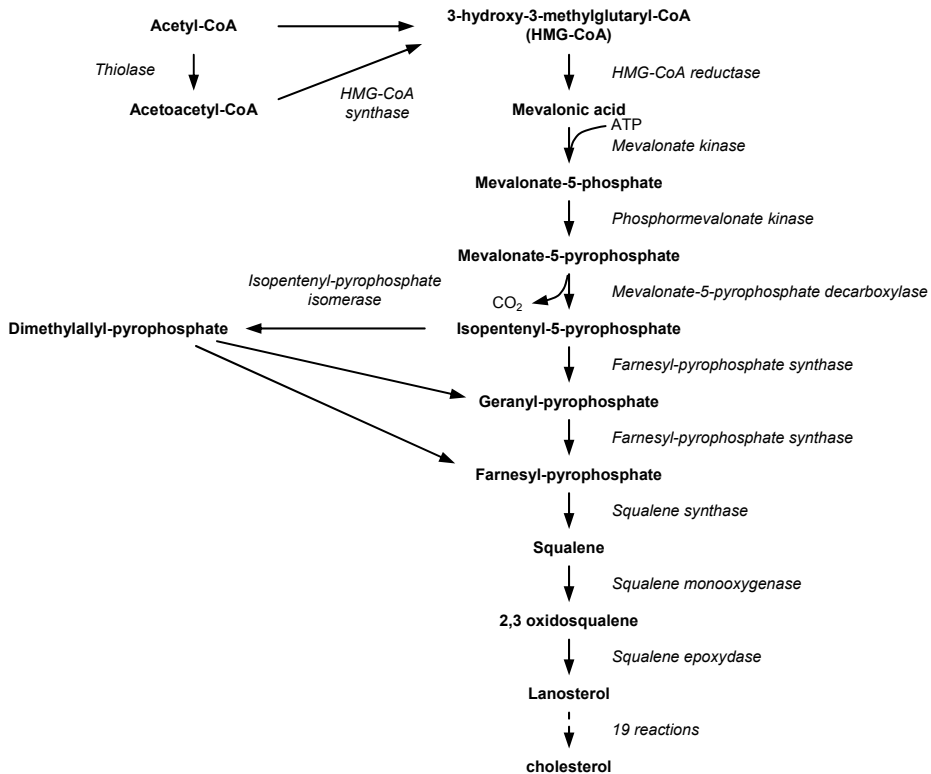
**Figure 3. Reverse cholesterol transport.** ApoA-I is secreted from the intestine and liver, and is lipidated in the blood to form mature high density lipoproteins (HDL). Multiple proteins are important for HDL remodeling in the circulation including phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), hepatic lipase (HL), endothelial lipase (EL), and lecithin:cholesterol acyltransferase (LCAT). VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein.

## Regulation of cholesterol

Approximately 1 g cholesterol is synthesized every 24 hours in humans through the reactions shown in Figure 4. When sterol synthesis was investigated *in vivo* in 18 tissues from squirrel, monkey, guinea pig, rabbit, hamster and rat it was found that when expressed as a percentage of total body synthesis, the liver of the rat produced 51%, while this figure was much lower in the monkey (40%), hamster (27%), rabbit (18%), and guinea pig (16%)<sup>90</sup>. It was concluded that most sterol utilized by extrahepatic tissues is largely synthesized locally within those

tissues. In addition to synthesis, cholesterol can also be supplied by the food. The typical diet in Western countries contains 200-600 mg cholesterol per day<sup>91</sup>. In the intestine, cholesterol absorption values range widely from approximately 30-80%<sup>92</sup>. Thus, cholesterol production constitutes the main source of cholesterol. To regulate the cholesterol content in the body, excessive cholesterol is excreted with the bile into the intestinal lumen, where it is partly re-absorbed and partly excreted with faeces. The internal cholesterol synthesis is regulated by the SREBPs, which belong to the basic helix-loop-helix-leucine zipper family of transcription factors. In the liver, cholesterol and fatty acid synthesis are regulated by three SREBPs; SREBP-1a, 1c and 2<sup>93</sup>. SREBP-1a and c are encoded by the same gene and differ in the start site of transcription leading to two different exon 1. SREBP is produced as an inactive form, which needs proteolytic activation before it can activate transcription of target genes. This proteolytic activation involves the sterol sensing protein SREBP cleavage-activating protein (SCAP). SCAP escorts SREBP from the endoplasmatic reticulum (ER), where it is translated, to the Golgi apparatus. In Golgi, SREBP is cleaved with the Site-1 and -2 proteases and the active N-terminal part of the protein is released from the membrane-bound part. The active N-terminal part of SREBP is then transported into the nucleus where it activates transcription through binding to sterol response elements in promoters and enhancer regions of target genes.

The different forms of SREBP have been found to preferentially activate different target genes. SREBP-1c for example preferentially activates enzymes in the fatty acid synthesis. SREBP-2 preferentially activates enzymes in the cholesterol synthesis. In contrast, SREBP-1a is a potent activator of all SREBPs target genes. In liver, SREBP-1c and 2 are more abundant compared to SREBP-1a. When the cholesterol content in the liver is high, insulin induced gene 1 (Insig1) binds SCAP, resulting in an inhibition of the escort of SREBP to the Golgi apparatus by SCAP. Consequently, SREBP is not proteolytically activated by Site-1 and 2 proteases and therefore does not activate transcription of genes involved in the cholesterol synthesis. 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in the cholesterol synthesis (Figure 4), is one of the genes regulated by the SREBP.



**Figure 4. Cholesterol synthesis.** Enzymes and products involved in the cholesterol synthesis are shown. HMG-CoA reductase is the rate-limiting step in the cholesterol synthesis.

## Genetics of complex disease

Complex diseases are characterized by a complex etiology and are caused by an interaction between multiple genes and environmental factors. Identification of susceptibility genes for complex diseases is difficult due to the presence of:

- **Incomplete penetrance** - A gene with incomplete penetrance will not always result in the phenotype with which it has been associated.
- **Phenocopies** – Phenocopies are individuals with the phenotype investigated, caused by environmentally factors (nonhereditary variation).
- **Locus heterogeneity** - If mutations or polymorphisms at a number of different loci result in the same phenotype.
- **Epistasis** – Genotypes at two or more unlinked loci can interact e.g. when one gene affects the expression of another gene.
- **Pleiotropy** - A locus or loci may predispose to more than one phenotype.
- **Effects of environmental factors**

To identify susceptibility genes for complex disease two approaches have been used; the candidate gene and the phenotype driven. In the candidate gene approach, genetic variations in genes with known important functions are investigated. In the phenotype-based approach, genome-wide scans and association studies are used to search for new genes.

Most forms of atherosclerosis, hypertension, dyslipidemia and type 2 diabetes are complex diseases. However, there are monogenic disorders that confer susceptibility to these diseases including familial hypercholesterolemia caused by mutations in the gene for the LDL-receptor, resulting in the inability to mediate binding, uptake and degradation of LDL<sup>94</sup> and thereby confer risk of developing atherosclerosis. In addition, a mutation in apolipoprotein B also causes hypercholesterolemia through diminished LDL-uptake<sup>95</sup>. Environmental factors e.g. smoking, diet and exercise affect the outcome in individuals with these mutations. In addition, numerous genes that confer susceptibility to the complex form of cardiovascular disease have been identified including apoE, paraoxonase-1, and 5-lipoxygenase activating protein<sup>7,96,97</sup>.

Quantitative trait loci (QTL) analysis has been used in humans and mice to identify new genes that regulate blood lipids. These analyses have resulted in identification of multiple QTLs controlling HDL-cholesterol, VLDL/LDL-cholesterol and plasma triglycerides<sup>98,99</sup>. Several of the QTLs present in humans are also present in the homologous regions in mice, suggesting that the same genes are important in humans and mice. However, the complexity of the traits complicates the identification of susceptibility genes<sup>100</sup>. The best results have been achieved using inbred mice, in which the origin of the parental alleles are known and all animals have the same genetic background. In these studies, environmental factors can be controlled. Nevertheless, several common polymorphisms have been found to be associated with dyslipidemia. For example, polymorphisms in apolipoprotein E could explain as much as 8% of the variation in LDL-cholesterol concentrations<sup>101</sup>.

