Tenomodulin, serum amyloid A and the serum amyloid A receptor selenoprotein S – implications for metabolic disease

Academic dissertation

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ABSTRACT

Obesity and obesity-related metabolic diseases are associated with a low-grade inflammation, including slightly increased serum levels of the acute phase protein serum amyloid A (A-SAA). A-SAA is one amongst several adipose tissue (AT) produced proteins suggested to influence development of metabolic diseases. The A-SAA protein may have pro-atherogenic functions, and release of A-SAA from the AT may contribute to the development of cardiovascular disease. Furthermore, A-SAA is functionally linked to insulin resistance via one of its receptors, selenoprotein S (SELS). The tenomodulin (TNMD) gene is expressed in adipose tissue, but its role in obesity is unclear. The overall aim of this thesis was to increase our understanding of how TNMD, A-SAA and SELS relate to obesity and obesity-associated metabolic diseases. An additional aim was to establish a mouse model mirroring the human A-SAA production in AT.

To achieve these goals, we (1) investigated TNMD gene expression in human AT by DNA microarray and real-time PCR analysis; (2) analyzed serum levels of A-SAA in a cohort with a wide range in body mass index and metabolic parameters; (3) analyzed SELS gene expression and genotyped three SELS polymorphisms, previously associated with serum levels of inflammatory markers, in a case-control study of coronary heart disease and (4) generated a mouse model with transgenic over-expression of the human SAA1 (hSAA) gene in AT.

The TNMD gene was highly expressed in human AT, with a higher expression in obese compared to lean subjects. Furthermore, TNMD gene expression was down-regulated during diet-induced weight loss. These data suggest that TNMD plays a role in the adipose tissue.
Inflammatory markers and measures of glycemic control were strongly associated with serum levels of A-SAA. The strongest associations were found in women, and serum levels of A-SAA were associated with adipocyte size in women only. These data suggest that sex-specific factors have to be considered when analyzing serum levels of A-SAA in relation to metabolic disease.
Gene expression of SELS in AT was associated with measures of obesity. Furthermore, genetic variants in the SELS gene were associated with serum levels of glucose, measures of insulin resistance and blood pressure. These findings suggest that SELS plays a role in the development of metabolic disease.
In the hSAA mouse model, hSAA was specifically expressed in AT and plasma levels of hSAA were increased in obese mice. The hSAA protein was found to be co-localized to high-density lipoprotein containing fractions of plasma.

In conclusion, the results of this thesis suggest that TNMD, A-SAA and SELS have metabolic effects that should be further explored. The established hSAA transgenic mouse model opens the possibility to further explore the effects of AT-derived A-SAA on cardiovascular disease.

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LIST OF PAPERS

This thesis is based upon the following papers and manuscripts:

I Tenomodulin is highly expressed in adipose tissue, increased in obesity, and down-regulated during diet-indued weight loss.
Saiki A[1], Olsson M[1], Jernås M, Gummesson A, McTernan PG, Andersson J, Jacobson P, Sjöholm K, Olsson B, Yamamura S, Walley A, Froguel P, Carlsson B, Sjöström L, Svensson P-A, Carlsson LMS.
J Clin Endocrinol Metab. 2009;94(10):3987-94.

II Association of serum amyloid A levels with adipocyte size and serum levels of adipokines: differences between men and women.
Sjöholm K, Lundgren M, Olsson M, Eriksson JW.
Cytokine. 2009;48(3):260-6

III Expression of the selenoprotein S (SELS) gene in subcutaneous adipose tissue and SELS genotype are associated with metabolic risk factors.
Olsson M, Olsson B, Jacobson P, Thelle DS, Björkegren J, Walley A, Froguel P, Carlsson LMS, Sjöholm K.
Submitted for publication

IV Establishment of a transgenic mouse model specifically expressing human serum amyloid A in adipose tissue.
Olsson M, Ahlin S, Olsson B, Svensson P-A, Ståhlman M, Borén J, Carlsson LMS, Sjöholm K.
Manuscript

[1] these authors contributed equally

ABBREVIATIONS

A-SAA	acute phase serum amyloid A
aP2	fatty acid binding protein 4, adipocyte (Fabp4)
Apo	apolipoprotein
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
CHD	coronary heart disease
ChM-I	chondromodulin-I (LECT1)
CRP	C-reactive protein
DEXA	dual energy X-ray analysis
DNA	deoxyribonucleic acid
ER	endolasmatic reticulum
ELISA	enzyme-linked immunosorbent assay
F2	filial 2 (second generation)
FFA	free fatty acids
FPLC	fast protein liquid chromatography
HDL	high-density lipoprotein
HF	high fat
HOMA-IR	homeostasis model assessment of insulin resistance
hSAA	human serum amyloid A1
IL	interleukin
LDL	low-density lipoprotein
MetS	metabolic syndrome
mRNA	messenger ribonucleic acid
NC	normal chow
PCR	polymerase chain reaction
RNA	ribonucleic acid
SAA	serum amyloid A
SD	standard deviation
SELS	selenoprotein S
SEM	standard error of mean
SNP	single nucleotide polymorphism
SOS	Swedish Obese Subjects
SVF	stromal vascular fraction
T2D	type 2 diabetes mellitus
TNF-α	tumor necrosis factor alpha
TNMD	tenomodulin
tRNA	transfer ribonucleic acid
VLCD	very low calorie diet
WHO	World Health Organization
WHR	waist to hip ratio

1 INTRODUCTION

1.1 Obesity

Obesity is defined by excess accumulation of body fat and is associated with adverse health effects. Estimated normal percentages of body fat are within the ranges of 15 to 20 percent in men and 25 to 30 percent in women [1]. When energy intake exceeds the energy expenditure the surplus energy is stored as fat in the body. In obesity, total body fat may increase to 50 percent of the total bodyweight due to long periods of excess fat accumulation. A convenient measure of relative weight commonly used to estimate obesity is the body mass index (BMI), calculated as weight divided by squared height (unit kg/m^2). According to the BMI classification from the World Health Organisation (WHO; Table 1) a BMI value of 30 kg/m^2 defines the lower limit of obesity [2].

Table 1. BMI ranges of weight classifications in adults [2].

Classification	BMI (kg/m2)
Underweight	< 18.5
Normal weight	18.5 – 24.9
Overweight	25 – 29.9
Obesity class I	30 – 34.9
Obesity class II	35 – 39.9
Obesity class III	≥ 40

During the past decades, the incidence of obesity has increased globally [2]. According to the WHO estimate in 2005, there were approximately 400 million of obese adults (age 15+) and an additional 1.6 billion overweight adults, and the estimated number for 2015 is 700 million obese adults globally [3]. In the Swedish population it was recently estimated that 12 percent of women and 13 percent of men are obese, and that 27 percent of women and 41 percent of men are overweight [4].

Obesity is associated with increased mortality, and comorbidities such as type 2 diabetes mellitus (T2D), cardiovascular disease and some types of cancer [5, 6]. It is also well accepted that obesity is associated with a low grade inflammation with elevated levels of inflammatory and acute phase markers in the circulation [7].

A popular hypothesis of why humans are prone to obesity was published by Neel in the year of 1962. According to "the Thrifty Genotype Hypothesis" there has been an evolutionary positive selection of genetic variants that promote efficiency of energy intake and storage, due to a survival advantage during periods of famine when energy resources are sparse [8]. Although appealing and well cited, this theory was recently criticized, and alternative hypotheses of "Drifty Genes" were proposed, suggesting that as obesity-related genetic variants never have been under evolutionary pressure, increased frequencies of mutations that are harmful in the obese state, are the result of random genetic drift [9]. However, as evolutionary forces are slow in progress it is widely accepted that the increased obesity incidence during the last decades can not be

explained by genetics. Rather, the increase in obesity at a population level is more likely due to the increased welfare, which is associated with reduced physical activity in combination with altered eating behaviour and excess energy intake.

Although obesity at a population level may not be explained by evolution, for an individual the genetic heritage influences susceptibility to develop obesity in response to an unhealthy environment. This is evident from the fact that genetic factors contribute to inter-individual variation in obesity susceptibility [10-12]. Obesity is classified as a complex disease, influenced by several environmental and genetic factors. Accordingly, genes with common obesity risk variants, associated with modest increase in bodyweight, have been identified [11, 13, 14]. However, there are also rare monogenetic variants of morbid obesity [11]. Furthermore, large chromosomal deletions were recently shown to be associated with highly penetrant inheritable obesity [15, 16].

Management of the obesity epidemic involves both prevention and treatment strategies and a multi-approached strategy is required to reintroduce physical activity in daily life in concert with healthy eating [2, 17]. However, for treatment of severe obesity, lifestyle modifications have so far not been successful. Bariatric surgery is the only treatment that results in large long-term weight losses and reduced mortality and morbidity [18-20]. As it is not an option to treat all obese subjects with bariatric surgery, there is a strong demand for novel treatment strategies to improve health and reduce mortality in obese patients. New drug targets and improved treatment strategies will hopefully arise from increased understanding of how obesity develops and how it influences the associated comorbidities.

1.2 Insulin Resistance

Obesity is associated with the development of insulin resistance [21]. Insulin is a circulating anabolic hormone that is released from the pancreas in response to increased blood glucose levels. The primary target tissues of insulin are the liver, the adipose tissue and the muscle, and its main function is to promote glucose uptake. Insulin also promotes glycogen storage in the liver and in the muscles. In addition, it inhibits glucose production in the liver, and lipolysis (resulting in release of free fatty acids and glycerol) in the adipose tissue.

