Adipose tissue–derived serum amyloid A and adipose tissue macrophages in metabolic disease

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Till mina älskade föräldrar

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ABSTRACT

Obesity is associated with a low-grade chronic inflammation with increased levels of proinflammatory markers in the circulation and local inflammation in the adipose tissue. In this thesis, two aspects of obesity-related inflammation have been studied. Macrophages in the adipose tissue and the moderately elevated serum levels of serum amyloid A (SAA) derived from the adipose tissue have both been suggested as possible players behind obesity-related comorbidities in humans. The aim of this thesis was to investigate if there are obesity-independent links between adipose tissue macrophages and metabolic dysfunction and to examine if adipose tissue-derived SAA contributes to the development of insulin resistance, obesity-related inflammation and atherosclerosis.

Adipose tissue gene expression of macrophage markers was analyzed in relation to anthropometric and metabolic parameters in the Swedish Obese Subjects (SOS) Sib Pair study. The gene expression of macrophage markers was associated with insulin sensitivity and serum lipid levels and these associations remained, although weakened, when the analysis was adjusted for BMI. The link between adipose tissue macrophages and insulin sensitivity was confirmed by showing that the adipose tissue gene expression of macrophage markers was increased in patients with type 2 diabetes mellitus compared to their BMI-matched non-diabetic controls.

To investigate the effects of adipose tissue-derived SAA, a hSAA$^{+/-}$ transgenic mouse model (hSAA1 mice) where human SAA1 is specifically expressed in the adipose tissue was established. When the hSAA1 mice were fed normal chow or high fat diet, the serum levels of SAA resembled those in lean and obese humans, respectively. The circulating SAA was found in the high density lipoprotein (HDL)-containing FPLC fractions indicating an association of SAA to high density lipoprotein. This is an important finding since lipid-free SAA has other functions than SAA associated with HDL. The hSAA1 mice fed a high fat diet displayed similar glucose and insulin responses during an oral glucose tolerance test compared to their wild type littermates. In addition, the adipose tissue mRNA levels of genes related to insulin sensitivity were not decreased. Circulating levels of proinflammatory markers and gene expression of macrophage markers in the adipose tissue were not increased in hSAA1 mice. Hence, the hSAA derived from adipose tissue did not affect local and systemic insulin sensitivity or obesity-related

inflammation in our mouse model. To investigate possible effects of adipose tissue-derived hSAA1 on the development of atherosclerosis, the hSAA1 mice were crossbred with ApoE$^{-/-}$ mice. Analyses of *en face* prepared aortas from hSAA$^{+/-}$/ApoE$^{-/-}$ mice displayed similar atherosclerotic lesion areas in all sections of the aorta compared to wild type mice.

In conclusion, adipose tissue gene expression of macrophage markers is increased in type 2 diabetes mellitus and linked to insulin sensitivity and serum lipid levels independent of obesity. Furthermore, despite extensive research and several different experimental setups, we find no evidence for a causal role of adipose tissue-derived hSAA in the development of insulin resistance, obesity-related inflammation or atherosclerosis in hSAA1 mice.

SAMMANFATTNING PÅ SVENSKA

Fetma är associerat med en kronisk låggradig inflammation i fettväven med ökad infiltration av makrofager och med måttligt förhöjda nivåer av akutfasproteinet serum amyloid A (SAA) i blodet. De förhöjda nivåerna av SAA har visat sig härstamma från fettväven hos feta individer. Både makrofager i fettväven och de förhöjda nivåerna av SAA skulle kunna vara delar av orsaken till varför fetma ofta leder till metabola störningar såsom insulin resistens och kärlförkalkning men det finns också en möjlighet att de bara är inerta markörer för metabol dysfunktion. Syftet med studierna i denna avhandling var att klargöra om fettvävsmakrofager är associerade till metabola störningar oberoende av fetmagrad och att studera om fettvävsproducerat SAA kan bidra till utvecklingen av metabol sjukdom.

Genuttrycket av markörer för makrofager mättes i fettväv och analyserades mot metabola parametrar i Swedish Obese Subject (SOS) Sib Pair study. Analysen visade att uttrycket av makrofagmarkörer i fettväv var kopplat till insulinkänslighet samt till kolesterol- och fettnivåer i blodet även när analysen justerades för inverkan av fetma. Sambandet mellan fettvävsmakrofager och insulinkänslighet undersöktes vidare genom att analys av genuttrycket av makrofagmarkörer i fettväven hos patienter med typ 2 diabetes jämfördes mot uttrycket hos friska BMI-matchade kontroller. Här kunde sambandet mellan insulinkänslighet och fettvävsmakrofager stärkas ytterligare då genuttrycket av makrofagmarkörer i fettväv var högre hos patienterna med typ 2 diabetes.

För att studera hur fettvävsproducerat SAA påverkar metabol sjukdom utvecklades en transgen musmodell med specifikt uttryck av humant SAA i fettväven. Musmodellen uppvisar liknande cirkulerande nivåer av humant SAA som de som finns hos smala och feta människor. Det humana proteinet återfinns associerat till high density lipoprotein (HDL) vilket är ytterligare en viktig faktor för att musmodellen ska efterlikna människa. Möss som utfodrats med högfettsdiet genomgick ett oralt glukostoleranstest för att få en uppfattning om hur mössen reagerade på en hög dos glukos. Glukos- och insulinnivåerna som uppmättes påvisade inte någon insulinresistens hos de transgena mössen jämfört med en grupp kontrollmöss. Den lokala insulinkänsligheten i fettväven undersöktes genom att analysera genuttrycket av gener viktiga för insulinsignalering. De transgena mössen uppvisade inga sänkta genexpressionsnivåer av dessa gener vilket tyder på att insulinresistensen i fettväven inte förvärrades av den lokala produktionen av SAA. De hSAA1 transgena mössen undersöktes även med avseende på

infiltration av makrofager i fettväven och nivåer av inflammatoriska markörer i blodet som vanligtvis är förhöjda vid fetma. Genuttrycket av makrofagmarkörer var oförändrat i fettväv hos transgena möss och de uppvisade inte heller några förhöjda nivåer av inflammatoriska markörer i blodet jämfört med en grupp kontrollmöss. För att undersöka huruvida fettvävsproducerat SAA påverkar kärlförkalkning avlades de transgena mössen mot en musstam som saknar apolipoprotein E och som spontant utvecklar kärlförkalkning. De transgena mössen uppvisade dock ingen skillnad i omfattning av kärlförkalkning jämfört med kontrollmöss.

Sammanfattningsvis har denna avhandling visat att fettvävsmakrofager är länkade till metabol dysfunktion oberoende av fetma och att diabetiker uppvisar förhöjda nivåer av fettvävsmakrofager i jämförelse med en frisk BMI-matchad kontrollgrupp. Avhandlingen rapporterar även om utvecklingen av en ny transgen musmodell som har ett specifikt uttryck av humant SAA i fettväven och som uppvisar nivåer av SAA som hos smala och feta individer. Studier i denna musmodell har dock inte kunnat påvisa att fettvävsproducerat SAA kan orsaka nedsatt insulinkänslighet eller ge upphov till fetmaassocierad inflammation. Ett kausalt samband mellan fettvävsproducerat SAA och kärlförkalkning har ej heller kunnat påvisas. Detta indikerar att de förhöjda nivåerna av SAA som finns vid fetma inte är en orsak till metabol sjukdom utan fungerar som en markör för metabol sjukdom.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **Macrophage gene expression in adipose tissue is associated with insulin sensitivity and serum lipid levels independent of obesity**
    Ahlin S, Sjöholm K, Jacobson P, Andersson-Assarsson J. C, Walley A, Tordjman J, Poitou C, Prifti E, Jansson P-A, Borén J, Sjöström L, Froguel P, Bergman R. N, Carlsson L. M. S, Olsson B, Svensson P-A.
    Obesity (Silver Spring). 2013 Mar 20. (Epub ahead of print)

II. **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 L. M. S, Sjöholm K.
    PLoS One. 2011;6(5):e19609. Epub 2011 May 18. (Open access)