## AIMS

The overall aim of this thesis was to identify susceptibility genes important in the development of obesity-associated metabolic disease. Strategies to achieve this aim have been developed and applied in the different articles and manuscripts included in this thesis. The specific aims were:

To identify a suitable reference gene for gene expression studies in human adipose tissue. (Paper I)

To identify genes predominantly expressed in omental adipocytes. (Paper II)

To identify genes with altered expression in subcutaneous adipose tissue from obese subjects with the metabolic syndrome compared with obese controls and link them to metabolic dysfunction. (Paper III-V)

## METHODOLOGICAL CONSIDERATIONS

### ***Subjects***

This thesis included participants from the Very Low Calorie Diet 1 (VLCD-1) study, VLCD-2 study, Quebec Family Study, Swedish Obese Subjects (SOS) Reference Study, Intergene, Stockholm Coronary Atherosclerosis Risk Factor (SCARF), and Dallas Heart Study.

### ***VLCD-1 study***

In the VLCD-1 study, subjects were originally included in the SOS study<sup>102</sup>. A subgroup were recruited from the SOS study to evaluate the weight loss maintenance after very low calorie diet (VLCD), and diet and behavioural support<sup>103,104</sup>. From this subgroup, 14 obese subjects with the metabolic syndrome according to slightly modified WHO criteria<sup>24</sup> and age-, sex-, and BMI-matched controls were selected for the gene expression analysis with DNA microarray in Paper I, III and IV. In Paper III-IV, genes with altered expression in subcutaneous adipose tissue from obese subjects with the metabolic syndrome compared with obese controls were identified. Table 2 shows extensive patient characteristics for the subjects included in this analysis. Biopsies and blood samples for this study were collected before (week 0), after 8 weeks (week 8) and 2 weeks after completed 16-weeks VLCD (week 18). All of the subjects with the metabolic syndrome but none of the controls had type 2 diabetes. In addition to type 2 diabetes, the subjects with the metabolic syndrome also had elevated blood pressure and/or dyslipidemia as defined by WHO (Table 1). We did not have measurements for microalbuminuria and could therefore not include this parameter in the classification.

### ***VLCD-2 study***

In total, 40 subjects (34 men and 6 women) were recruited among patients treated at the Department of Body Composition and Metabolism, Sahlgrenska University Hospital and from advertisements in the local press. The criteria for inclusion were set to BMI  $\geq$  30 and age 25-60. Subjects were divided into two groups; individuals with the metabolic syndrome according to slightly modified WHO criteria<sup>24</sup> and age-, sex-, and BMI-matched controls. Exclusion criteria were: medication (except antihypertensive therapy in the group with metabolic syndrome), pregnancy, breast feeding, type 1-diabetes mellitus, serious

**Table 2. Characteristics of subjects from the VLCD-1 study used for expression analysis with DNA microarray.**

Characteristic	Subjects with the metabolic syndrome			Controls		
	Week 0	Week 8	Week 18	Week 0	Week 8	Week 18
<i>n</i> (men/women)	14 (5/9)			14 (5/9)		
Age (years)	47.0±10.1			46.7±9.4		
BMI (kg/m <sup>2</sup> )	40.5±9.1	35.9±8.3	34.7±8.7	40.0±8.9	35.4±8.7	33.2±9.9
WHR	1.0±0.1			1.0±0.1		
Sagittal diameter (cm)	29.4±4.7			29.0±3.7		
SBP (mmHg)	159±29	143±21	150±23	130±15	120±16	131±16
DBP (mmHg)	92±20	85±14	85±12	86±11	76±9	77±9
b-Glucose (mmol/L)	9.6±2.3	7.4±2.9	7.6±2.5	4.3±0.6	4.3±0.7	4.4±0.9
Insulin (pmol/L)	26.6±15.6	14.9±6.6	16.1±9.7	14.4±9.4	11.3±8.3	11.8±10.6
Cholesterol (mmol/L)	5.7±0.7	5.3±1.1	5.5±0.9	6.0±2.1	4.8±1.0	5.3±1.2
HDL-C (mmol/L)	1.1±0.4	1.1±0.4	1.2±0.3	1.3±0.5	1.1±0.3	1.3±0.3
TG (mmol/L)	2.5±1.3	1.9±1.1	2.1±1.1	1.8±1.0	1.3±0.5	1.3±0.4

Obese subjects with the metabolic syndrome and controls matched for BMI, age and sex were treated with a very low calorie diet for 16 weeks followed by 2 weeks of gradual reintroduction of their ordinary diet. The metabolic syndrome was diagnosed according to slightly modified WHO criteria<sup>24</sup>. BMI, body mass index; HDL-C, high density lipoprotein cholesterol; TG, triglyceride.

psychiatric disorder, established coronary heart disease, malignant arrhythmias, participation in any other ongoing weight reduction study, eating disorder, history of bariatric surgery or cancer treatment, drug abuse, insufficient compliance, other significant somatic disease, smoking or unwillingness to participate. The subjects with the metabolic syndrome had diabetes, impaired glucose tolerance, or impaired fasting glucose according to WHO<sup>24</sup> and at least one of the following risk factors: (i) elevated arterial (systolic/diastolic) pressure  $\geq 140/90$  mm Hg (either value) or use of blood pressure medication; (ii) raised triglycerides ( $\geq 1.7$  mmol/L) and/or low HDL cholesterol ( $<0.9$  mmol/L). Subjects were treated with VLCD during 16 weeks followed by two weeks of gradual reintroduction of the ordinary diet. During this study period biopsies and blood samples were collected at week 0, 8, 16 and 18. At these time points, anthropometric measurements and computer tomography were also performed. In a small subgroup of the patients, sampling and measurements were also performed at three additional time points; day 3, and week 2 and 4. Data, from subjects included in the VLCD-2 study, at week 0, 8, 16, and 18 were used in Paper II, IV and V. Table 3 shows extensive patient characteristics.

Table 3. Characteristics of subjects with the metabolic syndrome and matched obese controls in the VLCD-2 study

Characteristic	Subjects with the metabolic syndrome				Controls			
	Week 0	Week 8	Week 16	Week 18	Week 0	Week 8	Week 16	Week 18
<i>n</i> (men/women)	21 (18/3)				19 (16/3)			
Age (years)	49.7 ± 9.4				44.5 ± 9.9			
BMI (kg/m <sup>2</sup> )	36.7 ± 5.0	30.8 ± 4.3	27.8 ± 4.2	27.9 ± 3.8	37.0 ± 3.9	31.2 ± 3.3	28.2 ± 3.4	28.5 ± 3.2
WHR	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Sagittal diameter (cm)	29.7 ± 2.8	23.9 ± 2.6	21.7 ± 2.3	22.0 ± 2.5	29.8 ± 3.7	24.8 ± 3.2	22.3 ± 2.7	22.3 ± 2.9
SBP (mmHg)	143 ± 17	122 ± 15	118 ± 11	127 ± 14	129 ± 14	118 ± 11	115 ± 15	116 ± 13
DBP (mmHg)	89 ± 14	74 ± 11	71 ± 10	76 ± 11	86 ± 11	76 ± 11	72 ± 11	74 ± 0
Relative ZAG expression*	26 ± 26	27 ± 24	51 ± 41	43 ± 32	40 ± 40	45 ± 59	63 ± 67	62 ± 64
ZAG (µg/mL)	160 ± 51	151 ± 28	162 ± 50	165 ± 48	179 ± 75	153 ± 37	183 ± 69	157 ± 60
Adiponectin (µg/mL)	6.7 ± 3.4	9.2 ± 4.1	11.3 ± 5.0	13.7 ± 6.9	10.0 ± 5.7	11.9 ± 8.6	14.1 ± 7.0	14.7 ± 8.1
Cholesterol (mmol/L)	5.9 ± 1.0	3.9 ± 0.9	4.3 ± 0.8	4.9 ± 0.8	5.4 ± 1.1	4.0 ± 0.9	4.6 ± 0.6	4.8 ± 0.7
HDL-C (mmol/L)	1.3 ± 0.4	1.2 ± 0.3	1.3 ± 0.3	1.4 ± 0.2	1.3 ± 0.4	1.2 ± 0.3	1.4 ± 0.3	1.4 ± 0.3
LDL-C (mmol/L)	3.6 ± 0.8	2.3 ± 0.9	2.5 ± 0.7	3.0 ± 0.7	3.4 ± 1.0	2.3 ± 0.7	2.7 ± 0.5	2.9 ± 0.6
TG (mmol/L)	2.5 ± 1.2	1.1 ± 0.2	0.9 ± 0.2	1.3 ± 0.4	1.4 ± 0.5	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.5
p-Glucose (mmol/L)	6.6 ± 1.4	4.6 ± 0.8	4.6 ± 0.8	5.3 ± 1.1	5.1 ± 0.5	4.5 ± 0.6	4.3 ± 0.4	4.6 ± 0.4
Insulin (mU/L)	19.5 ± 7.8	5.4 ± 2.1	4.1 ± 1.6	6.5 ± 3.3	14.9 ± 7.9	7.5 ± 4.8	4.4 ± 2.7	7.1 ± 4.4