In peripheral tissues, an inadequate response to normal amounts of insulin is termed insulin resistance. Insulin resistance is defined as the lowest quartile of insulin sensitivity or the highest quartile of insulin resistance or fasting insulin levels in a population [22]. Environmental and genetic risk factors contribute to the development of insulin resistance. Furthermore, molecules released from the adipose tissue may influence disease progression. Although the pancreas may for some time be able to increase the insulin production to compensate for the insulin resistance, the β-cells may eventually become dysfunctional, and insulin resistance develops into T2D with hyperglycemia [23].

1.3 The Metabolic Syndrome

Insulin resistance is often present in a cluster of metabolic disturbances, including T2D, obesity, hypertension and dyslipidemia. This cluster of phenotypes is referred to as the metabolic syndrome (MetS), although several other names and classifications have been suggested. The MetS is associated with increased risk of developing cardiovascular disease [24]. However, the usefulness of establishing MetS diagnosis has recently been criticized, due to ambiguous criteria and varying thresholds [25].

1.4 Atherosclerosis

Atherosclerosis is the main cause of cardiovascular disease and is characterized by lipid accumulation in the vessel wall of the larger arteries. Atherosclerotic risk factors are included in the MetS and additional risk factors include age, smoking, sex and genetic susceptibility. Hyperlipidemia is included in the definition of the MetS and conventionally assessed from low-density lipoprotein (LDL; the "bad" cholesterol) and high-density lipoprotein (HDL; the "good" cholesterol) levels. The HDL has a central role in reverse cholesterol transport, in which cholesterol is transported from peripheral tissues to the liver. Recently, the ratio between serum levels of apolipoprotein ApoB, the primary LDL associated apolipoprotein, and ApoA-I, a HDL associated apolipoprotein, was reported as a stronger predictor of acute myocardial infarction than serum levels of cholesterol [26, 27].

The atherosclerotic lesions develop over a long period of time and are influenced by both genetic and environmental factors. Atherosclerosis is regarded as an inflammatory disease [28], but the initial triggers are poorly understood. According to the "response to retention" hypothesis it is believed that LDL lipoproteins are retained behind the endothelial layer, through binding to proteoglycans [29, 30]. Retained LDL particles are susceptible to oxidization, and once oxidized they may become proatherogenic, thereby contributing to production of adhesion molecules by endothelial cells and recruitment of inflammatory cells, primarily monocytes. Lipid accumulation in the vessel wall leads to formation of fatty streaks, that mainly consist of lipid-loaded macrophages. An advanced plaque contains a lipid core with cholesterol crystals and necrotic cells, and can be covered by a fibrous cap.

Although some plaques never cause any complications, a plaque rupture can have severe consequences and may lead to occlusive thrombus formation. Interruption of blood flow in the coronary arteries can rapidly damage the heart muscle leading to myocardial infarction. Also, non-ruptured plaques can cause obstruction of blood flow in coronary arteries and this may result in angina pectoris characterized by chest pain due to insufficient supply of blood and oxygen to the heart muscle.

1.5 Adipose Tissue

Adipose tissue is predominantly composed of adipocytes, cells with the main function of storing and releasing energy. Mature adipocytes are characterized by a single lipid droplet, in which energy is stored mainly as triglycerides. The adipocytes are differentiated from preadipocytes within the adipose tissue and it is estimated that 10 percent of the adipocytes are renewed yearly in adults independent of age and

obesity [31]. Fully differentiated adipocytes can become hypertrophic (i.e. increased in size) and adipocyte diameter may increase 20-fold. Enlarged adipocytes are regarded as more metabolically unfavourable than smaller adipocytes [32]. In addition to adipocytes, the adipose tissue contains endothelial cells, fibroblasts and interspersed immune cells including macrophages [33, 34] and T cells [35-37].

Apart from energy storing/releasing functions, the subcutaneous adipose tissue that accounts for approximately 80 percent of total body fat, also serves as a thermal insulator. The regional distribution of body fat differs between men and women. In general, women store more fat in the subcutaneous depot, especially as lower body obesity (a gynoid distribution), whereas men tend to store more fat in the central or visceral region [38]. The central, visceral obesity is strongly related to obesity associated comorbidities [39, 40]. As the visceral adipose tissue is drained by the portal vein, visceral adipose tissue-derived free fatty acids and cytokines are delivered to the liver and may thereby contribute to impaired liver metabolism [41]. In the WHO definition of MetS [22], central obesity is defined as a waist-to-hip ratio (WHR) over 0.90 in men and 0.85 in women.

In adult obesity, the adipose tissue expands primarily through an increase in adipocyte size, rather than through an increased number of adipocytes [42]. Recently, it was described that the total number of adipocytes may be determined at an early age [31], which could provide an explanation for limits in adipose tissue depot expansion [43]. Inability to store surplus energy within the adipose tissue may cause ectopic fat accumulation in muscle and liver, thereby further promoting the development of insulin resistance [44, 45]. Furthermore, impaired angiogenesis during adipose tissue expansion has been suggested to cause adipose tissue hypoxia, which may further promote inflammation and insulin resistance [46, 47]. In addition, immune regulating functions of adipose tissue T-cells were recently found to be impaired by obesity [35-37]. Adiposity is also known to correlate with the amount of adipose tissue macrophages [33, 34], further contributing to both the local and systemic inflammation that is associated with obesity.

1.5.1 Adipokines

Although the adipose tissue was earlier regarded as a location for passive energy storage, it is today well recognized as being an active endocrine organ. The adipose tissue is known to release both cytokines and hormones, referred to as adipokines, thereby providing signals both within the adipose tissue and to other parts of the body. Initial discoveries of adipokines were made in mid 1990s, with the discovery that proinflammatory tumor necrosis factor-alpha (TNF-α) gene expression was high in adipose tissue from obese rodents and that the release of TNF-α was higher from adipose tissue explants from obese compared with lean mice [48]. As neutralization of circulating TNF-α improved insulin sensitivity, the adipose tissue release of TNF-α was suggested to be a link between obesity and insulin resistance [48]. Another early discovered adipokine was leptin. The leptin gene is mutated in the obese (ob/ob) mouse model, a mutation that arose spontaneously in the 1950s. The gene was cloned in 1994, and in contrast to wild type mice, the adipose tissue of the ob/ob mice were

shown to produce a non-functional leptin due to a premature stop codon in the ob-gene [49]. Later it was discovered that leptin regulates food intake in an endocrine manner by affecting appetite, explaining why absence of either leptin or the leptin receptor (db/db mice) is associated with obesity.

Today, many adipokines have been described and they constitute signals from the adipose tissue to other tissues, in order to modulate several different processes. These include energy regulation, lipid metabolism, glucose homeostasis, angiogenesis and inflammation. Several adipokines are known to be dysregulated in obesity and obesity-associated comorbidities. Although obesity results in increased synthesis of most adipokines, this is not true for adiponectin. Adiponectin is an anti-inflammatory adipokine and circulating adiponectin levels are negatively correlated with BMI [50]. Furthermore, adipokine release from adipose tissue is known to be dependent on additional factors including sex, depot/adipose tissue location, adipocyte size and genetic variations. This can be exemplified by leptin which displays increased circulating levels in women and enhanced expression in enlarged adipocytes [51, 52]. Most adipokines are produced by cells in the stromal vascular fraction (i.e. non adipocyte cells). For example, adipose tissue macrophages produce the majority of proinflammatory TNF-α, and a substantial proportion of interleukin (IL)-6 and IL-1 [34]. The TNF-α can locally stimulate preadipocytes to synthesize monocyte chemoattractant protein-1 (MCP-1), thereby possibly recruiting more macrophages [33]. Within the adipose tissue, TNF-α and IL-1 can induce adipocyte production of serum amyloid A (SAA) [53], suggested as a proinflammatory adipokine [53, 54]. Furthermore, when released into the circulation, TNF-α and IL-6 are able to induce hepatic and endothelial expression of acute-phase proteins, including C-reactive protein (CRP) and SAA [55], thereby further contributing to systemic obesity-associated low grade inflammation [56].

The identification of novel adipokines may reveal new information regarding how the adipose tissue contributes to the development of obesity-related metabolic diseases. Previously, our group has combined gene expression profiles from multiple tissues and cell types to identify genes predominantly expressed in the adipocytes or in the adipose tissue. The rationale behind this is that genes specifically expressed in a certain cell type may be involved in yet unknown functions that are specific for the cell type of interest. When using this strategy, the SAA gene was unexpectedly identified as having a predominant gene expression in the adipose tissue in the non acute phase [57]. Recently, the tenomodulin (TNMD) gene was identified as highly expressed in adipose tissue in a separate search for adipocyte-specific genes. Although its role in adipose tissue has not been investigated, TNMD contains a putative cleaving motif suggesting that its C-terminal peptide may be released from adipose tissue. TNMD will be further discussed in section 4.1.