III. **No evidence for a role of adipose tissue-derived serum amyloid A in the development of insulin resistance or obesity-related inflammation in hSAA1$^{+/-}$ transgenic mice**
    Ahlin S, Olsson M, Olsson B, Svensson P-A, Sjöholm K.
    PLoS One. 2013;8(8):e72204. Epub 2013 August 15. (Open access)

IV. **Adipose tissue-derived human serum amyloid A does not affect atherosclerotic lesion area in hSAA1$^{+/-}$/ApoE$^{-/-}$ mice**
    Ahlin S, Olsson M, Wilhelmson A. S, Skålén K, Borén J, Carlsson L. M. S, Svensson P-A, Sjöholm K.
    In manuscript

CONTENT

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ABBREVIATIONS

ABCA1   ATP-binding cassette transporter A1

ABCG1   ATP-binding cassette transporter G1

Adipoq   Adiponectin

aP2   Fatty acid binding protein 4

ApoAI   Apolipoprotein AI

ApoE   Apolipoprotein E

A-SAA   Acute phase serum amyloid A

ATP   Adenosine triphosphate

AUC   Area under the curve

BAT   Brown adipose tissue

BMI   Body mass index, kg/m^2

BP   Blood pressure

CCR2   C-C chemokine receptor 2

CRP   C-reactive protein

C-SAA   Constitutive serum amyloid A

CXCL1   Chemokine (C-X-C motif) ligand 1

DEXA   Dual energy x-ray absorptiometry

DNA   Deoxyribonucleic acid

ELISA   Enzyme-linked immunosorbent assay

eWAT   Epididymal white adipose tissue

FPLC	Fast protein liquid chromatography
GLUT4	Glucose transporter type 4
HDL	High density lipoprotein
HFD	High fat diet
IFN-γ	Interferon γ
Ikkβ	I kappa B kinase β
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
IL12p70	Interleukin 12, subunit p40 and p35
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
LDL	Low density lipoprotein
MCP-1	Monocyte chemoattractant protein 1
NC	Normal chow
PCR	Polymerase chain reaction
PPAR-γ	Peroxisome proliferator-activated receptor γ
RNA	Ribonucleic acid
rWAT	Retroperitoneal white adipose tissue
SAA	Serum amyloid A

SR   Sedimentation rate

SV40   Simian virus 40

TNF-α   Tumor necrosis factor α

WHR   Waist-to-hip ratio

wt   Wild type

1 INTRODUCTION

1.1 Obesity

1.1.1 Definition and measures of obesity

Obesity is a condition with excess accumulation of body fat [1]. Normal percentages of body fat range between 15-20 percent in men and 25-30 percent in women [1]. In obesity, individuals can have more than 50 % body fat. There are many methods that can be used to estimate body fat such as magnetic resonance imaging, computed tomography, and dual energy x-ray absorptiometry (DEXA). However, these methods are time consuming and expensive. Alternatively, anthropometric measures such as sagittal diameter, waist-to-hip ratio (WHR) and body mass index (BMI) can be used to estimate body composition [2].

Today, BMI is one of the most common methods used to estimate obesity. BMI is calculated by dividing weight (kg) with height squared (m^2). Despite individual variations, there is a good correlation between BMI and body fat in large populations [1]. The World Health Organization uses weight classification based on BMI where BMI < 18.5 = underweight, BMI 18.5-25 = normal weight, BMI 25-30 = overweight, BMI > 30 obese and BMI > 40 = morbidly obese [3]. Many epidemiological studies of obesity are also based on this classification.

1.1.2 Epidemiology

Over the last 30 years there has been a dramatic increase in obesity world-wide [4-6]. Reports have estimated that 1.46 billion adults are overweight and of these, 205 million men and 297 million women are obese [6]. As much as 33 % of the US population is considered as obese [7] whereas prevalence data for Sweden show that 15 % of men and 14 % of women are obese and an additional 28 percent of women and 42 % of men are overweight [8].

1.1.3 Etiology

Obesity is caused by an imbalance between energy intake and energy expenditure where the surplus energy is stored mainly as fat [9].The changes in human life style during the last decades, with a more sedentary life style and easily accessible food have contributed to the development of an obesity epidemic. However, studies in twins have shown that much of the individual variation in adiposity is due to genetic factors [10-12]. There are

monogenetic causes of obesity such as defects in the leptin signaling system but these are rare and the majority of obesity development is probably a combination of environmental changes and genetic susceptibility.

Many hypotheses have been presented to explain why humans are prone to develop obesity. According to the "thrifty genotype hypothesis" presented in 1962, genetic variants that promote efficient energy uptake and storage of surplus energy as fat had survival benefits in times of starvation [13]. An alternative hypothesis suggests that obesity-related genotypes have never been under evolutionary pressure until now since humans never lived under conditions of food excess. Thus, genetic variants causing obesity should be considered to be a result of genetic drift [14].

1.1.4 Obesity-related comorbidities

Obesity is part of the metabolic syndrome which is a clustering of metabolic disturbances often occurring together. There are different classifications of the metabolic syndrome but all include central obesity, prediabetes, hypertension and dyslipidemia and are all risk factors for cardiovascular disease [15-23]. Several other severe conditions such as fatty liver disease, cancer, atherosclerosis and sleep apnea are also linked to obesity [24-26] and obesity is associated with reduced life expectancy [27].

Insulin resistance is a part of the development of type 2 diabetes mellitus [28] and in parallel with increasing obesity prevalence, the prevalence of diabetes has increased as well [29]. The global prevalence of type 2 diabetes mellitus was estimated to 171 million people in 2000 and 366 million people are predicted to be affected in 2030 [30]. Both obesity and type 2 diabetes mellitus are risk factors for atherosclerosis, the major underlying cause of cardiovascular disease, a common cause of death worldwide [31,32]. However, the molecular mechanisms behind obesity-related disorders are, despite intensive research, still only partly understood.

1.1.5 Treatment strategies

Obesity is a condition that is difficult to treat. Treatment of obesity involves lifestyle modifications with restricted caloric intake and increased physical activity. However, it is hard to achieve long-term weight reduction using caloric restriction and a majority of people later regain the lost weight [33,34]. Today, bariatric surgery is the most effective way to obtain a long-term weight reduction [34]. Bariatric surgery also improves metabolic risk factors, prevents type 2 diabetes mellitus, lowers the risk for cancer, prevents fatal and non-fatal cardiovascular events and prevents premature death [34-

38]. However, it is neither possible nor desirable to operate all obese patients and molecular mechanisms behind obesity and obesity-related disorders need to be further investigated in order to find alternative treatment strategies.

1.2 Insulin action and insulin resistance

Systemic insulin resistance is a result of impaired insulin action in metabolically active tissues and a part of the development of type 2 diabetes mellitus [28].

Insulin is an anabolic hormone that induces glucose uptake in metabolically active tissues such as skeletal muscle, adipose tissue and the liver. Insulin is produced in and released from the β-cells in the pancreas and mediates glucose uptake via the binding to the insulin receptor (IR) on the cell surface. The binding of insulin to its receptor initiates a signaling cascade with subsequent tyrosine phosphorylation of insulin receptor substrate-1 and insulin receptor substrate-2 (IRS-1 and IRS-2). The cascade results in a translocation of glucose transporter type 4 (GLUT4) to the cell membrane and promotes glucose uptake [28,39]. The glucose is either used for ATP production or processed and stored as glycogen or fat within the cell. Insulin also regulates other processes such as protein translation and cellular hypertrophy and has the ability to suppress lipolysis in the adipose tissue [40].

In insulin resistance, there is a decreased glucose transport into the cells and insulin has an impaired ability to inhibit lipolysis in the adipose tissue [41]. The insulin resistance in the liver also leads to reduced suppression of gluconeogenesis and stimulation of free fatty acid production. This results in the hyperglycemia and hypertriglyceridemia that are characteristic of type 2 diabetes mellitus.