Obese subjects with the metabolic syndrome and controls matched for BMI, age and sex were treated with a very low calorie diet for 16 weeks followed by 2 weeks of gradual reintroduction of their ordinary diet. The metabolic syndrome was diagnosed according to slightly modified WHO criteria<sup>24</sup>. BMI, body mass index; HDL-C, high density lipoprotein cholesterol; TG, triglyceride; VLCD, very low calorie diet. \* in subcutaneous adipose tissue.



## Quebec Family Study

Subjects from the Quebec Family Study (QFS) were included in Paper III. In this study, resting metabolic rate was measured by indirect calorimetry in a ventilated-hood system and adjusted for age, sex, stature, and body composition (fat mass and fat free mass). For these adjustments, body density was measured by hydrodensitometry i.e. underwater weighing and total fat mass was derived from the equation of Siri<sup>105,106</sup>: % body fat = [(4.95/body density)-4.5]\*100. Fat-free mass was obtained by subtracting fat mass from body mass. In the Quebec Family Study (QFS), evidence for linkage to resting metabolic rate (RMR) was observed on chromosome 1q21.1 – q21.2<sup>107</sup>. Only 56 of the total 119 families were informative and contributed to this QTL in the QFS. Table 4 shows characteristics for the subjects included in QFS and used in the analysis in Paper III.

**Table 4. Patient characteristics for subjects in the QFS.**

Characteristics	All QFS subjects	Informative	Non-informative
N (men/women)	721 (327/394)	199 (85/114)	522 (242/280)
Age (years)	38.6 ± 14.8	38.7 ± 15.7	38.2 ± 14.5
BMI (kg/m <sup>2</sup> )	26.9 ± 7.0	27.6 ± 7.7	26.6 ± 6.7
RMR (kJ/min)	1.1 ± 0.2	1.1 ± 0.3	1.1 ± 0.2
Fat mass (kg)	21.9 ± 14.0	23.5 ± 15.6	21.3 ± 13.2
Fat free mass (kg)	52.4 ± 11.1	52.8 ± 12	52.4 ± 10.6
WHR	0.84 ± 0.10	0.85 ± 0.10	0.84 ± 0.10
SBP (mmHg)	114 ± 12	113 ± 12	114 ± 13
DBP (mmHg)	71 ± 12	70 ± 13	71 ± 11
Cholesterol (mmol/L)	4.9 ± 1.1	4.9 ± 1.0	4.9 ± 1.1
HDL-C (mmol/L)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
LDL-C (mmol/L)	3.0 ± 0.9	3.1 ± 0.8	3.0 ± 0.9
TG (mmol/L)	1.5 ± 1.5	1.6 ± 0.8	1.5 ± 1.6
Glucose (mmol/L)	5.0 ± 0.9	5.0 ± 0.8	5.0 ± 1.0
Insulin (pmol/L)	72.0 ± 60.8	77.3 ± 72.7	70.2 ± 56.1

Informative subjects contributed to the suggestive linkage to RMR on chromosome 1q21. BMI, body mass index; RMR, resting metabolic rate; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride.

## ***Intergene***

The Intergene study was designed to investigate which candidate genes that could explain the hereditary part of CAD in the population from the west part of Sweden. This study was also designed to investigate the interaction between susceptibility genes for CAD and external factors such as life style and environment as well as understanding the function of the candidate genes in the pathogenesis. The study is a combined control and cohort study of two thousand consecutive patients with coronary artery disease from hospitals situated in the western part of Sweden. The control group was selected from relatives of the patients and approximately 10.000 healthy individuals randomly selected from the population. Subjects were between 25 and 75 years old and lived in the western part of Sweden at the time of recruitment. Sampling took place between 2001 and 2004. More information regarding the Intergene-study is available on <http://www.sahlgrenska.gu.se/intergene/eng/index.jsp>. Table 5 shows the characteristics of cases and controls included in Paper IV.

**Table 5. Characteristics for subjects in the Intergene-study**

<b>Characteristic</b>	<b>Intergene</b>	
	<b>Case</b>	<b>Healthy subjects</b>
<i>n</i> (men/women)	411 (296/115)	411 (296/115)
Age (years)	61.2 ± 8.5	61.3 ± 8.5
WHR	0.95 ± 0.07	0.91 ± 0.08
BMI (kg/m <sup>2</sup> )	27.5 ± 3.9	26.6 ± 3.5
SBP (mmHg)	133 ± 21	142 ± 22
DBP (mmHg)	82 ± 11	85 ± 10
Glucose (mmol/L)	5.6 ± 1.1	5.3 ± 0.9
Cholesterol (mmol/L)	4.6 ± 1.0	5.7 ± 1.0
HDL-C (mmol/L)	1.3 ± 0.4	1.5 ± 0.4
LDL-C (mmol/L)	2.6 ± 0.9	3.6 ± 0.9
TG (mmol/L)	1.6 ± 1.1	1.5 ± 0.8

Characteristics for subjects without diabetes in the Intergene divided into CAD-cases and healthy subjects and used in Paper IV to study the possible association between polymorphisms in the ZAG-gene and CAD.

## ***Expression analysis with DNA microarray***

Sequencing of the human genome<sup>108,109</sup> has enabled development of methods for genome-wide analysis. Analysis of expression and polymorphisms using DNA

microarray are examples of such methods. These methods allow rapid genome-wide analysis using an unbiased exploration strategy.

Affymetrix GeneChip microarrays consist of small DNA fragments (probes), chemically synthesized at specific locations on a coated quartz surface. The probes are designed to determine whether or not the complementary sequence of RNA or DNA is present in the sample. They usually consist of 25 nucleotides and have high specificity for the target sequence. Normally 22 probes for each target transcript are used for gene expression measurements. For each probe on the array that perfectly matches its target sequence, one paired “mismatch” probe is present. This mismatch probe contains a single mismatch located directly in the middle of the 25-base probe sequence. The mismatch probe is used as a control for non-specific binding to the perfectly matched probe. For gene expression analysis, RNA is extracted from the samples, amplified, labelled, and hybridized to the array. The amount of a specific transcript is then measured.

Different microarrays have been used to analyze gene expression in this thesis including HuGeneFL, HG-U95A, and HG-U133A. HuGeneFL was released in 1998 and enabled the relative monitoring of mRNA transcripts of approximately 5600 full-length genes. Since then HG-U95A and HG-U133set have been released, enabling monitoring of mRNA transcripts of 10,000 and 38,500 full-length genes, respectively. This illustrates the rapid development of this technique.

### ***Expression analysis with real-time PCR***

To investigate mRNA levels using real-time PCR, mRNA is isolated and reverse transcribed into cDNA. The resulting cDNA is then used as template in the quantitative real-time PCR (TaqMan). For the gene of interest, a probe and primer set is designed. The forward and reverse primers are preferably designed to span an exon-exon boundary to allow amplification of cDNA but not genomic DNA. The probe is designed to be complementary to part of the sequence between the primers. The TaqMan-probes consist of an oligonucleotide containing a reporter dye (i.e. FAM™ or VIC®) at the 5' end, and a nonfluorescent quencher dye at the 3' end. During the exponential phase of the PCR amplification, the probe breaks down and the fluorescence increases due to increased distance between reporter dye and quencher, allowing determination of the number of copies of the target transcript in the starting mRNA. The Threshold Cycle (Ct), reflecting the PCR cycle number at which the fluorescence reaches a fixed threshold, is used to compare the number of copies of the target transcript in the different samples. Endogenous reference genes are commonly

used in quantitative real-time PCR analysis to adjust for sample variation such as different amount of starting RNA and different efficiency of cDNA synthesis.