1.6 Serum amyloid A (SAA)

1.6.1 Early studies of the SAA protein

Amyloidosis is defined by deposits of fibrillar protein aggregates that may disrupt tissue structure and function. In year 1971, Benditt and Eriksen reported that there were different classes of amyloidosis, and that the "amyloid A class" was associated with the presence of an inflammatory condition [58]. The year after, amino acid sequences of the amyloid A component of secondary or familial amyloid fibrils were reported [59-62]. At that time, the consensus sequence of the 76 amino acids comprising the amyloid A protein was unique to all known human proteins [63]. Antisera raised against the amyloid A protein reacted towards sera from seven percent of normal subjects and 50 to 80 percent of subjects with diseases associated with amyloidosis [63]. Furthermore, the protein was suggested to be a normal serum constituent that, in situations of considerably increased levels, would act as an amyloid fibril precursor [64]. The protein was named SAA, short for serum amyloid A-related component by Rosenthal and Franklin in the year 1975 [65]. They used a more sensitive radioimmunoassay and detected SAA in sera from all subjects analysed. The study demonstrated that serum levels of SAA increased with age and that certain groups of patients had elevated serum levels, especially subjects with acute infections. SAA was suggested to be released into the circulation as an acute phase reactant [65] and the human SAA protein was later detected in the same fractions as HDL_3, ApoA-I and ApoA-II [66]. Acute-phase SAA in mice was shown to be an apolipoprotein associated with HDL [67]. The liver, and more specifically the hepatocytes, were demonstrated as a location for inducible SAA production in 1980 [68].

1.6.2 The SAA gene family

The human acute phase SAA protein (A-SAA) is transcribed from the SAA1 and the SAA2 genes, and the corresponding proteins share more than 90 percent sequence homology [69, 70]. The human SAA gene family also includes SAA3, which is a pseudogene as it contains a premature stop codon [71], and the SAA4 gene is regarded as a constitutively expressed gene [72]. They are all located in a cluster at chromosome 11p15.1. The SAA gene family is phylogenetically conserved and, apart from in mammals, the SAA proteins have been found in echinoderm and fish [73, 74]. In mice, the A-SAA protein is coded by the SAA1 and the SAA2 genes, and two additional functional SAA proteins exists, the SAA3 and the SAA4 protein [75].

1.6.3 SAA levels in the circulation

SAA is part of the acute phase response, which serves as a primary response towards inflammation, infection or tissue damage. The acute phase response involves an altered hepatic production of several plasma proteins and a modified lipid metabolism with decreased circulating HDL levels [76, 77]. In the acute phase response, A-SAA is synthesized and released from the liver and serum levels can rise substantially (from a few μg/mL to mg/mL). Similar to CRP, a more well known acute phase reactant, the SAA response time after acute phase stimulation is eight hours and serum levels peak after two days [78]. A-SAA has a short plasma half-life; in mice the half life is reported to be 75-80 min [79]. Thus, to maintain high levels in the circulation, the synthesis of

SAA in the liver during the acute phase is substantial, and in mice, A-SAA synthesis during the acute phase response comprises 2.5 percent of total hepatic protein synthesis [80]. The hepatic synthesis of SAA is induced by the cytokines IL-1β, IL-6 and TNF-α.

In line with being an acute phase protein, A-SAA levels are elevated in a number of infectious diseases, as well as postoperatively and after trauma [65, 81]. Furthermore, markedly raised SAA levels are found in several chronic inflammatory conditions that can be complicated by secondary amyloid A (AA) amyloidosis, including rheumatoid arthritis and familial Mediterranean fever [82]. The AA amyloid fibrils are formed from N-terminal A-SAA fragments [83]. A post mortem examination of subjects suffering from rheumatoid arthritis revealed secondary amyloidosis in 30 percent of the samples [84], and AA amyloidosis has been estimated to be the underlying cause of death in five to 17 percent of the subjects with rheumatoid arthritis [85]. Furthermore, in rheumatoid arthritis subjects the risk of cardiovascular disease mortality is increased [86]. Modestly elevated serum levels of SAA are present in conditions associated with a low level of inflammation, including obesity, insulin resistance and T2D [54, 56, 65], conditions that are also associated with increased risk of cardiovascular disease. Both SAA and CRP levels are reported to predict future cardiovascular events [87, 88], and in some studies SAA is suggested to be a better predictor than CRP [87]. After a myocardial infarction serum levels of A-SAA increase, and peak within a few days [65, 89]. A-SAA levels are also increased at the site of plaque rupture compared to levels in the systemic circulation, suggesting a local A-SAA production [90].

1.6.4 SAA expression in adipose tissue and association with circulating SAA levels

Although the liver is the main source of SAA during the acute phase reaction, widespread extrahepatic expression has been reported in humans [91]. However, during the non-acute phase, adipocytes are major producers of A-SAA in obese subjects, as shown by our research group and others [57, 92]. In both studies, A-SAA serum levels and expression in adipose tissue were reduced in response to weight loss, suggesting that adipose tissue derived A-SAA contributes to circulating A-SAA levels [57, 92]. Furthermore, A-SAA expression was higher in subcutaneous compared to omental adipose tissue, women displayed higher A-SAA expression compared to men [57], and A-SAA expression and serum levels were higher in obese compared to normal weight subjects [54, 92]. *In vitro* experiments have shown a positive correlation between BMI and amounts of A-SAA released into media from adipose tissue fragments [54, 92]. Thus, it is likely that in human obesity both increased fat mass and increased expression in adipose tissue contributes to elevated A-SAA levels in the circulation. However, *in vivo* release of A-SAA from adipose tissue has not been reported in the literature.

1.6.5 Suggested roles of A-SAA

The A-SAA protein has been studied in various contexts. Even though there is a well established role of A-SAA within the innate immune system, as an acute phase reactant, the function of A-SAA remains incompletely understood after more than

30 years of research. For instance, both pro and anti-atherogenic roles of A-SAA have been suggested (further discussed in section 4.4). In the circulation, A-SAA is mainly associated with HDL, thereby altering the HDL composition, which may affect reverse cholesterol transport. A-SAA has been suggested to mediate recycling of cholesterol from phagocytosed cell membranes [78], and recombinant A-SAA can also bind free cholesterol [93]. Several receptors have been shown to mediate cholesterol efflux to recombinant A-SAA (Table 2). Various effects of recombinant A-SAA have been demonstrated, including stimulation of inflammatory cytokine production [54, 94], lipolysis [54] and chemotaxia of neutrophils and monocytes via the formyl peptide receptor 2 (FPR2) receptor (Table 2). Furthermore, recombinant A-SAA mediates functional signalling from several immune system related receptors (e.g. toll like receptors; Table 2), and opsonization of bacterial components was recently added to the list of putative A-SAA functions within the innate immune system [95]. Several receptors mediating the above mentioned effects have been identified (Table 2). However, the majority of the information has been obtained in *in vitro* experiments and it is unknown how this information translates to the *in vivo* situation. In obesity, the elevated levels of A-SAA in the circulation may originate from both hepatic production and from the adipose tissue. In addition, the role of adipose tissue produced A-SAA *in vivo* has previously not been studied.

Table 2. Suggested SAA receptors and binding molecules

Receptor / Binding protein[1]	Process	References	Recombinant A-SAA[3]
SELS	Glucose homeostasis, ER stress	96	no
ABCA1	Cholesterol efflux	97 98 99 100	yes
ABCA7	Cholesterol efflux	97	yes
SCARB1	Cholesterol efflux / RCT	101 102 99	yes
CD36	Inflammatory signalling	103	yes
TLR2	Inflammatory signalling	104 105	yes
TLR4	Inflammatory signalling	106	yes
CST3	Inflammatory signalling	107	yes
FPR2	Chemotaxis, immune cell activation	108 109 110 111 112	yes
AGER	Amyloidosis	113	yes
OmpA[2]	Opsonization	95	yes

SELS, Selenoprotein S; ABCA1, ATP-binding cassette, sub-family A (ABC1), member 1; ABCA7, ATP-binding cassette, sub-family A (ABC1), member 7, SCARB1, scavenger receptor class B, member 1; CD36, CD36 molecule (thrombospondin receptor); TLR2, toll-like receptor 2; TLR4, toll-like receptor 4, CST3, cystatin C; FPR2; formyl peptide receptor 2; AGER, advanced glycosylation end product-specific receptor; OmpA, outer membrane protein A; RCT, reverse cholesterol transport; ER, endoplasmatic reticulum; [1]represented by human homologue; [2]OmpA is located to bacterial membranes; [3]recombinant human SAA has been used in the study, discussed further in section 4.5.

1.6.6 The putative SAA receptor selenoprotein S (SELS)

A yest-2-hybrid screening for Tanis interacting proteins identified A-SAA as the only interacting ligand [96]. Tanis was identified as a dysregulated gene in the liver of a T2D/MetS rodent model [96]. The human homologue of Tanis, called selenoprotein S (SELS), is a member of the selenoprotein family [114]. Selenoproteins are characterized

by the presence of a selenocystein amino acid (Figure 1). Selenocystein is the 21st amino acid encoded by the genetic code. When the UGA codon, normally encoding a stop signal, is associated with the specific SECIS (selenocystein inserting sequence) mRNA stem loop structure, a selenocystein can be delivered from a specific tRNA and incorporated into the synthesized protein. In total, 25 selenoproteins are defined in the human genome and the selenocystein residue is suggested to be essential for protein activity [114]. Whereas some selenoproteins are involved in redox and antioxidative regulation, SELS has been suggested to play a role in endoplasmatic reticulum (ER) stress regulation [115]. An association between SELS genetic variants and levels of circulating proinflammatory markers [116] suggests that SELS may be involved in the development of obesity-associated comorbidities.

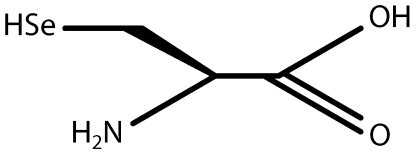

Figure 1. Selenocystein, the 21st amino acid encoded by the genetic code.

2 AIMS

The overall aim of this thesis was to increase our understanding of how factors related to adipose tissue contribute to obesity-associated metabolic disease.