1.3 Dyslipidemia and reverse cholesterol transport

Dyslipidemia associated with obesity and the metabolic syndrome is characterized by hypertriglyceridemia and low levels of high density lipoprotein (HDL) cholesterol [17,20]. These changes are also accompanied by an increase in small dense LDL-particles. The hypertriglyceridemia in obesity-related dyslipidemia is partly due to insulin resistance as described in 1.2.

HDL is considered to have atheroprotective properties [42] and a low level of HDL cholesterol is a risk factor for cardiovascular disease [43]. HDL and its structural protein apolipoprotein AI play a major role in the reverse cholesterol transport [42] where cholesterol from peripheral tissues are transferred to HDL and ApoAI via the ATP-binding cassette transporters ABCA1 and ABCG1 for transport to the liver [44]. Cholesterol can not be degraded in peripheral tissues and has to be transported to the liver for biliary secretion. Hence, reverse cholesterol transport is considered important for protecting the peripheral tissues from lipid overload.

1.4 Atherosclerosis

Atherosclerosis is a progressive disease where atherosclerotic lesions are formed in the vessel wall [45]. The early stages of atherosclerotic lesions consist of small lipid deposits in the arterial intima that progress into "fatty streaks". The fatty streaks consist of lipoproteins and cholesterol-esters and are characterized by the presence of lipid loaded macrophages, "foam cells", in the intimal layer of the vessel. These initial lesions are present early in life and can develop into more advanced lesions. [45]. In the more advanced atherosclerotic lesions there are more extracellular lipid deposits with calcium that deform the intimal layer of the vessel and this can also affect the media and adventitia. In addition, foam cells, macrophages and lymphocytes without intracellular lipid accumulation are found within the lesion. Furthermore, in advanced stages of atherosclerotic lesions an overlying fibrous tissue cap is formed. Advanced atherosclerotic lesions can lead to clinical consequences, especially when there is a disruption of atherosclerotic lesion surface that causes thrombosis or emboli with a subsequent occlusion of the vessel [46].

The subendothelial proteoglycans are considered to play a role in the retention of the lipoproteins in the vessel wall [47,48]. Within the vessel wall, the lipoproteins are modified by oxidation which further contributes to the lesion formation [49]. The accumulation of lipoproteins in the vessel wall is more pronounced in regions exposed to mechanical forces [45] indicating that mechanical stress is an important factor for atherosclerosis development. Macrophages and lymphocytes are present in the atherosclerotic lesion [46] but the triggering signals for infiltration in the vessel wall and what triggers the transformation of macrophages into foam cells are not clear. Hence, the development of atherosclerotic lesions is complex and more studies are needed to identify mechanisms leading to atherosclerosis development.

1.5 The Adipose Tissue

1.5.1 Adipose tissue function

One of the main functions of the adipose tissue is to serve as a long-term reserve of energy [50]. In times of positive energy balance, surplus energy can be stored as triglycerides in the adipose tissue [50]. During starvation, the adipose tissue is able to hydrolyze the triglycerides into free fatty acids and glycerol providing an energy source for other tissues [50,51]. Lipolysis is catalyzed by adipose triglyceride lipase and hormone sensitive lipase. The lipolysis is normally inhibited by insulin and stimulated by catecholamines [52] but is also known to be regulated by other hormones such as glucocorticoids [53].

The adipose tissue has many other functions in the body. It is considered to function as a thermal insulation and a protection against mechanical injuries [50]. The adipose tissue is also involved in the metabolism of steroid hormones such as glucocorticoids and sex hormones [54].

1.5.2 Adipose tissue depots and adipose tissue expansion

The adipose tissue does not only contain adipocytes but also a variety of other cell types such a endothelial cells, fibroblasts, preadipocytes and pericytes as well as inflammatory cells including macrophages, mast cells and lymphocytes [55]. The white adipose tissue is distributed in different depots that can be divided into intra-abdominal ("visceral") and subcutaneous adipose tissue. Men and women have a tendency to store fat in different adipose tissue depots. Men store their fat to a larger extent intraabdominally than women, who have a tendency to store their fat in the subcutaneous depots.

In obesity, adipose tissue expands and is remodeled due to increasing demands in fat storing capacity. The fat storing capacity can be increased as a result from hyperplasia with increased number of adipocytes or from hypertrophy of adipocytes [55-57]. There is a stronger association between hypertrophic adipocytes and metabolic disease such as insulin resistance than hyperplastic adipose tissue with an increased number of smaller adipocytes [58]. It has been speculated that a hypertrophic adipocyte may be a signal showing that lipid storage capacity has reached its limit. A storage limit in adipose tissue could lead to lipid accumulation in other tissues with subsequent lipotoxicity and metabolic dysfunction [59,60]. This hypothesis is

further supported by the fact that also very little adipose tissue, lipodystrophy, causes hyperglycemia and hyperinsulinemia probably due to limited fat storing capacity [61-65]. These observations pinpoint the importance of the adipose tissue as a factor in glucose homeostasis and metabolic disease.

1.5.3 The adipose tissue as an endocrine organ

The adipose tissue is not only capable of releasing free fatty acids to provide energy for other organs but is also able to produce and release proteins involved in body metabolism, blood hemostasis and inflammatory responses [54,66-72]. The endocrine function of the adipose was put into focus by the discovery of leptin in 1994 when the mutant gene causing obesity in the ob/ob mice was found [66]. The adipose tissue is the main source of circulating leptin in both mouse and human and is increased in obesity [67,68]. Leptin has a major impact on the central control of energy balance in the hypothalamus where it suppresses food intake and stimulates energy expenditure [73-75]. The role of leptin in human energy balance is well illustrated by the few cases of patients who develop early on-set, morbid obesity due to leptin deficiency or non-signaling leptin receptors [76].

Several other proteins secreted from the adipose tissue have been detected since the discovery of leptin [70,72,77,78]. The expression and secretion of these proteins are often influenced by adiposity and hypertrophic adipocytes are known to have an altered production of many adipokines [72,77,79-81]. The altered expression of these proteins is suggested to play a role in the development of obesity and obesity-related disorders. However, the full extent of adipokine impact on metabolic disease is not clear.

1.6 Obesity–related inflammation

Obesity is an inflammatory condition with moderately increased levels of inflammatory markers, including IL-6, C-reactive protein and serum amyloid A (SAA) [82-89]. In addition, obese individuals display a low-grade chronic inflammation locally in the adipose tissue, including increased infiltration of macrophages [90,91]. The metabolic dysfunction associated with obesity has been suggested to be caused by the obesity-related inflammation [72,90,92]. Several studies have investigated different aspects of the obesity-related inflammation but more studies are needed to elucidate all mechanisms behind this link.

1.6.1 Adipose tissue macrophages

Macrophages are present in the adipose tissue of lean and obese individuals but numbers are increased in obesity [90,91,93,94]. The macrophages in adipose tissue of obese individuals are often found surrounding dying adipocytes in so called "crown-like" structures whereas macrophages in lean individuals are more interspersed in the adipose tissue [95]. Adipocyte death is suggested to be induced by hypoxia and subsequent endoplasmic reticulum stress that occur in the expanding adipose tissue in obesity [95-100]. The adipocyte death could trigger signals that mediate macrophage infiltration in the adipose tissue. Transgenic adipose-tissue specific expression of chemoattractants has been shown to have an impact on macrophage infiltration in the adipose tissue [101,102] but more studies are needed to examine what causes macrophage infiltration into the adipose tissue and what role the adipose tissue macrophages play in metabolic dysfunction.

1.6.2 Serum Amyloid A (SAA)

SAA gene family

SAA is an apolipoprotein and the SAA gene family members are, similar to other apolipoproteins, structured with a four exon and three exon organization [103]. Mature SAA proteins contain 104-112 amino acids [104] and the SAA gene family is phylogenetically conserved and found in many other species [105-108]. The SAA gene family is divided into two groups, acute phase SAA (A-SAA) and constitutive SAA (C-SAA) depending on activation in response to inflammatory stimuli [104].