## ***Genetic association studies***

Genetic association studies are commonly used to study the correlation between a genetic variant and a phenotype, usually the presence or absence of a disease or a quantitative disease-associated trait e.g. serum cholesterol levels. In the studies presented in this thesis, we have analyzed single nucleotide polymorphisms (SNPs). In SNPs, a particular base varies between two or more different nucleotides. However, SNPs are not the only genetic variant used in association studies. So-called tandem repeats or microsatellites, consisting of repeated DNA-sequence built up by di-, tri- or tetranucleotide repeats, are also used. The DNA sequence variation is said to be polymorphic if the most common variant is present in less than 99% of the population.

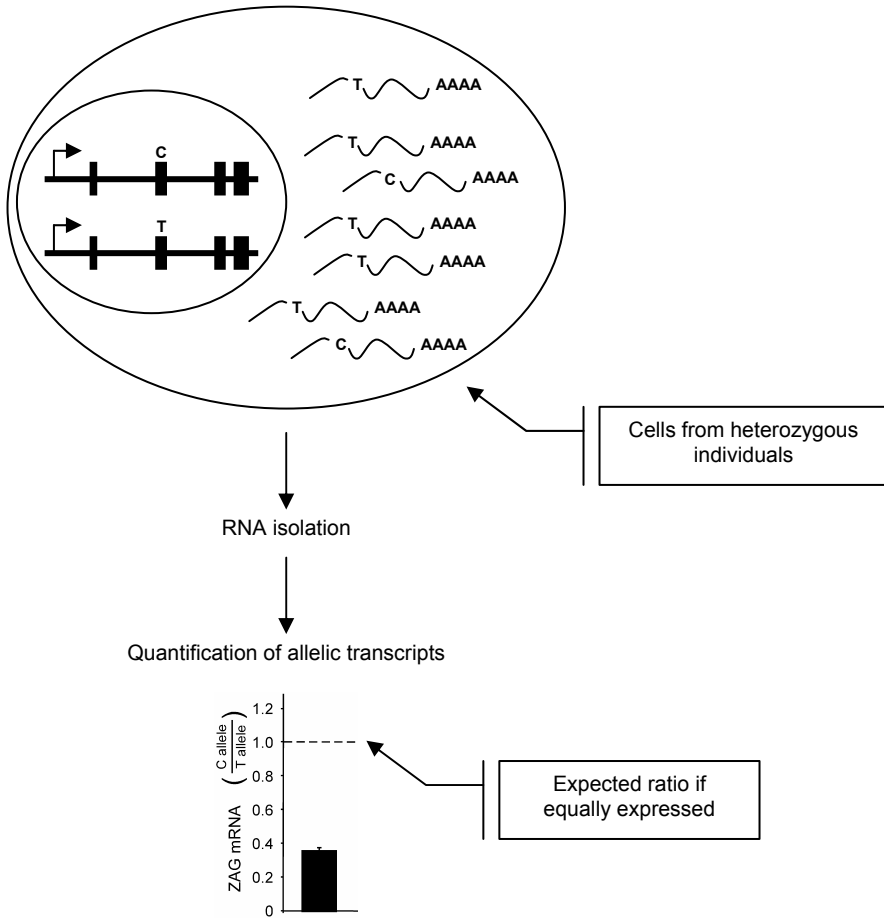
Several methods are available for genotyping SNPs including allele discrimination using flourophore-based PCR (TaqMan) and minisequencing (SNaPshot). There are advantages and drawbacks with both methods. TaqMan is a rapid genotyping method generating high-confidence results using as little as 2-10 ng DNA. However, this method is not suitable when the genotyped SNP is located close to another SNP. Primer and probe design may also be difficult depending on the DNA-sequence surrounding the SNP. Primers for the more time- and DNA-consuming method, minisequencing, can often be more easily designed. Several SNPs may also be genotyped simultaneously using a multiplex minisequencing protocol, which can make this method more time-effective.

## ***Allelic imbalance***

Identification of genetic variants that affect gene regulation is expected to have an important role in the molecular characterization of complex traits<sup>110</sup>. Several different approaches have been applied to identify polymorphisms associated with gene expression. Analysis of transcriptional activity in an allelic-specific quantitative real-time PCR is a relatively easy way to study association between SNPs and gene expression. However, this approach requires that the mRNA contains at least one SNP. A drawback with this method is that it does not directly identify the cis-acting polymorphism or mechanism that is responsible for the variation in gene expression.

We developed a real-time PCR-based assay to determine the relative expression of the two alleles of a SNP in the coding region of Zn-alpha2-glycoprotein

(ZAG). When individuals are heterozygous for an exonic polymorphism, it is possible to detect the relative abundance of allelic transcripts (Figure 5).



**Figure 5. Strategy for studying the relative expression of the C and T alleles of the SNP rs4215, located in the coding region of the Zn-alpha2-glycoprotein gene.** RNA was isolated from heterozygous individuals and the number of transcripts containing the C and T-alleles were determined using a real-time PCR assay. Genomic DNA and a cDNA standard, containing equal amount of the C and T-transcripts, were used as standard.

## Genetically modified mice

Mice have during the last century become the most widely used animal model for studies of human complex disease. Certain mouse strains develop, in similarity to humans, complex diseases including cancer, obesity and type 2 diabetes. Furthermore, several other conditions that are not spontaneously developed in

mice can be induced by modifications of the mice genome and/or environment. There are additional reasons for choosing mice as animal model including the tools available for studying mice, the price, the short generation time, the size of the animal and the large number of animals per litter. Genetically modified mice that overexpress or lack one or more genes make it possible to study the gene function and possible implication in disease development. Techniques and knowledge now allow genetic modifications to be tissue-specific and to be induced at a chosen time point. We used mice with ZAG-deficiency to study the function of ZAG in cholesterol metabolism (Paper IV). The ZAG-deficient mice were crossed with human apoB100 transgenic mice to obtain mice with a lipoprotein profile that resembles the human profile<sup>111</sup>. The ZAG-deficiency was confirmed using RT-PCR on liver and Western blot analysis of serum. Further studies of the mice included techniques such as enzyme-linked immunosorbent assay (ELISA), DNA microarray, determination of liver cholesterol content, and serum cholesterol profiles.

## ***Adenovirus***

There are different ways to transfer genes into cells including transduction, transfection, direct gene transfer, and bacterial gene transfer. In this thesis, we used transduction i.e. virus-mediated gene transfer to study the effects of overexpression of *C/EBP $\alpha$*  in 3T3-L1 cells (Paper V).

Members of the adenovirus family infect a great variety of post-mitotic cells through a receptor-mediated process. After infection, adenovirus delivers their genome to the nucleus and the viral genes are then transcribed, leading to replication of the genome<sup>112</sup>. For studies using virus-mediated gene transfer, parts of the viral genome have been removed in order to make the adenovirus less toxic for the cells. The removal of these genes inhibits the amplification of the virus. To amplify the virus before use, Ad-293 cells that contain the “missing genes” are used.

## RESULTS AND DISCUSSION

### ***Evaluation of reference genes for studies of gene expression in human adipose tissue***

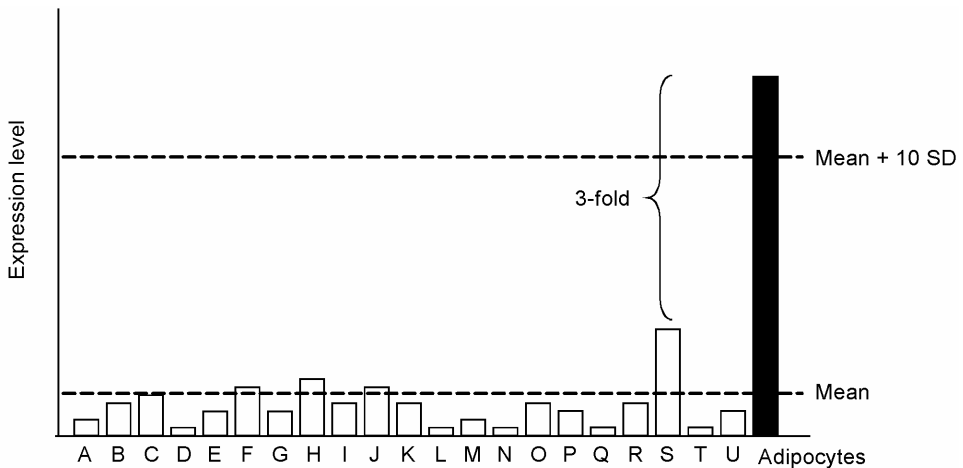
Reference genes are commonly used in quantitative real-time PCR analysis to adjust for sample variation such as different amount of starting RNA and different efficiency of cDNA synthesis. Other approaches have also been used to adjust for sample variations. For example, gene expression has been related to total RNA or cell count<sup>113</sup>. However, these approaches lack control of the cDNA synthesis step, and cell counts can only be applied in studies performed on cells and not tissues. Other strategies, such as spiking exogenous in vitro synthesized RNA<sup>114,115</sup>, are time-consuming, which is why the majority of published studies use an endogenous reference gene.