The specific aims were:

- To investigate expression of the TNMD gene in human adipose tissue in relation to obesity *(Paper I)*.

- To explore the associations of serum levels of A-SAA with adipocyte size, serum levels of inflammatory markers and measures of glycemic control *(Paper II)*.

- To investigate if SELS gene expression in human adipose tissue, and SELS genetic variants are associated with metabolic risk factors *(Paper III)*.

- To develop a mouse model with production of human A-SAA in adipocytes that can be used to investigate metabolic effects of adipose tissue-derived A-SAA *(Paper IV)*.

3 STUDY COHORTS AND METHODOLOGICAL CONSIDERATIONS

This section provides an overview of the cohorts analyzed and the purpose is to discuss some of the methods used for analysis. Detailed descriptions of the cohorts and methods used are available in the papers and manuscripts.

3.1 Subjects

This thesis includes participants from the Depot Study, the Very Low Calorie Diet Study I and II, the Swedish Obese Subjects (SOS) Sib Pair Study, the "Umeå cohort" and the INTERGENE Study. Study protocols were approved by Regional Ethics Committees in Göteborg, in South Birmingham and at Umeå University.

3.1.1 The Depot Study

Paired abdominal subcutaneous and abdominal omental adipose tissue biopsies were collected from women undergoing elective surgery. Subjects were divided into a lean (BMI, 23.0±1.2 kg/m^2; n=5) and an obese (BMI, 33.2±3.1 kg/m^2; n=5) group.

3.1.2 The Very Low Calorie Diet Study (VLCD) I and II

The VLCD studies were designed to investigate the effects of diet-induced weight loss. Two separate VLCD studies were used:

VLCD-I. This study was designed to identify gene expression changes during diet-induced weight loss. Obese subjects (BMI, > 30 kg/m^2) were recruited and divided into subgroups with and without the metabolic syndrome (MetS$^+$ and MetS$^-$, respectively). The metabolic syndrome was diagnosed according to WHO criteria [22], except for that albuminuria and insulin resistance were not included. MetS$^-$ subjects were matched by BMI, sex and age. In total, 40 subjects were treated with a very low calorie diet (450 kcal/day) for 16 weeks, followed by two weeks when regular food was reintroduced. The mean weight loss after 18 weeks of VLCD was 24 percent. During the two weeks of refeeding the average body weight was unchanged. Anthropometrical measurements, fasting blood samples and abdominal subcutaneous adipose tissue biopsies were taken at baseline, and at 8, 16 and 18 weeks after the start of the VLCD treatment. DNA microarray expression data from subjects in the VLCD-I study, where data from all 4 time points were available (n=24) are included in *Paper I*.

VLCD-II. The VLCD-II study comprises 20 female and 8 male obese subjects (BMI, >30 kg/m^2) that were treated with VLCD (450 kcal/day) for 12 weeks. The mean weight loss after 12 weeks of VLCD was 19 percent. Anthropometrical measurements, fasting blood samples and abdominal subcutaneous adipose tissue biopsies were taken at baseline, and after 2 and 12 weeks of treatment. Adipose tissue gene expression in VLCD-II samples is included in *Paper I*.

3.1.3 The Swedish Obese Subjects (SOS) Sib Pair Study

The SOS Sib Pair Study was designed to study the genetics of obesity. The cohort consists of 154 families with BMI discordant sib pairs (BMI difference > 10kg/m^2), resulting in a total of 732 subjects, including parents and siblings. Families were recruited from all over Sweden and were examined in Gothenburg. Characterization included anthropometrical measurements, blood pressure measurements, blood chemistry, measurements of body composition and sampling of abdominal subcutaneous adipose tissue. In this study, when comparisons between lean and obese siblings were made, only the most extreme BMI discordant sib pairs in each family were used and sex discordant siblings were excluded. In *Paper I* and *Paper III,* gene expression in abdominal subcutaneous adipose tissue from the extreme sib pairs is presented in relation to clinical parameters.

3.1.4 The "Umeå cohort"

This cohort, which has previously been described by Lundgren *et al.* [117], and was designed to investigate how fat cell size relates to insulin sensitivity and adipokine levels. The subjects in the cohort displayed a wide range of age (19-88 years) and BMI (16-49 kg/m^2). Characterization of subjects included anthropometrical measurements, blood chemistry, abdominal subcutaneous adipose tissue samples, omental adipose tissue samples (a subset) and insulin sensitivity assessed by 2 h-euglycemic-hyperinsulinemic clamp (a subset). In *Paper II*, subjects from the original cohort was included if serum samples were available for analysis. As a consequence, the analyzed population consisted of 167 subjects (87 women and 80 men), including 53 subjects with T2D.

3.1.5 The INTERGENE Study

The INTERGENE study is a population based research programme assessing the INTERplay between GENEtic susceptibility and environmental factors for the risk of chronic diseases in western Sweden. The study population consists of 3610 subjects randomly selected from all inhabitants aged 25-75 years living in the region of Västra Götaland, Sweden. Samples were obtained between April 2001 and December of 2004. Survivors suffering from acute coronary heart disease (CHD) (myocardial infarction and unstable angina pectoris) from the same source population were sampled separately during the above time period and 617 were included in a case-control study. Control subjects (n=617) were matched for age and sex and selected from the 3610 randomly selected subjects. Characteristics of the case and control cohorts are described in Table 3. The study procedure is further detailed in previous articles [118, 119] and at www.sahlgrenska.gu.se/intergene/.

Table 3. Characteristics of CHD cases and controls in the INTERGENE study

Characteristics	CHD Cases (n=618)	Controls (n=618)
Men/Women (n)	453/165	453/165
T2D (n)	150	60
Age (year)	61.8 ± 8.4	61.9 ± 8.3
BMI (kg/m^2)	27.9 ± 4.2	26.7 ± 3.5
WHR	1.0 ± 0.1	0.9 ± 0.1
Systolic BP (mmHg)	134.1 ± 21.0	143.1 ± 21.7
Diastolic BP (mmHg)	81.6 ± 11.3	85.1 ± 10.4
Triglyceride (mmol/L)	1.7 ± 1.3	1.5 ± 0.8
Total cholesterol (mmol/L)	4.6 ± 1.1	5.8 ± 1.0
HDL cholesterol (mmol/L)	1.4 ± 0.4	1.6 ± 0.4
LDL cholesterol (mmol/L)	2.5 ± 0.8	3.5 ± 0.9
Glucose (mmol/L)	6.1 ± 2.4	5.5 ± 1.3
Insulin (mU/L)	14.5 ± 16.7	8.4 ± 6.6
Hs-CRP (mg/L)	5.1 ± 11.2	2.3 ± 3.9

BP, blood pressure; Hs, Highly sensitive. Values are mean ± SD.

3.2 Ribonucleic acid (RNA) extraction

Obtaining high quality RNA is important for gene expression analyses. Since RNA molecules can rapidly be degraded by stable ribonuclease enzymes (RNases), cells and tissues to be used in RNA preparations should be rapidly stabilized (e.g. immediately frozen in liquid nitrogen) and stored in -80 °C. RNA extractions should be performed in an RNase free environment. Today, the most commonly used RNA extraction protocols are based on the protocol published by Chomczynski and Sacchi year 1987, a protocol which has also been developed into commercial kits [120, 121]. RNA extractions were performed in *Paper I*, *Paper III* and *Paper IV*.

3.3 Gene expression analysis

Gene expression can be measured using different techniques. Gene expression of a limited number of genes can for instance be analysed using Northern blot, *in situ* hybridization, competitive reverse transcription polymerase chain reaction (PCR) analysis or real-time PCR. Gene expression profiling of a larger number of genes can be performed using microarray analysis, expressed sequence tag (EST)-sequencing or serial analysis of gene expression (SAGE) analysis. This thesis includes gene expression analysis of in-house generated DNA microarray datasets (*Paper I* and *Paper III*), a publicly available microarray dataset (*Paper I*) and analysis by real-time PCR (*Paper I*, *Paper III* and *Paper IV*).

3.3.1 Real-time PCR analysis using TaqMan chemistry

Briefly, the real-time PCR analysis is performed using a cDNA template from reversed transcribed total RNA. The TaqMan expression analysis is based on a PCR amplification that is monitored in real-time, accomplished by use of sequence specific probe and primers. The probe contains a 5´ fluorophore reporter dye (e.g. FAM) and 3´

quencher molecule (NFQ). The quencher suppresses the fluorescent signal from the reporter molecule as long as they are in close proximity. In each PCR extension step, the 5´-3´ exonuclease activity of the polymerase causes release of the quencher from the probe and an increase in fluorescent signal in the sample. As the probe is displaced from the template a full length amplicon is produced. The fluorescent signal is monitored in each temperature cycle step in the thermal cycler (e.g. Applied Biosystems 7900HT Sequence Detection System). The PCR cycle number in which the fluorescent signal reaches a specified threshold cycle (C_T) is directly related to amount of starting material. In *Paper I*, *Paper* III and *Paper IV* gene expression was analyzed using TaqMan chemistry using the relative standard curve method. All samples were related to a standard curve and to the expression of a reference gene to be able to normalize for variations in amount and quality of starting template in each reaction. The relative gene expression is presented as a ratio between the gene of interest and the reference gene. Ideally, the reference gene should be the perfect housekeeping gene with an equal expression in all cells, and should be chosen carefully [122].