The human SAA gene family comprises at least four members, all clustered at chromosome 11p15.1 [109]. SAA1 and SAA2 are the acute phase forms of the SAA gene family and display 90% sequence homology [110]. SAA4 is the constitutively expressed form of the human SAA gene family and does not share the same high sequence homology as SAA1 and SAA2 [111]. SAA3 is considered to be pseudogene as it contains a premature stop codon and is not transcribed [112].

In mouse, the SAA gene family is located in the proximal part of chromosome 7 [113-115]. Saa1 and Saa2 are considered to be the evolutionary homologues to SAA1 and SAA2 and are the acute phase forms of mouse SAA [104]. However, in the mouse there is a third acute phase form, Saa3 that does not share the same high sequence homology as Saa1 versus Saa2 and has no specific hepatic expression [116-118]. Saa4 is the

constitutive form of mouse SAA and is the evolutionary homologue of the human SAA4 [110,115].

SAA expression and induction

The acute phase forms of SAA are expressed in the liver as a response to inflammatory stimuli such as infection or tissue damage [119,120]. The hepatic A-SAA expression is induced by the proinflammatory cytokines IL-1β, TNF-α and IL-6 and the serum levels of SAA can rise 1000-fold during acute phase [119-125]. However, extrahepatic expression of A-SAA has also been reported in both mouse and human [126-129].

We and others have shown that the adipose tissue is the main producer of A-SAA in obese humans in absence of a systemic inflammatory response [128,129]. SAA expression is increased in obese individuals and hypertrophic adipocytes and obese individuals display moderately elevated serum levels of SAA [128,130]. Both SAA gene expression in adipose tissue and circulating levels of SAA decrease during weight reduction [128,129]. In addition, *in vitro* studies have shown that SAA release from adipose tissue biopsies is correlated with BMI [131]. These data indicate that the elevated levels of SAA in obesity are derived from the adipose tissue in humans. The chronic moderately elevated levels of SAA found in obesity are linked to both insulin resistance and atherosclerosis [84,86,89,132,133]. Several mechanisms behind this link have been suggested but the actual role of adipose tissue-derived SAA in metabolic disease is still unknown.

Suggested functional roles of SAA

The fact that the SAA gene family is highly phylogenetically conserved and that there is a rapid and massive induction as a response to inflammatory stimuli during the acute phase indicate that SAA has important functions in the body [110]. Many different studies have therefore been performed to investigate the possible roles of SAA.

SAA is an apolipoprotein and associates with HDL in the circulation (HDL-SAA) [134-136]. It has been suggested that SAA may play a role in the reverse cholesterol transport during the acute phase (Figure 1). However, the role for SAA in reverse cholesterol transport is disputed. HDL-SAA displays reduced affinity for hepatocytes and higher affinity for macrophages [137]. This has been interpreted as HDL being redirected to activated macrophages in the acute-phase. One hypothesis states that SAA affects reverse cholesterol transport in order to provide damaged cells with lipids for regeneration [138,139] and it has been found that overexpression of human SAA1 in mice impairs HDL-mediated reverse cholesterol transport [140]. Others suggest

that SAA removes excess cholesterol from sites of injury [141]. Both administration of mouse SAA2 and peptides of mouse SAA2 in a liposomal solution promote cholesterol efflux from macrophages both *in vivo* and *in vitro* [142-144], suggesting that SAA may facilitate reverse cholesterol transport. The suggested SAA-induced changes in reverse cholesterol transport may also have anti- or proatherogenic effects if it affects the extent of lipid accumulation in the vessel wall. Other suggested functions for SAA during the acute phase include a role in tissue repair. Recombinant human SAA1/2 induces the expression of matrix metalloproteinases that degrade the extracellular matrix *in vitro* [145,146]. Recent studies have reported mRNA levels of SAA in human and mouse atherosclerotic lesions [147-149]. A local production of SAA in the vessel wall could lead to atherosclerotic lesion rupture and subsequent thrombosis if SAA is able to induce matrix metalloproteinases *in vivo*.

Several other mechanisms where SAA could affect atherogenesis development have been suggested and are summarized in Figure 1. SAA may promote lipoprotein retention in the vessel wall. SAA has a binding site for heparan sulfate [150] and mouse acute-phase SAA facilitates the binding of HDL to proteoglycans *in vitro* [151]. In addition, SAA stimulates proteoglycan synthesis in vascular smooth muscle cells *in vitro* and *in vivo* [152], which potentially could lead to increased lipoprotein retention in the vessel wall. Moreover, recombinant human SAA1/2 induces foam cell formation in cultured mouse macrophages [153,154], which could promote atherosclerosis development.

The association of SAA to HDL is suggested to affect the protective anti-inflammatory properties of HDL in atherosclerosis. Recombinant human SAA1/2 in association with HDL reduces HDL's ability to inhibit MCP-1 expression in vascular smooth muscle cells [155] and the recombinant protein stimulates cytokine and chemokine expression in both macrophage and endothelial cell lines [156,157]. In addition, recombinant SAA1/2 up regulates cell adhesion molecules in cultured endothelial cells [157,158] and exhibits chemoattractant effects on monocytes and neutrophils [159-161]. This could lead to the recruitment of macrophages in the vessel wall and contribute to the vessel wall inflammation.

The reported proinflammatory properties of SAA may also be of importance for obesity-related inflammation with increased levels of inflammatory markers in the circulation and increased infiltration of macrophages in the adipose tissue. Increased macrophage infiltration into adipose tissue may affect insulin resistance [101,102]. Hence, SAA could indirectly affect

insulin sensitivity if SAA acts as a chemoattractant in the adipose tissue. However, SAA may also directly affect insulin resistance as recombinant SAA reduces mRNA levels of genes related to insulin sensitivity in adipocytes [162-164].

Some of the functions of SAA presented here could be mediated via different SAA receptors [165-173]. Reports have suggested importance of SAA receptors in processes such as cholesterol efflux, glucose homeostasis, chemotaxis and inflammatory signaling. However, most of these receptors are not specific for SAA and most of the receptors have been identified as receptors for the recombinant SAA1/2 and not the endogenous SAA.