Since the expression of the gene of interest is divided by the expression of the reference gene, the choice of reference gene is very important and will affect the results. The aim of Paper I was to select stably expressed genes in human adipose tissue using microarray data and to evaluate these genes using real-time PCR and compare them to established reference genes. However, as discussed by Vandesompele et al.<sup>116</sup>, the evaluation of an optimal reference gene based on the real-time PCR data becomes a circular problem because of the lack of an absolute reference point. Hence, Vandesompele et al developed a strategy to identify the most stably expressed genes by comparing the expression of all genes and then exclude the least stably expressed gene in a stepwise fashion. In this study, we extended the method developed by Vandesompele et al. by applying a bootstrap procedure, which enabled us to differentiate the expression stability of the two highest ranked genes. In addition, this extended method also gave us a certainty of the ranking. In this study, low density lipoprotein-related receptor 10 (LRP10) was found to be the most stably expressed gene. Our data indicate that LRP10 is a more suitable reference gene for gene expression studies of human adipose tissue compared to the established reference genes beta-actin, 18S ribosomal RNA, and glyceraldehyde-3-phosphate dehydrogenase.

### ***Searching for genes predominantly expressed in human omental adipocytes***

Adipose tissue, especially omental (intra-abdominal) adipose tissue, is tightly linked to components of the metabolic syndrome<sup>117</sup>. Due to the specialized function of adipose tissue, it can be assumed that adipocytes express a specific

set of genes that other cells types do not express or only express at low levels. Identification of genes predominantly expressed in adipocytes could give us new insights into adipocyte function and may thereby provide important information about genes involved in the development of obesity and obesity-associated metabolic disease. In Paper II, genes predominantly expressed in omental adipocytes were therefore identified. In this search, two criteria were used: 1) the genes should have an expression level of 10 standard deviations higher than the mean of all the other tissues, and 2) the genes should have at least a 3-fold higher expression level than the tissue with the second highest expression (Figure 6). These two criteria allowed us to identify genes that have an expression level that is clearly separated from both the average expression level of all the tissues, but also from any other highly expressing tissue or cell type<sup>118</sup>. Using this approach, we identified 28 genes predominantly expressed in omental adipocytes. Several of these genes were expected including adiponectin<sup>59,119-121</sup>, aquaporin 7<sup>122</sup>, peroxisome proliferative activated receptor  $\gamma$ <sup>70</sup>, and adipsin<sup>123</sup>. However, the finding that acute-phase SAA (A-SAA) was predominantly expressed in adipocytes during nonacute-phase was unexpected. Previous published studies report the highest expression of A-SAA in the liver<sup>124,125</sup>.



**Figure 6. Selection criteria for genes predominantly expressed in human omental adipocytes.** A-U illustrates expression in other tissue or cell types.

### ***Serum Amyloid A***

Three functional serum amyloid A (SAA) genes are present in humans: SAA1, SAA2 and SAA4. The SAA1 and SAA2 are referred to as “acute-phase SAA” (A-SAA) and these genes display a very high homology at both the RNA and protein levels, which makes it difficult to distinguish between the two isoforms.



SAA4 is constitutively expressed and referred to as a “housekeeping” gene<sup>125</sup>. The third member, SAA3, has previously been detected in mouse adipose tissue<sup>126,127</sup>. However, most studies refer to human SAA3 as a pseudogene<sup>128</sup>. In addition, we were not able to detect SAA3 in human adipose tissue, adipocytes, or liver using RT-PCR or real-time PCR, suggesting that this gene is not expressed in these tissues.

In similarity to CRP, SAA increases up to 1000-fold in the circulation within 1-2 days in response to inflammation<sup>129</sup>. Modestly increased levels of acute-phase proteins, including SAA and CRP, are independent risk factors for coronary artery disease in both men and women<sup>20-22</sup>. Serum levels of SAA and CRP are highly correlated<sup>130</sup> and both SAA and CRP mRNA expression are induced by interleukin-1 $\beta$  (IL-1 $\beta$ ) in synergy with IL-6 in hepatoma cells<sup>131-133</sup>. In addition to effects on the transcription, data suggest that the induction of SAA mRNA by these cytokines involve a posttranscriptional mechanism such as increased mRNA stability<sup>133</sup>. SAA mRNA expression has also been found to be induced by oxidized LDL in monocytes/macrophages<sup>134</sup>. Multiple functions for SAA during inflammation have been suggested including acting as a chemoattractant for inflammatory cells such as monocytes, granulocytes and T-lymphocytes<sup>135,136</sup>. In addition, *in vitro* and *in vivo* studies indicate that SAA can induce the expression of proteinases, which may degrade extracellular matrix and be important in host defence<sup>137-139</sup>.

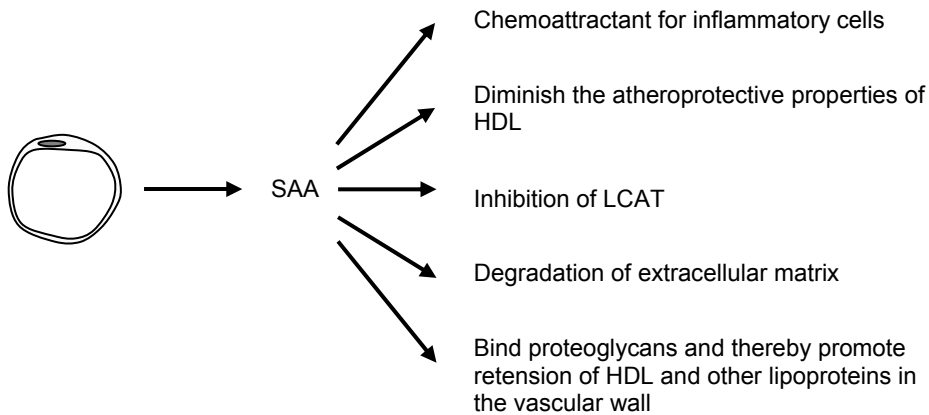
Although most studies have focused on SAA as a marker of inflammation, results suggest that SAA has several atherogenic effects. As described above, inflammatory cells are present in atherosclerotic plaques. Studies have shown that SAA is expressed in the plaque<sup>140</sup> and may function as a chemoattractant leading to accumulation of inflammatory cells in the vascular wall. In addition, the ability of SAA to induce expression of extracellular matrix-degrading proteinases may contribute to instability of atherosclerotic plaque<sup>141</sup>. There are also indications that SAA influences HDL function. In mice, approximately 90% of SAA in plasma is found in the HDL fraction<sup>142</sup>. SAA is associated with HDL also in humans<sup>143</sup>. The role of SAA in HDL-metabolism is unknown but it has been suggested that SAA replaces apoA-I in the HDL-particle and thereby diminish the atheroprotective properties of HDL<sup>144</sup>. Since apoA-I containing HDL particles appear to contain the majority of LCAT<sup>145</sup>, it is possible that increased SAA may inhibit the LCAT-mediated esterification of cholesterol. It has also been speculated that increased levels of SAA are responsible for the decreased HDL levels seen during the acute-phase response and that SAA decrease the ability of HDL to protect LDL from oxidation.<sup>146</sup> However, in

transgenic mice overexpressing SAA no decrease in HDL-cholesterol or replacement of apoA-I in HDL was observed<sup>147</sup>.