In TaqMan analysis, the efficiency of both the preceding reverse transcription reaction and PCR amplification may vary between different transcripts. Furthermore, specificity and efficiency of primers and probes may influence the amplification. Thus, annealing of primers and probes is sensitive to genetic sequence variation. The amplified region is preferably located over an exon junction to minimize influence of possible contamination by genomic DNA. However, it is estimated that as many as 95 percent of multiexon genes may undergo alternative splicing [123]. As a consequence, the strength of exon junction-based quantifications is that different splice forms can be quantified separately, and the drawback is that only an unknown proportion of the transcripts from a gene are quantified. Although such a putative amplification bias is not inferred in techniques without amplifications (e.g. Northern blot), real-time PCR analysis has a major advantage in that it allows for small amounts of starting material.

3.3.2 Gene expression analysis using GeneChip technology

Microarray analysis of gene expression is based on hybridization of the analyzed sample to a microarray consisting of densely spaced oligonucleotides attached to a solid surface. The GeneChip technology, commercialized by the Affymetrix Company, uses a light-directed technique where oligonucleotides are synthesized, nucleotide by nucleotide, at specific positions on the array. The oligonucleotide probes synthesized on the microarrays are designed to include as many transcripts as possible. Since the technique is dependent on sequence information available at the time of design, only transcripts described in databases are included on microarrays. The oligonucleotide probes are densely spaced on the microarrays. The Human Genome U133A Plus 2.0 GeneChip contains 47,000 oligonucleotide probe sets, covering most of the human genes.

Briefly, RNA is reversed transcribed to cDNA and the cDNA is then reverse transcribed to biotinylated cRNA. Fragmented biotinylated cRNA is allowed to hybridize to the microarray. The biotin molecule is a high affinity ligand for the

streptavidin molecule. This is utilized in the detection step as streptavidin molecules, each linked to fluorescent phycoerythrin, are allowed to bind to the biotin molecules. The fluorescence is measured with a confocal laser scanner at each position of the microarray. The amount of hybridized cRNA for each oligonucleotide species is calculated from the fluorescent signal.

Obtaining the most reliable expression information from DNA microarray data analysis has been, and still is, challenging to the biostatistical discipline. In 2003 Irizarry *et al.* published a technique called robust multi-chip average (RMA) that provided a robust way of preprocessing signal data [124]. The RMA software was released as a freely available R package and is the most commonly used preprocessing algorithm for Affymetrix analysis today [125]. The RMA algorithm was used for microarray data analysis in the Depot Study (*Paper I*), the VLCD-I Study (*Paper I*) and the SOS Sib Pair Study (*Paper I* and *Paper III*).

3.4 Genetic variation in the human genome

The human genome consists of DNA that is built from about 6 billion nucleotides organized in 23 pairs of chromosomes. In 1990 the Human Genome Project was initiated. It was aimed at sequencing the human genome, and when planned, the project was controversial due to limitations in sequencing capacity and lack of data analysis software [126]. However, improved nucleotide sequencing techniques and data processing made it possible to sequence the whole human genome before the set time frame of 15 years. It was revealed that the haploid (unpaired) human genome codes for approximately 20,000 genes, corresponding to only a few percent of the whole genome [127-129]. The remaining part of the genome consists of regulatory sequences, introns, repeated elements, transposons and non-coding RNA species. The DNA with unknown function is classified as junk DNA.

Genetic variation can arise spontaneously by mutations, but can only be passed on to the next generation if germ line cells are mutated. There are different classes of genetic variation. The most frequent form of variation is when only one nucleotide varies. When this variation in a population is larger than can be explained by spontaneous mutation, with a frequency of the rarest variant above one percent, the position is classified as polymorphic. This genetic variant type is denoted a single nucleotide polymorphism (SNP). The human genome is estimated to have one SNP per 300 base pairs, adding up to a total of 10 millions of SNPs [130].

One way to search for genetic variations that result in disease is to compare allele frequencies between affected cases and healthy controls. In a case-control study, the healthy controls are matched against the cases. A difficult confounder in such genetic association studies is if the study cohort has a mixed genetic background. A difference in SNP frequencies and disease prevalence in merged subpopulations can mask a true association or provide a false positive result [131]. Genetic variants can also associate with other nearby variants due to low frequency of recombination events between them. A region of DNA with genetic variants that are likely to be inherited together is called a haploblock. An array with the linear order of associated alleles in a haploblock

is called a haplotype. The HapMap project (www.hapmap.org) was initiated year 2002 with the aim of providing a HAPLOtype MAP of the human genome. The strategy of the project has been to genotype a large number of SNPs in a small number of subjects within populations of different genetic background. As a result of publicly available datasets, SNP information is easily accessible and genotyping technologies have improved.

Several different techniques are available for genotyping analysis. Although the genotyping technology is evolving rapidly with reduced cost per analyzed genotype in high throughput systems, the analysis of choice is of course depending on the variation to be analyzed, number of loci, amount of starting material, and budget. One way of genotyping SNP variations is to use the TaqMan SNP genotyping technique.

3.4.1 Genotyping using TaqMan SNP technology

The TaqMan SNP genotyping technique is similar to that of the TaqMan gene expression chemistry. The method is PCR-based with specific primers and two probes. Both probes are designed to span the location of the SNP of interest and specifically hybridizes to one of the allelic variants. The probes are labelled with a 5´ flourescent reporter (VIC or FAM) and a 3´ NFQ. A genotype specific signal is obtained by comparison of the amount of fluorescent signals from the FAM and VIC reporters, seen after the exonuclease release of quencher molecules. From a heterozygous subject, both VIC and FAM signals are generated. TaqMan SNP genotyping provides a robust way of genotyping SNP variations using only a small amount of template. The method is fast and reliable since, in general, prevalidated assays work well, and when combined with pipetting robots (e.g. Beckmann Biomek FX robot) and 384 well format analysis, the risk of pipetting errors is reduced. However, due to the necessary binding specificity, probes can not be designed if there are other SNPs located close to the SNP of interest. In *Paper III*, three SNPs were chosen to be analyzed by TaqMan SNP genotyping in the SELS gene, based on the reported association with circulating levels of inflammatory markers [116].

3.5. Animal Experiments

3.5.1 The use of transgenic mice

The most commonly used laboratory mouse strain is the C57BL that can be back tracked to 1921 to a mate between female 57 and male 52 from Miss Abbie Lathrop's animals [132]. The former school teacher Miss Abbie Lathrop started to breed mice for sale as pets, at a similar time as researchers were unravelling inheritance of mice coat color. As the academic interest in genetic research increased, she also began to supply universities with mice, and many of the inbred strains used in research today are derived from her mouse farm [133].

During *in vivo* experiments both environmental and genetic factors contribute to experimental variation. Environmental variability can be reduced by standardized housing conditions. By using inbred mice the genetic variance is minimized, thereby also reducing the experimental variation further. Mice have several advantages as model animals, such as a short generation time with relatively large litter sizes, they

are relatively inexpensive, easy to handle and easy to house. Since mice have been used in experiments for decades their physiology has been extensively studied and standardized experimental protocols are available. Furthermore, the mouse genome has been sequenced as part of the human genome project. As it is possible to modify the mouse genome by introducing foreign DNA [134, 135], and create gene specific alterations (e.g. mutations or deletions) by homologous recombination, new gene specific mouse strains have been developed (www.jax.org). However, although laboratory mice serve as well controlled model systems they have a different physiology compared to humans and one has to be careful when extending findings from mouse experiments to human physiology. Furthermore, one has to be aware that different mutations and variations have been fixed in the genome in the inbred strains, thereby causing strain specific phenotypes.

Using animals that are able to sense both pain and stress in research provides an ethical dilemma. Therefore, each experiment has to be evaluated and approved by the local Animal Ethics Committee. The rule of thumb in all animal experiments is to follow the three R´s: refine the experimental set up, reduce number of animals in experiments and replace animal experiments if possible.

3.5.2 Transgenic mice over-expressing human SAA in adipose tissue

We hypothesized that adipose tissue-produced A-SAA has metabolic effects both locally and systemically. As complex interplays between different tissues can not be studied *in vitro*, we developed an animal model in which the effects of adipose tissue-derived human A-SAA can be studied.

The promoter/enhancer of the adipocyte fatty acid binding protein (Fabp4 or aP2) gene generates adipose tissue-specific gene expression [136]. We constructed an artificial gene with the aP2 promoter/enhancer (kindly donated by Professor B. Spiegelman) to obtain adipose tissue-specific expression of human SAA1 (hSAA; Figure 2). The C57BL/6 mouse strain was selected as a background since these animals tend to develop obesity over time, and because there are atherosclerosis prone mouse strains on C57BL6 background that we wanted to cross our strain with. The artificial gene was injected into fertilized C57BL/6 eggs using pronuclei injection, and injected eggs were inserted into pseudopregnant foster mothers. In pronuclei injection, the DNA is integrated at a random position in the genome. Transgenic mice were identified using a PCR assay optimized to detect transgenic insert when at least one copy of construct was inserted per genome. Since the integration is done after initial cell division, the offspring is partially transgenic and not all transgenic offspring may be transgenic in germ lines. The founder animals in the first generation of transgenic animals were identified by the ability to produce transgenic offspring. Founders were bred against wild type C57BL/6 mice purchased from Charles River (Sulzfeld, Germany), thus all transgenic animals were heterozygous. Animals from the F2 or later generations were used in experiments in *Paper IV*.