Taken together, SAA has been suggested to be involved in many different processes including cholesterol transport, insulin resistance, inflammation and the development of atherosclerotic lesions. However, many studies investigating SAA function have been performed using a recombinant and/or de-lipidated SAA [145,154-157,159-163]. The recombinant protein is a consensus SAA molecule which corresponds to SAA1α except for an N-terminal methionine and substitution of asparagine for aspartic acid at position 60 and arginine for histidine at position 71. We and others have shown that the recombinant protein does not share the same functions as the endogenous SAA [174,175] and the physiological relevance of the recombinant protein is unclear. There are a limited number of *in vivo* studies investigating the role of SAA in metabolic disease and the role of adipose tissue-derived SAA is still obscure. Hence, further research is needed to elucidate if SAA plays a causal role in the development of metabolic disease.
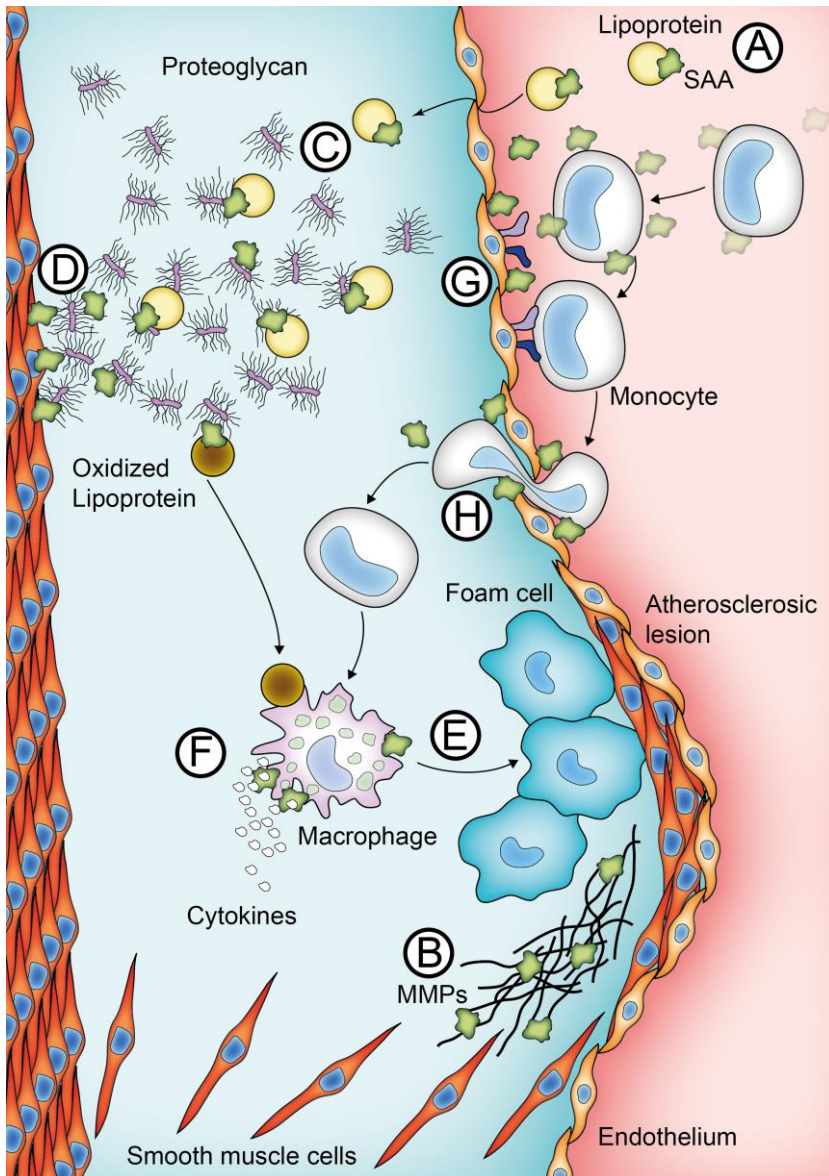
Figure 1. Suggested proatherogenic functions of SAA. A. Interaction with the reverse cholesterol transport. B Induction of matrix metalloproteinases that are able to degrade the extracellular matrix. C. Promoting binding of HDL to proteoglycans in the vessel wall. D. Stimulation of proteoglycan synthesis in the vessel wall. E. Induction of foam cell formation. F. Stimulation of cytokine and chemokine synthesis. G. Induction of endothelial adhesion molecules production. H. Acting as a chemoattractant for monocytes and neutrophils. *Picture by J. Carlsten*

AIM OF THE THESIS

The overall aim of this thesis was to investigate factors within the adipose tissue that could contribute to the development of obesity-related disorders such as insulin resistance and atherosclerosis.

The specific aims of the different studies were:

I.   To elucidate whether there are obesity-independent links between macrophage gene expression in adipose tissue and metabolic dysfunction in humans.

II.   To establish a transgenic mouse model with adipose tissue-specific expression of human SAA1 as a tool for studies of the role of adipose tissue-derived SAA in metabolic disease.

III.   To test the hypothesis that adipose tissue-derived human SAA can act as a local and systemic regulator of insulin sensitivity and inflammation.

IV.   To investigate whether adipose tissue-derived human SAA plays a role in the development of atherosclerosis in mice.

2 STUDY SUBJECTS AND METHODS

2.1 Study subjects

2.1.1 Ethics statement

The regional Ethics Committee in Gothenburg, Sweden approved the human studies. All subjects received written and oral information about the study before giving written consent to participate.

All study protocols involving animals were approved by the local ethics committee for animal studies at the administrative court of appeals, Gothenburg, Sweden.

2.1.2 The Swedish Obese Subjects (SOS) Sib Pair study

The SOS Sib Pair study consists of 732 individuals in 154 nuclear families containing sibling pairs with a BMI difference of more than 10 units. Data from a group of parents (n = 88) and an offspring group (n = 357) were used in paper I. Only the most extreme siblings according to BMI in each family were used for comparisons between lean and obese which resulted in 78 pairs of sisters and 12 pairs of brothers. The supplementary data in paper I display the different study group characteristics in the SOS Sib Pair study.

The participants in the SOS Sib Pair study are very well characterized. Fasting blood samples were obtained and blood chemistry analyses were performed at the Department of Clinical Chemistry, Sahlgrenska University hospital. Frequent-sampling intravenous glucose tolerance test was performed and data were subjected to minimal model analysis to assess estimates of insulin sensitivity. Measurement of body composition was made with dual-energy x-ray absorptiometry (DEXA) at Sahlgrenska University Hospital. Subcutaneous adipose tissue biopsies were obtained by needle aspiration in the paraumbilical area during local anesthesia and were snap frozen in liquid nitrogen, stored at -80°C and subsequently used for mRNA extraction and DNA microarray analysis.

2.1.3 Patients with type 2 diabetes mellitus and BMI−matched controls

Six patients with recently diagnosed type 2 diabetes mellitus and seven BMI- and sex-matched non-diabetic controls were enrolled in this study. Blood samples were obtained from fasting participants, blood chemistry analyses were performed and adipose tissue biopsies were obtained as described above. RNA was extracted and DNA Microarray analysis was performed.

2.2 Methods

2.2.1 A transgenic mouse model specifically expressing human SAA1 in the adipose tissue

A transgenic mouse model with specific expression of the human SAA1 gene in adipose tissue was established. In brief, a gene construction with the human SAA1 gene under the control of the adipocyte-specific fatty acid binding protein 4 promoter (aP2) was created (Figure 2). A SV40 polyadenylation sequence was ligated to the construction downstream the aP2-hSAA1 sequence. A rabbit beta-globin intron was inserted as an internal control upstream of the polyadenylation SV40 sequence but downstream the aP2-hSAA1 sequence.The gene construction was injected into fertilized eggs from C57BL/6 females using pronuclei injection and the eggs were then implanted in pseudopregnant females. DNA was extracted from tail biopsies and transgenic mice were identified using polymerase chain reaction (PCR). Subsequent breeding was performed with C57BL/6 mice to generate heterozygous hSAA$^{+/-}$ mice and wild type littermates.
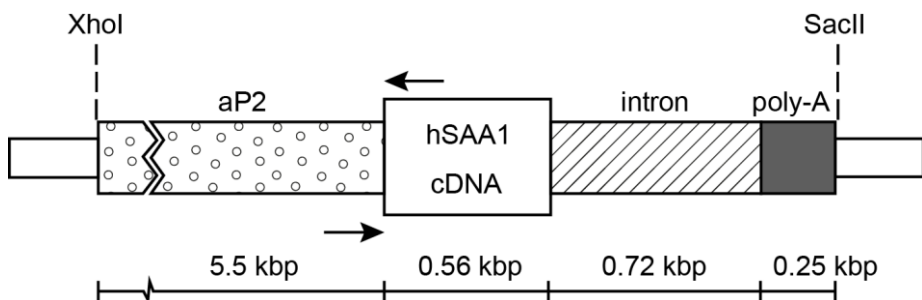

Figure 2. Schematic representation of the aP2 promoter/hSAA1gene construction. Dotted box represent the aP2 promoter, hatched box rabbit β-globin intron, grey box polyadenylation signal.

In the experimental set-up in paper II and paper III, groups of mice were given pelleted high fat diet (60 kcal% fat) from 13 or 10 weeks of age, respectively, until the end of the experiment. In paper IV, the hSAA1$^{+/-}$ mice were crossbred with apoE$^{-/-}$ mice to obtain hSAA1$^{+/-}$/apoE$^{-/-}$ mice to study the effect of adipose tissue-derived human SAA on the development of atherosclerosis in mice. The hSAA$^{+/-}$/apoE$^{-/-}$ mice and their wt/apoE$^{-/-}$ littermates were fed normal chow diet during the experiment.