Another possible atherogenic function of SAA is the ability of SAA to bind proteoglycans, which may favour the retention of HDL in the vascular wall<sup>148,149</sup>. From studies in rodents, it has been proposed that SAA is a ligand of the cell surface receptor Tanis<sup>150</sup>. Tanis has been suggested to be a link between diabetes, inflammatory response, and cardiovascular disease, because it is regulated by glucose and the expression is altered in a animal model for type 2 diabetes<sup>150</sup>. The rodent receptor Tanis displays homology with the human protein AD-015<sup>150</sup> also known as selenoprotein S.

In Paper II, we demonstrate that adipose tissue is a major site of A-SAA production during nonacute-phase. Because adipocytes can make a large contribution to total body weight, particularly in obese individuals, it is possible that the adipose tissue contributes substantially to the SAA levels in the blood. This is supported by the strong correlation between SAA serum levels and BMI. Interestingly, we found that SAA expression in adipose tissue and serum levels of SAA were regulated in response to diet-induced weight loss. This may be of importance because of the potential role of SAA in atherosclerosis. It is possible that SAA produced and secreted from the adipose tissue influences HDL properties (Figure 7). It may also function as a chemoattractant for inflammatory cells, which could lead to accumulation of these cells in adipose tissue. In addition, SAA from adipocytes could potentially affect the progression of atherosclerotic lesions by increased accumulation of inflammatory cells and degradation of the extracellular matrix. However, the role of adipose tissue-derived SAA in the pathogenesis of atherosclerosis needs to be further elucidated.

Previous studies indicate that adipose tissue is an important source of proinflammatory cytokines. Taken together, this suggests that adipose tissue may contribute to the low-grade systemic inflammation seen in obesity. Since SAA has been suggested to have multiple atherogenic effects, the production of SAA in adipose tissue may be a link between obesity and atherosclerosis.



**Figure 7. Possible atherogenic effects of SAA.** SAA produced and secreted from the adipocytes may influence HDL properties and LCAT. It may also function as a chemoattractant for inflammatory cells into the adipose tissue. In the circulation, SAA is associated with HDL. However, it is possible that SAA could dissociate from HDL and could then potentially also modify the progression of atherosclerotic lesion.

### ***Identification of potential susceptibility genes for obesity-associated metabolic disease***

It has been suggested that genetic variants involved in the development of complex disorders often are located in regulatory regions of the genome affecting the transcription rate of susceptibility genes. Polymorphisms affecting gene expression are common in humans<sup>151</sup>, and about 6% of all genes are estimated to have functional regulatory variants<sup>152</sup>. This suggests that susceptibility genes can be identified based on altered gene expression. However, a simple comparison of gene expression in individuals with disease and controls will identify all genes with altered expression. Thus, the genes with altered expression contributing to disease development can not be separated from the genes with altered expression as an effect of the disease itself. In the VLCD-1 study, when we compared gene expression in adipose tissue from obese women with and without the metabolic syndrome we identified 34 genes with reduced and 36 genes with increased expression in subjects with the metabolic syndrome compared to controls. Consequently, these differentially expressed genes may include susceptibility genes carrying sequence variations in regulatory regions responsible for the altered expression and potentially contributing to the disease. However, these genes may also include genes with altered expression by the disease itself e.g. regulated by insulin. In the VLCD-1 study, the insulin-regulated gene phosphoenolpyruvate carboxykinase 1 (PEPCK) was among the regulated genes at baseline. Insulin inhibits PEPCK transcription<sup>153</sup> and as expected, we found

that the expression of this gene was reduced in the subjects with the metabolic syndrome i.e. subjects with the highest insulin levels. To exclude genes that differ in expression between affected and healthy subjects as a consequence of the disease, we analyzed gene expression in samples before and after treatment that ameliorates the disease phenotype. Since weight loss results in marked improvement of several components of the metabolic syndrome, including insulin resistance, hypertension, and lipid disturbances<sup>154-157</sup>, subjects were treated with a very low calorie diet (VLCD; 450 kcal/day) for 16 weeks followed by 2 weeks of gradual reintroduction of ordinary food. Gene expression was analyzed before, during and after this treatment. An altered expression of a gene even after amelioration of the disease phenotype could reveal causal polymorphisms either in its regulatory regions or in transcription factors controlling its expression. These dysregulated genes may interact with other genetic and environmental determinants during development of disease. During healthy conditions, i.e. when individuals are lean, consume a healthy diet, and exercise, it is likely that the altered expression of these genes do not cause a disease phenotype. However, in physically inactive obese individuals, consuming a diet rich in fat and sugar, the dysregulation of these genes may be implicated in disease development.

Analysis of gene expression during the diet-treatment also gives us the advantage that the subjects then consumed the same nutrients. Several genes are regulated by the diet. For example, in rodents, dietary fatty acids has been found to regulate adipose tissue expression of multiple genes involved in lipid and glucose metabolism including CCAAT/enhancer binding protein alpha (C/EBP $\alpha$ ), lipoprotein lipase, fatty acid synthase, hormone sensitive lipase, PEPCK, leptin, adipsin, PPAR $\alpha$ , glucose transporter 4, fatty acid-binding protein, and thyroid hormone-responsive SPOT14<sup>158</sup>. Susceptibility genes with altered expression in affected individuals compared to controls are presumably easier to identify when diet-differences among subjects are diminished. Using this approach, S100 calcium binding protein A1, Zn-alpha2-glycoprotein and C/EBP $\alpha$  were identified as potential susceptibility genes due to reduced expression in adipose tissue from obese subjects with the metabolic syndrome compared to obese controls and therefore selected for further studies.

### ***S100 calcium binding protein A1***

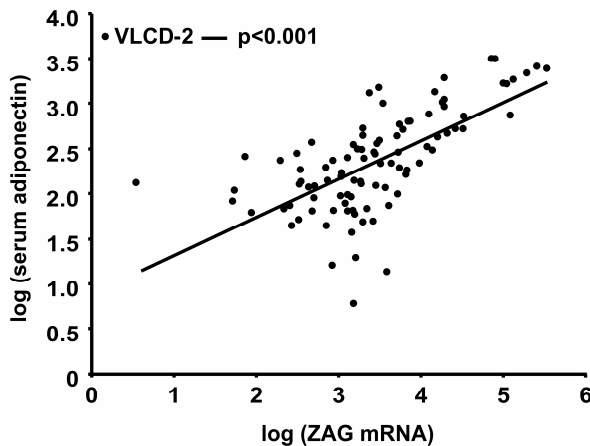
In Paper III, S100 calcium binding protein A1 (S100A1) was identified as a potential susceptibility genes for metabolic disease. S100 proteins are characterized by two Ca<sup>2+</sup>-binding EF-hands (helix-loophelix motif), located in the N- and C-terminal part of the protein, respectively. The two EF-hands are

connected through a region referred to as the hinge region. Several S100 proteins have been identified and differ from one another mainly in the hinge region and in the extended C-terminal part of the proteins<sup>159</sup>. Most S100 proteins forms homodimers and in some cases heterodimers. S100A1, a 21 kDa protein, is highly expressed in heart and skeletal muscle where it has been shown to be a regulator of muscle contractility. Previous results shows that S100A1 improves the contractile performance by both regulating sarcoplasmic reticulum calcium ion handling and myofibrillar calcium ion responsiveness<sup>160</sup>. S100A1 binds multiple proteins in the sarcomere including titin<sup>161</sup>, actin-capping protein Cap-Z<sup>162</sup>, nebulin<sup>163</sup> and the kinase domain of the titin-related protein twitchin<sup>164</sup>. S100A1 interacted with the kinase titin in a  $\text{Ca}^{2+}$ -dependent manner and thereby decrease the interaction between titin and filamentous F-actin<sup>161</sup>. It was proposed that  $\text{Ca}^{2+}$ /S100A1 reduce the titin-based inhibition of F-actin motility. Furthermore, S100A1 has been shown to bind to the  $\text{Ca}^{2+}$ -releasing channel SR-ryanodine receptor located in the sarcoplasmic reticulum<sup>165</sup> and increase the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release<sup>166,167</sup>. In accordance with these findings, S100A1-deficient mice have normal cardiac function under baseline conditions but have significantly reduced contraction rate and relaxation rate responses to  $\beta$ -adrenergic stimulation, associated with a reduced  $\text{Ca}^{2+}$  sensitivity<sup>168</sup>. S100A1 has also been shown to affect several glycolytic enzymes including fructose-1,6-bisphosphate aldolase<sup>169</sup>, phosphoglucomutase<sup>170</sup> and glycogen phosphorylase A<sup>171</sup>.