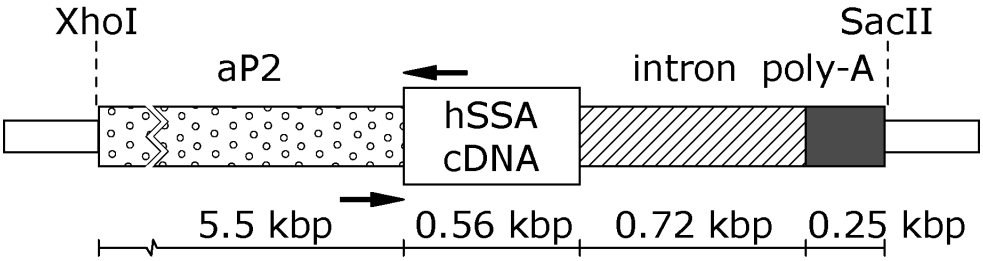

Figure 2. A schematic representation of the aP2 promoter-hSAA fusion gene. *Human SAA1 cDNA was ligated to the aP2 promoter/enhancer as described in Paper IV. The aP2 promoter/enhancer (dotted box), the rabbit β-globin intron (striped box) and the polyadenylation signal (grey box) are included in the figure. Restriction enzyme digestion with XhoI and SacII enzymes generated fragments of 7.2 kilobases (kbp) that were used in pronuclei injections to generate hSAA transgenic mice. Locations of genotyping primers are indicated with arrows.*

Mice were maintained with a 12 h dark/light cycle with *ad libitum* food and water. In the experimental setup, wild type and hSAA transgenic (hSAA) animals were housed 3-6 animals per cage and fed a normal chow (NC) diet or a high fat (HF; 60 kcal% fat, D12492, Research Diets, New Brunswick, NJ) diet. Body composition was measured on Isofluran (Baxter, Kista, Sweden) anesthetised animals using dual energy X-ray analysis (DEXA) in a Lunar PIXImus II (GE Healthcare, Waukesha, WI). The mice were sacrificed under Isofluran anesthesia. Blood and tissues were sampled as described in *Paper IV*. hSAA-induced phenotypes were evaluated by comparing the transgenic animals with wild type littermates as described in *Paper IV*. The tissue localization of hSAA expression was investigated in transgenic mice. Characterization of the animals included blood chemistry, body weight and adipose tissue depots weight measurements. A more detailed description of the characterization of the transgenic mice is available in *Paper IV*.

4 RESULTS AND DISCUSSION

4.1 Paper I

Tenomodulin is highly expressed in adipose tissue, increased in obesity, and down-regulated during diet-induced weight loss.

During a scan for genes specifically expressed in human adipose tissue we found that the TNMD gene was highly expressed in adipose tissue compared to other tissues. The TNMD gene was initially identified because it displayed a high sequence homology to the chondromodulin-I gene (ChM-I, also denoted LECT1) [137-139]. The ChM-I gene plays a role in chondrocyte differentiation and inhibition of angiogenesis.

We analyzed the human tissue distribution of TNMD gene expression in both a publicly available DNA microarray dataset (Geo database: GSE3526), and by real-time PCR analysis in RNA samples from different tissues. Subcutaneous adipose tissue was a major site of TNMD gene expression in both tissue panels. Although TNMD gene expression has previously been reported in human adipose tissue, earlier reports have not compared the TNMD gene expression in adipose tissue to other tissues [140, 141]. In a more recent publication, the tissue distribution of TNMD was evaluated in a human tissue panel including both tendons and adipose tissue and the expression was found to be approximately four times higher in human tendons compared to adipose tissue [142]. In mice, the Tnmd gene is highly expressed in hypovascular tissue such as tendons and cartilage [137-139, 143]. However, neither tendons nor cartilage were available for our tissue panel analysis. Available mouse tissue expression panels in SymAtlas/BioGPS suggest that the Tnmd gene is not expressed in mouse adipose tissue [144]. Absence of Tnmd gene expression has also recently been shown for rat adipose tissue [142]. Tnmd-deficient mice display no changes in body size or weight compared to normal mice, further suggesting that Tnmd does not play an important role in mouse adipose tissue.

In the analysis of DNA microarray data from the SOS Sib Pair Study, the TNMD gene expression was higher in obese subjects compared to lean subjects in both men and women. The TNMD gene expression was also increased in obese women compared to obese men. This verifies the previous results showing an association between TNMD gene expression with fat mass, and an increased TNMD gene expression in women compared to men [140]. Analysis of subcutaneous adipose tissue obtained during diet-induced weight loss in the VLCD-I and the VLCD-II studies showed that TNMD gene expression was reduced during treatment with a hypocaloric diet. These data verify the previously reported down-regulation of TNMD gene expression in human adipose tissue in response of weight loss [140, 141]. These earlier studies show a reduction in TNMD gene expression after 10 weeks or 8 months of life style changes, respectively. In the present study, the TNMD expression remained low between week 16 and 18 in the VLCD-I study. During that period, ordinary food was reintroduced and body weight remained stable, although there was a threefold increase in caloric intake. Our data therefore extend the previous findings [140, 141] by demonstrating that during diet-

induced weight loss the TNMD gene expression is not regulated by caloric intake but rather in response to changes in body weight. This is also further strengthened by the fact that BMI was an independent predictor of TNMD gene expression in the SOS Sib Pair Study.

The TNMD gene is located on chromosome Xq22 and its inheritance pattern is therefore different from genes positioned on autosomal chromosomes. Polymorphisms in the TNMD gene have been studied in relation to metabolic risk factors [145-147]. TNMD SNPs were initially found to be associated with diabetes risk and obesity but these findings could not be replicated in a larger cohort [145, 146]. Association between TNMD SNPs and serum concentrations of inflammatory markers, including CRP and SAA, were also found in a smaller population [147]. One TNMD SNP was associated with LDL and total cholesterol levels in the higher BMI ranges [146]. However, the mechanisms behind these associations remain unknown. Even though TNMD expression is higher in human tendons compared to adipose tissue [142], tendons are not as metabolically active and abundant as adipose tissue. Accordingly, it is more likely that genetic associations with the TNMD gene are related to its expression in adipose tissue than to TNMD expression in tendons.

The functional role of TNMD in adipose tissue is unknown. Anti-angiogenic effects have been suggested based on sequence homology with ChM-I. Angiogenesis is known to be regulated by many factors that are expressed in adipose tissue [148], and angiogenesis has been shown to be spatially co-localized with adipogenesis [47]. Induced destruction of adipose tissue vasculature has been demonstrated to reduce body weight by resorption of adipose tissue [149]. These studies suggest that angiogenesis and adipose tissue growth are functionally related. Increased expression of an anti-angiogenesis factor in obesity may contribute to metabolic disease in different ways. It can be hypothesized that a reduction in expanding capacity of the adipose tissue may lead to ectopic fat storage. It can also be hypothesized that reduced angiogenesis may cause insufficient blood flow and lead to hypoxia that promotes both inflammation and macrophage recruitment. However, while ChM-I has an established function as angiogenesis inhibitor mediated by a cleaved off C-terminal region, less is known about TNMD. Both proteins are transmembrane proteins [137] and a high sequence homology in the C-terminal region extends to conserved spatial distribution of eight cystein residues, known to be functionally important in ChM-I [137]. However, compared to ChM-I, an alternative putative cleavage motif is present in TNMD [137]. A C-terminal TNMD fragment has been found in mice tendons [143]. The C-terminal fragment mediated an anti-angiogenesis signal *in vitro* [150]. Despite this, impaired angiogenesis was not reported as a phenotype in a Tnmd-deficient mice [143]. Instead, reduced tenocyte density and increased collagen fibril diameter were reported [143]. It is unfortunate that the Tnmd-deficient mice were not metabolically characterized as other phenotypes might have been revealed if animals had been challenged with a westernized diet. So far, C-terminal TNMD cleavage has not been reported in human samples and consequently more extensive functional studies are needed to characterize the role of TNMD in human adipose tissue.

In summary, we report that TNMD gene expression is affected by obesity, and expression is reduced during diet-induced weight loss, suggesting that TNMD has a specific role in the adipose tissue.

4.2 Paper II

Association of serum amyloid A levels with adipocyte size and serum levels of adipokines: differences between men and women.

Nutritional overload has been suggested to contribute to metabolic disease in different ways. One proposed explanation is that the increase in fat mass causes both local and systemic inflammation [56]. Another explanation is that insulin resistance is promoted by ectopic fat storage, caused by insufficient storage capacity of the adipocytes [44]. Adipocytes are able to expand over thousand fold in volume and a low generation rate of new adipocytes was recently shown to be associated with hypertrophic adipocytes [151]. Hypertrophic adipocytes have been reported not only to be associated with insulin resistance but also to predict T2D [32], and to be associated with increased expression and secretion of adipokines [52, 152].

Our group has previously reported that gene expression of A-SAA is higher in large compared to small adipocytes [52]. Previous data also suggest that A-SAA is released from adipose tissue in humans (discussed in section 1.6.4). In this study we wanted to explore the association between adipocyte size and serum levels of A-SAA. Furthermore, associations between serum levels of A-SAA and measures of glycemic control and circulating levels of adipokines and inflammatory markers were investigated. Two studies have previously investigated correlations between adipocyte size and serum levels of A-SAA in small cohorts. Poitou *et al.* studied morbidly obese subjects (50 women and 10 men) and found that serum levels of A-SAA were associated both with adipocyte volume and A-SAA gene expression in subcutaneous adipose tissue [153]. However, no association was found between adipocyte size and serum levels of A-SAA in a study of 37 obese men and 38 obese women [154].