2.2.2 Genotyping by PCR

The polymerase chain reaction (PCR) is the basic step in genotyping methods. The PCR method was developed in the 1980s and allow rapid amplification of DNA sequences [176]. The PCR is based on thermal cycling. In the first step, the reaction solution heats up and allow the DNA template strands to open up and enables synthesis of the DNA region of interest. Primers, complementary DNA sequences, decide which region of the DNA template that will be amplified. The primers hybridize to the complementary DNA when the working solution is cooled down. Then, after heating up the working solution again, the DNA polymerase will create a complementary DNA strand from nucleotides. The newly synthesized DNA strands will function as DNA template which creates a chain reaction where the number of DNA transcript will increase exponentially.

In this thesis genotyping of mice was performed in paper, II, III and IV. The amplification products and a DNA-ladder were run on an agarose gel with ethidium bromide or SYBR-safe DNA gel stain. The DNA-ladder and the amplification product were detected by UV-light.

2.2.3 RNA extraction

RNA extraction is the first step in gene expression analysis. Many different protocols are provided for RNA extraction but most of them are based on the original Chomczynski protocol from 1987 [177-179]. The RNA is separated from DNA and proteins in the cell lysate by adding guanidium thiocanate, sodium acetate, phenol and chloroform and subsequent centrifugation. The RNA will then stay in the upper aqueous phase which can be collected, undergo further washing step and precipitation [178]. Today, the RNA extraction can be performed with commercially available kits.

The RNA extraction has to be performed in ribonuclease free environment otherwise the RNA will be degraded. For gene expression analysis complementary DNA (cDNA) is synthesized from the extracted RNA using

reverse transcriptase. RNA extraction for subsequent gene expression analysis was performed in all papers in this thesis.

2.2.4 Gene expression analysis

Expression analysis with DNA microarray analysis

The gene expression of many different genes can be measured simultaneously by DNA microarray analysis. DNA microarrays are based on the principle that DNA molecules hybridize to complementary DNA strands [180-182]. A DNA microarray chip is constructed so that oligonucleotides corresponding to a gene sequence are placed on a known position on a solid surface. The cDNA synthesized from the RNA extraction is transcribed into biotinylated cRNA which can hybridize to the oligonucleotides on the surface. In the detection step of the analysis, biotin binds streptavidin that are linked to fluorescent phycoerythrin and a fluorescent signal is emitted. The intensity of the fluorescence signal reflects the expression level the gene of interest.

DNA microarray analysis was performed in paper I in two study populations to investigate the gene expression of macrophage markers in subcutaneous adipose tissue in relation to metabolic and anthropometric measurements. In the SOS Sib Pair study, the Human Genome U133 Plus 2.0 Gene Chip was used which contains over 54 000 oligonucleotide probe sets which covers most of the human genes. The Gene 1.0 ST arrays from Affymetrix, containing over 28 000 oligonucleotide probe sets was used in the patients with type 2 diabetes mellitus and their BMI-matched healthy controls. The DNA microarray analysis data was analyzed using the robust multi-chip average (RMA), which is the most common algorithm for processing DNA Microarray gene expression data today [183].

Expression analysis with TaqMan real-time PCR

As with regular PCR, real-time PCR technique uses primers flanking the DNA sequence of interest and a DNA polymerase synthesizing the DNA sequence from nucleotides in the reaction mixture. However, the reaction mixture also contains a probe that binds to the middle of the DNA sequence. The probe contains a fluorescence reporter molecule and a quencher which inhibits the emission of fluorescence from the reporter. When the DNA-polymerase synthesizes the new DNA-strand, the quencher is separated from the reporter. This terminates the inhibition and emission of fluorescence can occur. Depending on the amount of target template cDNA in the sample, there is a massive increase of fluorescence in the sample at a certain time point [184]. Quantification of the gene expression can be performed using a

standard curve or with relative quantification with a housekeeping gene whose expression is relatively stable [184-186].

Gene expression analysis with TaqMan real-time PCR was performed in paper II, III and IV. The gene expression of human SAA1/2 and mouse Saa3 in adipose tissue were explored in paper II, III and IV. Additional analysis of the expression of macrophage markers and genes related to insulin sensitivity in the adipose tissue were performed in paper III.

2.2.5 Plasma measurements

Fast Protein Liquid Chromatography (FPLC)

The Fast Protein Liquid Chromatography (FPLC) technique is used to purify a mixture of proteins. The methodology is based on the phenomenon that proteins display different affinity for different materials. The two materials in a FPLC consist of a mobile phase (elution buffer) and a porous solid (stationary) phase. The protein mixture is applied to the solid phase and the proteins bind to this. A constant flow of elution buffer but with successively increasing concentration is then applied. During this process the proteins dissociate from the solid phase and are then found in the effluent fractions. The proteins are detected by absorption of UV-light. Each protein appears in the effluent as a peak in protein concentration and the eluate can be collected for further analysis. FPLC has been performed in paper II in order to measure the concentration of human SAA and cholesterol in different FPLC-fractions.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is used to measure antigen or proteins in a liquid sample. There are different examples of ELISAs but most set ups involve two antibodies. The primary antibody detects the antigen/protein of interest and the secondary antibody either binds the primary antibody or the protein of interest. The secondary antibody carries an enzyme. When the substrate of the enzyme is added to the working solution it will lead to a shift in color which is related to the amount of target protein in the sample. The color of the sample is measured by a spectrophotometer [187-189] and analyzed in relation to a standard curve with known concentration of the protein of interest.

Measurement of human SAA and mouse SAA in plasma was performed in paper II, III and IV. Blood levels of insulin were measured in paper II and paper III. In paper III, a multiplex assay was used to measure proinflammatory markers in plasma from male mice fed a high fat diet.

2.2.6 Measures of body composition

Dual Energy X-ray Absorptiometry (DEXA)

Dual energy x-ray absorptiometry is a method used to estimate total and regional percentages of body fat. The technology is based on two X-rays with different energies. It measures the photon attenuation, considered to reflect the tissue composition, in each pixel of a picture. The DEXA analysis can differentiate between fat mass, lean tissue mass and bone mineral mass. The method is non-invasive and relatively easy to perform and the radiation dose is small in comparison to computed tomography-scans [190].

Dual energy x-ray absorptiometry was performed in paper I, II and III to assess body composition in the SOS Sib Pair study participants and in hSAA1 mice and wild type controls.

2.2.7 Measurements of insulin sensitivity

Frequent-sampling intravenous glucose tolerance test and subsequent analysis with Minimal model

The minimal model was developed by Dr. Richard Bergman [191] and uses the mathematical relationship between glucose and insulin concentrations during an intravenous glucose tolerance test to assess insulin sensitivity. The parameters estimated in the minimal model have been shown to be highly concordant with the glycemic clamp that is considered to be the golden standard for insulin sensitivity estimates [192,193].

In the SOS Sib Pair study, an insulin modified frequent sampling intravenous glucose tolerance test was performed [194]. The study participants had to fast over night before the test. A bolous dose of glucose (300 mg/kg) was given intravenously over 2 minutes. An intravenous dose of insulin was administrated 20 minutes after the glucose administration (0.03U/kg for subjects with BMI below 35 kg and 0.05 U/kg for subjects with BMI over 35). Blood samples were frequently collected for measurements of plasma glucose and serum insulin determinations at scheduled time points. Data were then analyzed using the MINMOD computer program (Millennium 6.02 version, Los Angeles, CA) to assess estimates of insulin sensitivity, Si [195].

Oral glucose tolerance tests

An oral glucose tolerance test is commonly used in the clinic to measure glucose tolerance and to estimate insulin resistance. A single oral dose of glucose is administrated and blood glucose and blood insulin are measured at

pre-determined time points during two hours to see how the body responds to glucose and how quickly glucose is cleared from the circulation.

Oral glucose tolerance tests were performed in hSAA1 mice and wild type controls at 21 weeks of age in paper III. In our mice, a single dose of glucose (400 mg/ml, 2 g/kg) was administrated by oral gavage after a 4 h fast. Circulating levels of insulin and glucose was measured after 0, 5, 10, 15, 30, 60, 120 minutes.