In the Quebec Family Study (QFS), evidence for linkage to resting metabolic rate (RMR) was observed on chromosome 1q21.1 – q21.2 in close proximity to the S100A1 locus<sup>107</sup>. The RMR quantitative trait locus also co-localized with genomic regions previously linked to components of the metabolic syndrome<sup>172-174</sup>. Moreover, a strong positive correlation between RMR and the metabolic syndrome was observed in the QFS. We found that S100A1 genotype was associated with resting metabolic rate in QFS in subjects from families contributing to the linkage to RMR at 1q21. Thus, our data suggest that S100A1 influence resting energy expenditure and that the expression of S100A1 is dysregulation in obese subjects with the metabolic syndrome compared with controls. The role of S100A1 in adipose tissue is not clear. However, it is possible that S100A1 is a calcium sensor also in this tissue, which is interesting since it has been suggested that intracellular  $\text{Ca}^{2+}$  levels could play a role in the regulation of adiposity<sup>175</sup>. It is possible that S100A1 could in part account for the observed correlation between metabolic syndrome and energy metabolism.

### Zn-alpha2-glycoprotein

In Paper IV, Zn-alpha2-glycoprotein (ZAG) was identified as a potential susceptibility gene for metabolic disease. The expression of ZAG was reduced in adipose tissue from obese subjects with the metabolic syndrome compared with obese controls. ZAG has previously been identified as an adipokine, produced and secreted from adipocytes<sup>176,177</sup>. However, in our study ZAG expression in the adipose tissue was low compared with the expression in liver and prostate (unpublished data). Nevertheless, ZAG may have a local effect in adipose tissue. Interestingly, overexpression of ZAG in 3T3-L1 adipocytes has been reported to lead to an increase in adiponectin mRNA levels<sup>178</sup>. Moreover, we observed a strong correlation between ZAG mRNA levels in adipose tissue and serum adiponectin levels (Figure 8; unpublished data).



**Figure 8.** Correlation between adipose tissue ZAG mRNA levels and serum levels of adiponectin during diet induced weight loss in the VLCD-2 study.

The human ZAG gene spans approximately 9 kilobases, has four exons, and encodes a 41-kDa secretory protein. When sequencing the conserved regions of the ZAG gene, we identified 9 SNPs. Only one of these SNPs was previously not reported. Uria et al isolated two previously unknown isoforms of ZAG from rat liver, with 123 and 138 nucleotides inserted in the RNA between exon two and three, which resulted in a 41 and 46 amino acids insert in the protein, respectively<sup>179</sup>. However, we were not able to detect any ZAG isoforms in human liver or adipose tissue (unpublished data).

ZAG is a major histocompatibility complex (MHC) class I homolog<sup>180,181</sup>, produced in the liver, prostate, kidney, salivary glands, mammary glands, and

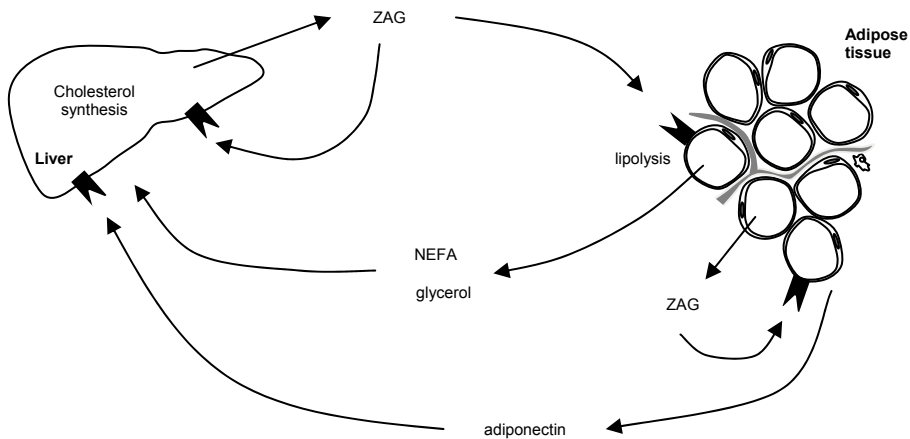
sweat glands<sup>182</sup>. ZAG was first isolated from plasma and has later been found in other body fluids such as urine, saliva and sperm. In similarity to MHC class I, ZAG has a binding pocket. However, in contrast to MHC class I, which binds peptides in its pocket, ZAG is thought to bind a hydrophobic molecule. Binding-studies have shown that ZAG has high affinity for unsaturated fatty acids including eicosapentaenoic acid and docosahexaenoic acid<sup>183</sup>. In similarity to other MHC class I proteins, it is likely that the binding of different ligands affects ZAG's function and its interaction with a putative receptor on target cells. Previously, ZAG has mostly been studied in cancer cachexia, the wasting syndrome causing depletion of adipose and muscle tissue. A tumour-secreted lipid-mobilizing factor (LMF) was identified and later found to be identical to ZAG<sup>184,185</sup>. When given to rodents, ZAG stimulates lipolysis in adipose tissue resulting in a release of free fatty acids and glycerol<sup>186</sup>. The animals consumed the same amount of food, but decreased their weight as a result of increased lipolysis and energy expenditure<sup>186</sup>. When body composition was analyzed, it was discovered that the animals reduced their weight solely by decreasing the adipose tissue mass. In rodents, ZAG's effect on lipolysis in adipose tissue is thought to be mediated by binding to the  $\beta$ 3-adrenergic receptor<sup>187</sup>.

Our study showed a relationship between ZAG and cholesterol metabolism. Serum levels of ZAG were positively correlated with serum levels of total cholesterol in humans. In agreement with these results, the ZAG rs4215 CC genotype was associated with reduced ZAG gene expression, reduced serum levels of ZAG and low serum total cholesterol levels in humans. ZAG-deficiency in mice resulted in reduced hepatic cholesterol content and decreased hepatic expression of HMG-CoA reductase but normal serum cholesterol levels. This is compatible with an increased retention time of cholesterol in plasma, indicating a decreased cholesterol turnover. Unexpectedly, we also observed that the ZAG rs4215 CC-genotype was associated with increased risk of coronary heart disease. This genotype was associated with lower serum levels of total cholesterol, but also lower levels of HDL-C. In addition, it is possible that the lower expression of ZAG in adipose tissue, associated with this genotype, can result in reduced serum adiponectin levels. In fact, individuals with the rs4215 CC-genotype have lower serum adiponectin levels compared with non-CC in the VLCAD-2 study (CC  $9.5 \pm 5.4$   $\mu$ g/mL, non-CC  $12.8 \pm 7.1$   $\mu$ g/mL,  $p=0.05$ ; unpublished data).

Recently, ZAG deficient mice were found to have reduced lipolysis in adipose tissue and increased body weight<sup>188</sup>. However, we did not observe a difference in body weight between ZAG-deficient mice (ZAG<sup>-/-</sup>/apoB100<sup>+/-</sup>) and their littermate controls (ZAG<sup>+/+</sup>/apoB100<sup>+/-</sup>). There were several differences between

these two studies that may explain the divergence including time of follow up, diet, number of generations backcrossed, and the use of an apoB100 transgenic background.

The function of ZAG has to be further elucidated. However, this and previous studies indicate that ZAG could play an important role in lipid metabolism i.e. potentially be involved in the regulation of both lipolysis and cholesterol synthesis (Figure 9). Interestingly, ZAG may also affect expression and serum levels of adiponectin.



**Figure 9. Possible effects of ZAG.** Previous studies have shown that ZAG stimulates lipolysis in adipose tissue, which increases the release of NEFA and glycerol. In addition, ZAG may have a local role in adipose tissue, where it potentially stimulates adiponectin expression and secretion. Our data indicates that ZAG can affect cholesterol synthesis.