We found that serum levels of A-SAA were increased in obese compared to lean subjects, higher in women compared to men, and levels were positively correlated with BMI and percent body fat, as previously reported by others [54, 153, 155]. Increased serum levels of A-SAA in women are in line with our previously published results showing an increased A-SAA gene expression in subcutaneous adipose tissue in women [52], together with the fact that women have larger subcutaneous adipose tissue depots than men. The mechanisms behind this sex related difference is unknown but may be due to hormonal status since estrogen replacement in postmenopausal women has been reported to increase A-SAA, CRP and IL-6 levels [156]. In women, we found a positive association with IL-6, possibly due to the fact that IL-6, in synergy with IL-1β or TNF-α, is able to induce A-SAA in hepatocytes [157]. Furthermore, an association between serum levels of A-SAA and leptin was found in women. There are several similarities between leptin and A-SAA, but the link behind these similarities is not entirely clear. However, the expression of both genes is increased in large compared to

small adipocytes [52], and in subcutaneous compared to omental adipose tissue [57, 158]. In addition, serum levels of both A-SAA and leptin are increased in women compared to men and also in obese compared to lean subjects [51].

A correlation between serum levels of A-SAA and adipocyte size was found in women only. Also, BMI and percent body fat were correlated with serum levels of A-SAA in women but not in men. When subjects were divided into BMI classes, adipocyte size and serum levels of A-SAA correlated in lean women only, whereas a borderline significance was found in overweight women. No association between adipocyte size and serum levels of A-SAA was found in obese women or in any of the BMI classes in men.

It has previously been reported that poor glycemic control in T2D subjects is associated with insulin resistance and with increased levels of CRP and TNF-α [159]. In our study, we found an association between serum levels of A-SAA with serum levels of CRP and with homeostasis model assessment of insulin resistance (HOMA-IR). When diabetics and non-diabetics were analyzed separately, serum levels of A-SAA were associated with insulin and HOMA-IR in non-diabetics but not in diabetics, suggesting that, in pre-diabetic subjects, a low level of inflammation is positively associated with the degree of insulin resistance. In a recent publication, TNF-α was investigated in subcutaneous adipose tissue [160]. In that study, *in vitro* secretion of TNF-α from adipose tissue was correlated with adipocyte hypertrophy, BMI and total body fat in lean women but not in obese women [160]. Equivalent associations were absent for IL-6, adiponectin and leptin in lean women and for TNF-α in obese women [160]. TNF-α was suggested to affect homeostasis by determining total fat mass and adipose tissue volume in lean women [160]. A-SAA was not included in that study, which is unfortunate since we found that serum levels of A-SAA correlated with adipocyte size in lean women. In our study, no association between serum levels of TNF-α and A-SAA was found, in line with what has also been reported by others [153]. This lack of association may be caused by disturbed regulation of adipokine release as a consequence of metabolic disease.

In conclusion, we found that adipocyte size was associated with serum levels of A-SAA in lean women only. The serum levels of A-SAA were associated with inflammatory markers as well as with measures of glycemic control. In this study, a majority of the correlations with serum levels of A-SAA were found in women. Accordingly, our results show that there are sex differences in associations with A-SAA levels and that sex-specific factors should be considered when analyzing A-SAA serum levels in relation to metabolic disease.

4.3 Paper III

Expression of the selenoprotein S (SELS) gene in subcutaneous adipose tissue and SELS genotype are associated with metabolic risk factors.

The SELS protein is one out of two predicted membrane spanning selenoproteins in the human proteome [114]. The SELS gene is located on chromosome 15q26.3, in a region subjected to copy number variation in a minority (five percent) of European descents [161]. However, the proportion of copy number variation in the Swedish population is unknown and it is unknown if copy number variation of SELS affects its gene expression levels. The SELS gene is known to be expressed in different cell types, including tissues important for glycemic control such as liver, muscle and adipose tissue [96, 162, 163]. The SELS protein is located both in the endoplasmatic reticulum (ER) and in the plasma membrane [164]. The ER plays a role in protein and lipid synthesis and, within the ER lumen, correct folding of newly synthesized polypeptides is assisted by chaperone proteins. Correctly folded proteins are transported to the Golgi organelle for further modifications or transport to their final destination. Cellular stress impairs the ER function, resulting in an accumulation of unfolded or misfolded proteins in the ER lumen. The cellular response that tries to resolve ER stress is called the unfolded protein response (sometimes denoted UPR). This response can be induced by factors that induce cellular stress; e.g. toxins, glucose deprivation, increased protein synthesis, imbalanced calcium levels and by presence of mutated or misfolded proteins [165]. Several of these factors are present in obesity, and obesity-induced ER-stress may be able to promote peripheral insulin resistance [166]. The unfolded protein response works through several mechanisms to restore proper ER function, including chaperone synthesis and increased ER-associated degradation of proteins. If ER function can not be restored, the unfolded protein response will eventually induce apoptosis.

It has previously been shown that the SELS protein is part of a membrane complex that mediates retro-translocation of misfolded proteins from the ER lumen to the cytosol, where the misfolded proteins are degraded [115]. Over-expression of SELS in macrophages provided protection against pharmacological ER stress agents and increased survival of macrophages *in vitro* [163]. SELS expression is induced by pharmacological ER stress agents, by proinflammatory cytokines in HepG2 and intestinal epithelial cells [167, 168] and by glucose deprivation in HepG2 cells [169].

SELS gene expression analysis

In *Paper III* we wanted to test the hypothesis that SELS is involved in the development of obesity-associated comorbidities. SELS gene expression was analyzed in subcutaneous adipose tissue in lean and obese siblings from the SOS Sib Pair Study. The obese subjects had slightly higher SELS expression levels than lean subjects, although signal values were not strong in either group. The SELS gene expression correlated with measures of obesity in both lean and obese subjects. A previous study indicates that T2D affects SELS gene expression in subcutaneous adipose tissue [162]. In the SOS Sib Pair Study only very few subjects are diagnosed with T2D, therefore we could not directly assess this association. However, in obese subjects, SELS gene

expression correlated positively with levels of insulin and with HOMA-IR. An interaction between SELS and A-SAA has been proposed to be a mechanistic link between T2D, inflammation and cardiovascular disease but it remains unknown if A-SAA interacts with SELS located in the plasma membrane or in the ER. A recent study shows that gene expression of both SELS and SAA1 is increased in the liver of diabetic rats compared to non-diabetic rats, and that SAA1 and SELS gene expression in the liver are positively correlated [170]. Furthermore, both SELS and A-SAA adipose tissue gene expression have been reported to correlate with A-SAA serum levels [57, 162], However, in our study, we found no correlation between SELS and A-SAA gene expression in adipose tissue, after adjusting for age and sex. To further investigate SELS in relation to glycemic control, SELS gene expression was analyzed after *in vitro* insulin stimulation of human subcutaneous adipocytes. We found that SELS gene expression increased after insulin stimulation.

Genotyping of SELS SNPs in the INTERGENE cohort

To test the hypothesis that genetic variants of SELS play a role in the development of cardiovascular disease, we investigated genetic variants in the SELS gene in the INTERGENE case-control cohort. Three SNPs (Table 4) previously shown to be associated with plasma levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α [116] were selected for genotyping.

Table 4. SELS SNPs reported to be associated with levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α [116]

SNP ID	dbSNP ID	Location in SELS gene
C-105T	rs28665122	5' UTR
C3705T	rs4965814	Intron 5
A5227G	rs4965373	3' UTR

db, database; UTR, untranslated region;

The 5227GG genotype was overrepresented among CHD cases with a Mantel-Haenszel Odds Ratio of 1.28 (95% confidence interval: 1.01-1.64), however C-105T and C3705T displayed no difference between cases and controls. Associations between SELS genotype and cardiovascular disease (CHD or stroke) have been reported by Alanne *et al.* [171]. In that study, they found strong associations in women with SNPs rs8025174 and rs7178239. However, when our study was initiated, the study by Alanne *et al.* had not been published. Therefore, these two SNPs were not included in our study. As the INTERGENE study contains few women we were not able to test if the associations were stronger in women than in men.

We next analyzed SELS genotypes in the full cohort in relation with metabolic parameters. Using multiple linear regression analysis, adjusting for diabetes status, sex and age, we found associations between the C3705T and measures of glycemic control (HOMA-IR and glucose levels), and between the A5227G genotype and levels of insulin and diastolic blood pressure. However, no associations were found for the C-105T SNP. When effects of dominant inheritance of the minor allele in A5227G

were analyzed (genotype AA+AG vs GG), associations were found to diastolic blood pressure, insulin and HOMA-IR. Analysis of the recessive effects of the minor allele (genotype GG+AG vs AA), revealed an association with only diastolic blood pressure.

In conclusion, SELS gene expression in human adipose tissue and SELS genotypes were associated with measures of glucose homeostasis. In addition, SELS gene expression was upregulated by insulin stimulation in isolated human adipocytes. Our results further support a role for SELS in metabolic disease.

4.4 Paper IV

Establishment of a transgenic mouse model specifically expressing human serum amyloid A in adipose tissue.

Several studies indicate that A-SAA may have metabolic functions, however the role of A-SAA in metabolism is unclear. Based on the assumption that, in humans, adipose tissue produced A-SAA enters the circulation (see section 1.6.4), we hypothesised that adipose tissue-derived A-SAA may influence the development of obesity-associated comorbidities, especially the development of atherosclerosis. To investigate this, we have generated a transgenic mouse model that mirrors the situation in human obesity with over-expression of hSAA in adipose tissue (see section 3.5.2).