2.2.8 Quantification of atherosclerotic lesions

Quantification of atherosclerotic lesions can be performed using lipid staining of the luminal surface of aortas prepared *en face* or with histological analysis of cross sectional parts of the aortic root. Different parts of the aortas display different susceptibility to develop atherosclerotic lesions. For quantification analysis the results are often reported separately for the different parts.

Lipid staining with Sudan IV has been performed in *en face* prepared aortas from hSAA1$^{+/-}$/ApoE$^{-/-}$ mice in paper IV to quantify extent of aortic lesions. Computer-assisted image analysis for quantification of atherosclerotic lesion area was performed with BioPix IQ 2.2.1 (Gothenburg, Sweden). The atherosclerotic lesion areas (expressed as the percentage of total area) were analyzed in total aorta, the aortic arch, the thoracic aorta and the abdominal aorta.

3 RESULTS

3.1 Paper I

Macrophage gene expression in adipose tissue is associated with insulin sensitivity and serum lipid levels independent of obesity.

Obesity leads to an increased macrophage infiltration in the adipose tissue in both mice and humans. In paper I, we have investigated whether markers for macrophages in the adipose tissue are associated with metabolic disturbances independent of obesity in humans. In addition, we have evaluated different macrophage markers that can be used for future studies of macrophages in adipose tissue.

Putative macrophage markers were selected from the literature. The evaluation of thirty-one putative macrophage markers was performed in two steps. First, putative macrophage markers were examined in a human immune cell transcriptome data set to ensure that no other immune cell displayed high expression of these genes. Nineteen genes displayed at least a two-fold higher expression in macrophage-like cell types compared to other immune cells and were selected for further evaluation. In the next step, the adipose tissue gene expression of putative macrophage markers was evaluated by pair-wise correlation analysis in the offspring group of the SOS Sib Pair study. In this step, one marker was removed due to low level of association with the other macrophage markers.

The adipose tissue gene expression of macrophage markers was analyzed in relation to obesity by comparing the adipose tissue expression in lean versus obese siblings in the SOS Sib Pair study. All of the macrophage markers displayed an increased expression in the adipose tissue of obese siblings compared to their lean counterparts. On average the expression of macrophage markers was increased two-fold in obese siblings.

The gene expression levels of the selected macrophage markers were then analyzed in relation to anthropometric and metabolic measurements in the offspring group of the SOS Sib Pair study. All anthropometric and metabolic measurements were significantly associated with the gene expression of macrophage markers. The strongest associations were found to measures of adiposity (BMI, fat mass and percentage body fat assessed by DEXA) and to serum levels of insulin and C-peptide. Furthermore there was a strong negative association between the expression of macrophage markers and
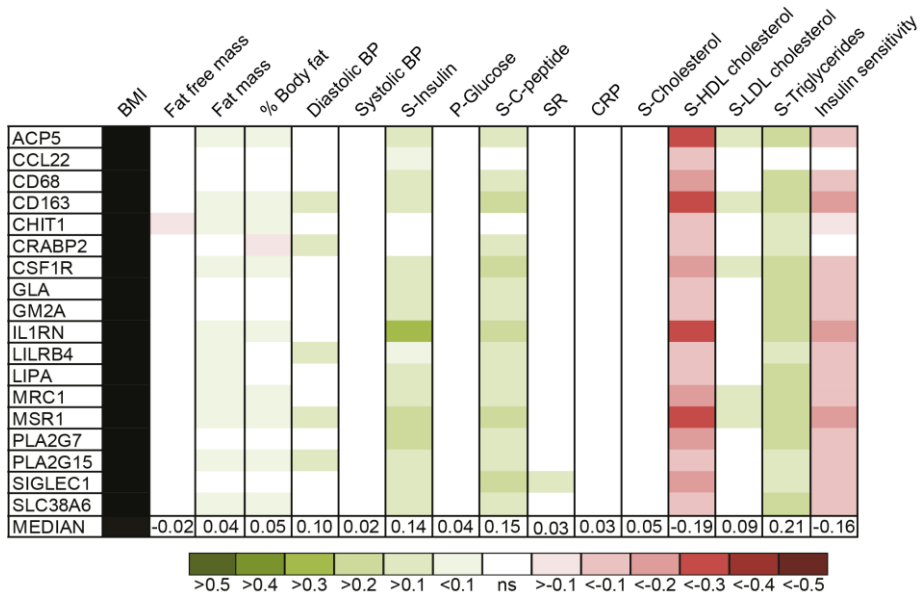
Figure 3. Associations between adipose tissue expression of macrophage markers and metabolic and anthropometric measurements in the offspring group of the SOS Sib Pair study. The analysis was adjusted for BMI, age, sex and non-independence among related individuals. Macrophage markers are indicated by their gene symbol. The colors represent the level of association (parameter estimate) of the various measurements. Green color represents a significant positive association and red color represents a significant negative association. White color represents a non-significant association. The median parameter estimate is shown in the bottom row. BP = blood pressure, SR = sedimentation rate, CRP = C-reactive protein

HDL-cholesterol and insulin sensitivity. The same analysis was repeated with a BMI-adjustment to investigate the influence of obesity (Figure 3). The associations were weakened but the expression of macrophage markers was still associated to insulin sensitivity, serum levels of insulin, C-peptide, HDL-cholesterol and triglycerides.

To further confirm the results, the analysis was repeated in the parent group of the SOS Sib Pair study. In line with the results from the offspring group, the gene expression of several of the macrophage markers was still significantly associated with insulin sensitivity, serum levels of insulin, HDL-cholesterol and triglycerides after BMI-adjustment.

The link between gene expression of macrophage markers and insulin sensitivity was further investigated by comparing the expression of the 18 macrophage markers in patients with type 2 diabetes mellitus and BMI-matched healthy controls. Fifteen of the eighteen macrophage markers displayed significantly higher expression in the patients with type 2 diabetes mellitus.

3.2 Paper II

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

Serum levels of SAA are linked to insulin resistance and atherosclerosis. Results from *in vitro* studies have suggested a role for SAA in these processes. In obesity, the majority of SAA is derived from adipose tissue during the non-acute phase. We therefore established a hSAA1 transgenic mouse model to investigate the role of adipose tissue-derived SAA in metabolic disease *in vivo*.

Using pronuclei injection, three hSAA1$^{+/-}$ transgenic founder mice on a C57BL/6 background were generated and one strain was later established. In this paper, only male mice of F2 generation or later were used. The mice were fed normal chow until 12 weeks of age when they were divided into four groups where two groups (hSAA1 mice, n = 7 and wild type mice, n = 8) were fed a high fat diet for 18 weeks and two groups (hSAA1 mice, n = 10 and wild type mice, n = 10) continued to be fed normal chow. The study was ended when the animals were 30 weeks of age.

Analysis of human SAA1 mRNA levels in hSAA1 mice revealed a specific expression of hSAA1 in the different adipose tissue depots but barely detectable mRNA levels in the other tissues investigated (muscle, kidney, heart and liver) (Figure 4). The mRNA levels of hSAA1 in the adipose tissue depots were similar in hSAA1 mice fed normal chow and mice fed a high fat diet. As expected, wild type littermates did not display any detectable hSAA1 mRNA levels in any of the investigated tissues.