### **CCAAT/enhancer binding protein alpha**

CCAAT/enhancer binding proteins (C/EBP) are members of the basic-leucine zipper class of transcription factors and acts as homo- or heterodimers<sup>189</sup>. Six members have been identified and four of these are expressed in adipose tissue: C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$  and C/EBP $\zeta$ <sup>189</sup>. Two isoforms of C/EBP $\alpha$  are formed from the same mRNA by a ribosomal scanning mechanism<sup>190,191</sup>. The full-length protein is 42 kDa. A fraction of the ribosomes ignore the first two AUG codons and initiate translation at the third AUG, 351 nucleotides downstream of the first AUG, generating a 30-kDa protein<sup>190,191</sup>. This shorter protein has an altered transactivation potential compared with the 42-kDa isoform<sup>190,191</sup>.

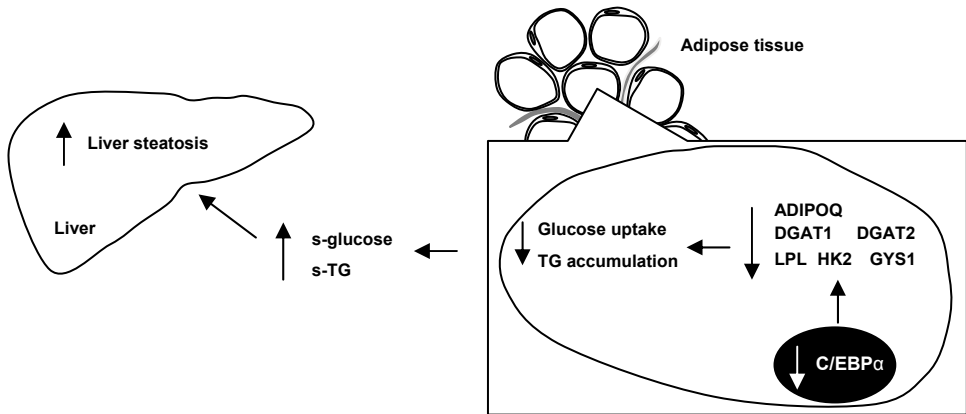


C/EBP $\alpha$  was identified as a potential susceptibility gene for metabolic disease in Paper V. Its expression in adipose tissue was correlated to serum glucose levels 2 hours after an oral glucose tolerance test. In addition, C/EBP $\alpha$  expression was lower in adipose tissue from obese subjects with the metabolic syndrome compared with controls during treatment with a very low calorie diet. The inter-individual variation in C/EBP $\alpha$  expression decreased during diet compared to baseline. It is possible that the larger inter-individual variation at baseline is a result of dietary effects on C/EBP $\alpha$  expression since C/EBP $\alpha$  is known to be regulated by dietary fatty acids<sup>158</sup>. This may explain why we (at baseline in VLCD-2) and others<sup>192-194</sup> do not observe significant differences in C/EBP $\alpha$  expression between individuals with insulin resistance/metabolic syndrome and controls.

C/EBP $\alpha$ -deficient mice die because of hypoglycemia within 8 hours after birth<sup>195,196</sup> demonstrating that C/EBP $\alpha$  has an important role in glucose metabolism. In these mice, expression of enzymes in the glycogen synthesis and gluconeogenesis were altered, which is likely to cause the hypoglycemia observed in these mice. Furthermore, adipocytes from these mice fail to accumulate lipids leading to diminished adipose tissue. In contrast to whole body C/EBP $\alpha$ -null mice, mice lacking hepatic C/EBP $\alpha$  have normal blood glucose levels and glycogen synthesis but are glucose intolerant and develop hepatosteatosis<sup>197</sup>. We found that C/EBP $\alpha$  regulates the glycogen synthase 1 promoter, which may be important for glycogen synthesis in adipose tissue. Furthermore, we found that insulin induces C/EBP $\alpha$  expression in human adipocytes. However, subjects with the metabolic syndrome i.e. with higher insulin concentration, have lower expression of C/EBP $\alpha$  in subcutaneous adipose tissue compared with controls in the VLCD-2 study. It is possible that individuals with insulin resistance, i.e. individuals in which insulin fails to evoke a normal response, can not up-regulate C/EBP $\alpha$  expression in response to insulin.

C/EBP $\alpha$  is a key regulator of adipogenesis<sup>198</sup> and promotes differentiation through binding of cdk2 and cdk4 which prevents them from binding cyclins leading to growth arrest<sup>199</sup>. Deletion of specific transactivation domains in C/EBP $\alpha$  leads to reduced adipogenesis in NIH3T3 fibroblasts<sup>200</sup>. In addition, overexpression of C/EBP $\alpha$  in 3T3-L1 preadipocytes induces their differentiation into mature adipocytes<sup>201,202</sup> and the inhibition of C/EBP $\alpha$  by antisense RNA in these cells blocks this process<sup>203</sup>. In concordance with these results, we found that C/EBP $\alpha$  regulates multiple genes in the lipid and glucose metabolism including adiponectin, hexokinase 2, lipoprotein lipase, diacylglycerol O-acyltransferase 1 and 2, CD36 antigen, ATP-binding cassette, sub-family D, member 2, acyl-CoA synthetase long-chain family member 1, hydroxysteroid 11-beta dehydrogenase

1, and glycogen synthase 1. Up-regulation of these genes may, among other things, result in increased hydrolysis of triglycerides in the circulation and increased uptake of free fatty acids, glycerol and glucose in adipocytes leading to increased triglyceride storage. The lower expression of *C/EBP $\alpha$*  observed in the subjects with the metabolic syndrome i.e. individuals with insulin resistance may contribute to the higher serum levels of TG and glucose found in these subjects (Figure 10).



**Figure 10. Possible effects of low *C/EBP $\alpha$*  expression in adipose tissue.** Lower expression of *C/EBP $\alpha$*  in adipose tissue from subjects with the metabolic syndrome compared to controls may lead to higher serum levels of triglycerides and glucose. *C/EBP $\alpha$* , CCAAT/enhancer binding protein  $\alpha$ ; ADIPOQ, adiponectin; DGAT, diacylglycerol o-acyltransferase; LPL, lipoprotein lipase; HK, hexokinase, GYS, glycogen synthase; s-glucose, serum levels of glucose; s-TG, serum levels of triglycerides.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVE

The overall aim of this thesis was to identify susceptibility genes important in the development of obesity-associated metabolic disease and to increase our understanding regarding the function of these genes. Two different expression-based strategies were applied in order to achieve this aim. Using the first approach, genes predominantly expressed in adipocytes were identified. In this study (Paper II), adipose tissue was found to be a major production site of SAA during nonacute-phase. Since it has been suggested that SAA have multiple atherogenic effects, it is possible that SAA from adipose tissue is a link between obesity and atherosclerosis.

Using the other approach, potential susceptibility genes for metabolic disease were identified based on altered expression in affected individuals compared to controls using DNA microarray. However, a simple comparison of gene expression in affected individuals compared with controls will identify all genes with altered expression. Thus, the genes with altered expression contributing to disease development can not be separated from the genes with altered expression as an effect of the disease itself. We therefore also investigated the gene expression in affected subjects and controls after amelioration of the disease phenotype. We found that this strategy reduce the number of genes with altered expression. In Papers III-V, S100A1, ZAG and C/EBP $\alpha$  were identified as potential susceptibility genes using this approach. In subsequent studies of humans and mice, these genes were linked to components of the metabolic syndrome or metabolic parameters associated with metabolic disease. Thus, our findings suggest that all three genes identified by this strategy are promising candidate genes for metabolic disease. We have applied this approach for identification of potential susceptibility genes for metabolic disease. However, the same strategy could also be used for other diseases.

To increase our knowledge regarding the role of these genes in the pathology of metabolic disease, functional studies are required including knock out mice and mice overexpressing the gene of interest. In Paper IV, the role of ZAG in cholesterol metabolism was investigated in ZAG-deficient mice. Future studies of ZAG will include a more thorough characterization of these mice during different conditions, such as starvation. Effects of different diets will be investigated. In addition, adenovirus for studies of ZAG overexpression *in vivo* has been generated. In addition to wild type ZAG, adenoviruses carrying the

ZAG cDNA with mutations in the sequence coding for the binding pocket have been generated to study the effects of the ligand-binding pocket. One of these mutations is thought to increase affinity for the ligand, while the other mutation is likely to decrease the affinity<sup>204</sup>. Taken together, this mouse model and these tools can help us to clarify the role of ZAG in metabolic disease. Functional studies will also be required to further elucidate the role of SAA, S100A1 and C/EBP $\alpha$  in the pathogenesis of metabolic disease.

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