Several findings indicate that A-SAA influences the development of atherosclerosis. The A-SAA protein may contribute to local and systemic inflammation as recombinant SAA1 has been shown to promote proinflammatory cytokine production [54]. In mouse adipose tissue, the SAA3 protein has been reported to interact with hyaluronan in the extracellular matrix [172]. Hyaluronan-SAA3 complexes are chemotactic towards monocytes, a property that is dependent on the SAA3 content in the complex [172]. Recombinant A-SAA has also been reported to induce chemotaxis of inflammatory cells (see Table 2 for references), thereby possibly recruiting monocytes to atherosclerotic lesions. In the circulation, the majority of A-SAA is found associated with HDL [66]. The SAA-HDL displays increased affinity for macrophages and reduced affinity for hepatocytes [173]. Accordingly, A-SAA has been suggested to impair reverse cholesterol transport.

Recently, adenoviral induction of mouse SAA production, in absence of systemic inflammation, was reported to be associated with reduced reverse cholesterol transport [174]. However, the mouse SAA2 protein has been reported to stimulate cholesterol efflux from macrophages, suggesting an antiatherogenic role as well. The effect was demonstrated to involve the intracellular balance between transportable de-esterified cholesterol and stored esterified cholesterol. The SAA2 protein interacts with two enzymes regulating this balance, thereby shifting the balance in favour of transportable cholesterol [175]. The functionally active domains in the SAA2 protein have been identified [175], and liposomal administration of the active SAA2 peptides to mice prevented and reversed atherosclerotic lesions [176]. Since the mechanisms were specific for peptides of the SAA2 isoform [175], it is unclear if this is a major effect of A-SAA *in*

vivo. Despite this, the effect is interesting, especially since administration of SAA2 peptides may translate into clinical use.

A-SAA contains proteoglycan binding domains [177] which may contribute to lipid and lipoprotein retention in atherosclerotic lesions [178]. *In vitro*, the ability of SAA-HDL to bind to proteoglycans has been demonstrated to correlate with the A-SAA content of HDL particles [179]. Retained HDL particles increase the lipid content in the vessel wall, and may also be more susceptible to oxidation, especially if the HDL has reduced anti-oxidative properties as is the case of HDL produced during the acute phase reaction [180]. A recent study showed that recombinant A-SAA may even directly promote synthesis of the vascular proteoglycan biglycan in a pro-atherogenic manner, and that a short-term adenoviral induced hSAA production was sufficient to obtain pro-atherogenic proteoglycan synthesis *in vivo* [181].

Circulating levels of A-SAA are increased in diet-induced obesity in mice [182, 183] similar to the situation in obese humans. Recently, A-SAA was investigated in relation to atherosclerosis in atherosclerosis-prone ApoE-deficient mice ($ApoE^{-/-}$). The HF fed $ApoE^{-/-}$ animals gained weight, had elevated plasma A-SAA levels and the A-SAA protein was found to be colocalized with ApoB in the atherosclerotic lesions [184]. However, gene expression of SAA gene family members differ in mice compared to humans. In the adipose tissue of obese mice, A-SAA expression is low and the SAA3 protein is the predominantly produced isoform [182, 183]. Absence of SAA3 in the serum of obese mice was recently demonstrated [183], indicating that in obese mice, circulating SAA is of hepatic origin. As a consequence, diet induced obesity in mice is not an optimal model for studies of the functional role of adipose tissue-derived A-SAA. One approach that has been used to analyze the effects of human A-SAA in mice without inducing an acute phase response is to use adenoviral-induced expression. However, the time span of adenoviral-induced expression is short and the production is localized to the liver. Accordingly, this technique can not accurately reflect the long term increase of A-SAA that is present in human obesity.

In *Paper IV* we describe the initial characterization of our hSAA transgenic mouse model. A tissue distribution analysis of hSAA expression confirmed that the hSAA was predominantly expressed in adipose tissue. The hSAA mice displayed no visible phenotype compared to wild type mice. Although hSAA mice had a slightly lower body weight at 11 weeks of age, compared to wild type mice, the growth rate during the 18 subsequent weeks of NC and HF diet did not differ between hSAA and wild type mice. The total amount of body fat was similar in hSAA and wild type mice, as determined by DEXA analysis after 17 weeks of HF or NC diet.

Plasma samples were analyzed for presence of hSAA protein using an enzyme-linked immunosorbent assay (ELISA) specific for human SAA. The hSAA was detectable in plasma, demonstrating that adipose tissue produced A-SAA is released into the circulation. The HF hSAA mice had increased plasma levels of hSAA compared to NC fed hSAA mice (Figure 3). The hSAA plasma levels in HF and NC fed animals were in the same range as in obese and lean human subjects [153, 185], respectively. In humans, the serum levels of A-SAA are correlated with measures of adiposity [57, 92]. Similarly,

in transgenic mice, plasma hSAA levels were strongly correlated with total fat mass in the HF fed animals. Furthermore, in humans and mice, A-SAA in the circulation is associated with HDL [66, 67]. To test if this was true also in the transgenic animals, pooled plasma samples were subjected to fast protein liquid chromatography (FPLC) separation. Analysis of the hSAA content in FPLC fractions using ELISA revealed that hSAA was found in the HDL containing fractions. Initial analysis of plasma samples showed that the hSAA association induced no size- or cholesterol-content changes in the lipoproteins. This is in line with previous studies of adenoviral transgenic expression of hSAA in mice [181, 186].

In vitro studies have suggested that A-SAA is able to displace ApoA-I from HDL particles in a ratio dependent way [76]. However, it is unclear to what extent this displacement occurs in vivo [187]. Although not yet investigated, our model opens up for the possibility to investigate if ApoA-I on the HDL particles is displaced by A-SAA during non acute phase situations.

In conclusion, the established mouse model has an adipose tissue-specific expression of hSAA under the control of the aP2 promoter/enhancer region. The adipose tissue derived hSAA is released into the circulation and, in plasma, the hSAA concentration was highest in the HDL containing fractions. Furthermore, plasma levels of hSAA in NC and HF fed animals were in the same range as in lean and obese humans, respectively. In contrast with previous studies with short term effects of adenoviral-induced liver-produced hSAA, our model enables the study of long term hSAA effects, especially when investigated in atherosclerotic prone mice strains. Taken together, this model displays all the important characteristics needed to study direct metabolic and vascular effects of adipose tissue-derived hSAA.

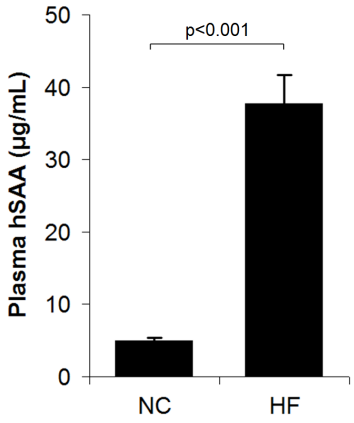

Figure 3. hSAA plasma levels in hSAA transgenic animals. *Plasma levels of hSAA were measured in normal chow (NC) (n=7) and high fat (HF) fed (n=10) animals. Values are given as mean ± SEM.*

4.5 A final note regarding SAA

In a recent study, it was suggested by Björkman *et al.* that the commercial recombinant human A-SAA protein does not have the same functional properties as the endogenous purified A-SAA protein [110]. Instead, endogenous A-SAA was shown to lack

proinflammatory effects, whereas recombinant A-SAA was proinflammatory [110]. The recombinant A-SAA molecule used was synthesized as a consensus molecule of SAA1 and SAA2. The rationale behind using a consensus molecule is that the more than 90 percent sequence homology between the SAA1 and SAA2 proteins, suggests similar functions of the isoforms. The recombinant A-SAA is based on the SAA1 sequence but has a N-terminal methionine and substitution of aspargine for aspartic acid at position 60 and arginine for histidine at position 71. This means that the C-terminal is homologous to the SAA2 protein. As a result, this peptide sequence does not correspond to the endogenous peptide sequence. Consequently, conclusions drawn from previously published *in vitro* experiments using the recombinant A-SAA may have to be reevaluated.

The recombinant A-SAA protein has not been used in the experiments that are presented in this thesis. Despite this, we have formed our hypothesis and drawn our conclusions based on previously published data. If the proposed discrepancy between the effects of the endogenous and recombinant molecules is true, it is unfortunate for this field of research that this molecule has been used extensively during the past 15 years (see Table 2). However, not all previous studies of A-SAA have used this recombinant protein, and in several studies *in vitro* data are complemented with *in vivo* data. It will be interesting to see if the findings by Björkman *et al.* [110] can be replicated by others, and also to see how far the suggested discrepancy extends.

5 SUMMARY

- The TNMD gene was highly expressed in subcutaneous adipose tissue and expression was equally high in adipocyte and stromal vascular fractions. TNMD gene expression was increased in obesity and reduced in response to weight loss. These findings suggest that TNMD plays a role in human adipose tissue.

- In a cohort with a wide BMI range, a correlation between serum levels of A-SAA and adipocyte size was found in women, and this correlation was most apparent in lean women,. The majority of associations between serum A-SAA and adiposity or serum markers were also found in women, demonstrating that serum levels of A-SAA may be influenced by sex specific factors.

- Gene expression of SELS correlated with serum levels of insulin and HOMA-IR in obese subjects. The SELS 5227GG genetic variant was slightly overrepresented in cases with coronary artery disease. SELS genotypes were also associated with measures of glycemic control. These data indicate that SELS may be involved in the development of obesity-associated comorbidities.

- A transgenic mouse model expressing hSAA in adipose tissue was established. Initial characterization indicates that necessary characteristics are present. The model will be used to study metabolic and vascular effects of adipose tissue-derived A-SAA.

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