The hSAA1 mice fed a high fat diet displayed increased levels of human SAA compared to the hSAA1 mice fed with normal chow (Table 1, p < 0.001). As expected, the human SAA protein was undetectable in the circulation of wild type animals. The hSAA1 mice fed with a high fat diet displayed a significant reduction in mRNA levels of the endogenous mouse Saa3 in the epididymal adipose tissue compared to their wild type controls on
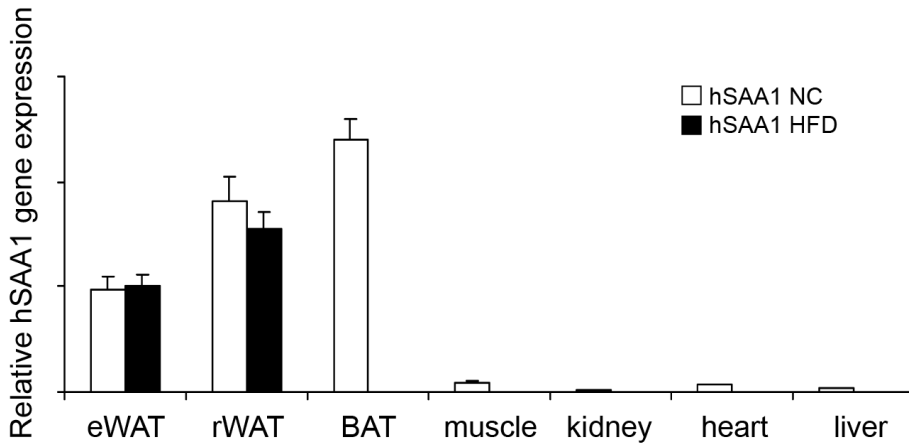
Figure 4. hSAA1 gene expression in epididymal white adipose tissue (eWAT), retroperitoneal white adipose tissue (rWAT), brown adipose tissue (BAT), muscle, kidney, heart and liver from hSAA1 mice fed normal chow (n = 7). hSAA1 gene expression in eWAT and rWAT from hSAA1 mice fed a high fat diet (n = 10).

the same diet (p < 0.05). However, a similar mRNA level of Saa3 was seen in hSAA1 and wt mice fed with normal chow. The circulating levels of mouse SAA were increased in the groups fed with a high fat diet (p < 0.001). However, no differences between hSAA1 and wild type mice in the same diet groups were found.

The hSAA1 mice displayed no visible phenotype but had a slightly lower body weight at 11 weeks of age (25.0 ± 2.1 g, n = 18) compared to their wild type littermates (26.3 ± 2.0 g, n= 19, p =0.046). Despite an initial weight difference, similar growth was observed in hSAA1 mice and wild type mice fed the same diet. In line with these results, similar amount of total body fat was seen in hSAA1 mice and wild type mice fed the same diet at 29 weeks of age.

At 28 weeks of age, the groups of animals fed a high fat diet were fasted for 4 hours and blood glucose and blood insulin were measured. Total cholesterol and triglycerides were measured in plasma samples at the end of the experiment. Similar levels of glucose, insulin, total cholesterol and triglycerides were seen in hSAA1 and wild type mice in the same diet groups (Table 1).

	wt NC	hSAA1 NC	wt HFD	hSAA1 HFD
hSAA, ug/mL	-	4.8 ± 0.5***	-	37.7 ± 4.0***
mSAA, ug/mL	5.5 ± 1.7***	3.7 ± 0.8***	25.8 ± 2.9***	25.6 ± 3.8***
Cholesterol, mmol/L	3.0 ± 0.2	3.0 ± 0.4	5.5 ± 0.9	5.3 ± 1.0
Triglycerides, mmol/L	0.7 ± 0.08	0.7 ± 0.09	0.52 ± 0.13	0.55 ± 0.09

Table 1. Plasma levels of human SAA, mouse SAA, total cholesterol and triglycerides in hSAA1 mice and wild type mice (wt) fed with normal chow (NC) or high fat diet (HFD). *** $p \geq 0.001$.

Plasma levels of human SAA were analyzed in relation to body fat assessed by DEXA, body weight, plasma levels of cholesterol and triglycerides, blood glucose and blood insulin. Plasma levels of human SAA were significantly associated with amount of body fat, body weight and levels of total cholesterol. Body weight and amount of body fat were also significantly associated with plasma levels of mouse SAA.

Lipoprotein cholesterol and triglyceride content were similar in hSAA1 mice and wild type controls when analyzed by FPLC gel filtration (Figure 5). The hSAA levels peaked in the HDL containing fractions from hSAA1 mice. There was a significant increase of hSAA levels in the HDL-containing fractions from hSAA1 mice fed high fat diet compared to those fed with normal chow. Furthermore, hSAA was also present in HDL fractions isolated by ultracentrifugation from hSAA1 mice fed with normal chow.
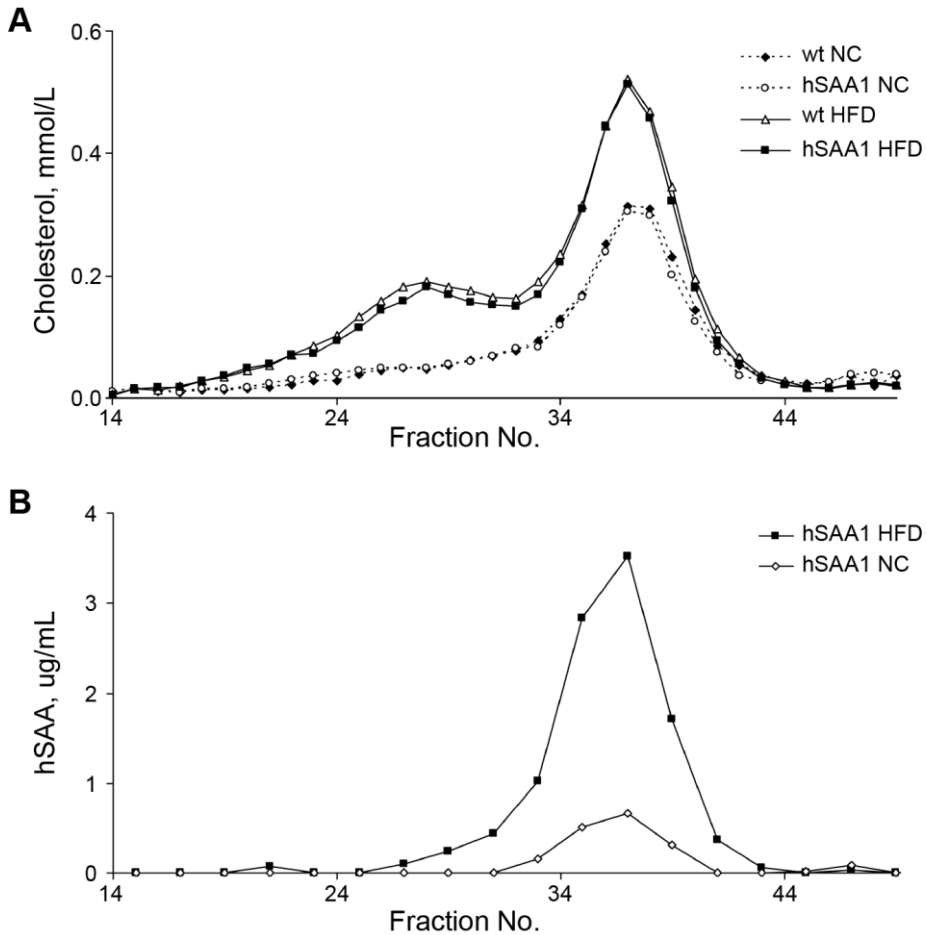
Figure 5. Distribution of cholesterol and human SAA in FPLC fractions. A. Cholesterol levels in plasma FPLC fractions from wild type mice (wt) and hSAA1 mice fed normal chow (NC) or a high fat diet (HFD). B. Levels of human SAA in plasma FPLC fractions from hSAA1 mice fed with normal chow (NC) or a high fat diet (HFD)

3.3 Paper III

No evidence for a role of adipose tissue-derived serum amyloid A in the development of insulin resistance and obesity-related inflammation in hSAA1$^{+/-}$ transgenic mice.

Recombinant human SAA1/2 is a chemoattractant for monocytes and neutrophils and serum levels of SAA are linked to insulin resistance. However, whether adipose tissue-derived SAA is a chemoattractant in adipose tissue, and/or a regulator of insulin resistance is not known.

In this paper, both female and male hSAA1 mice (females, n = 20, males, n = 17) and their wild type littermates (females, n = 20, males = 20) were used. The mice were fed a normal chow until 10 weeks of age and were then divided into groups fed normal chow or a high fat diet for another 12 weeks.

In line with our previous studies, adipose tissue mRNA levels of hSAA1 were present in both female and male hSAA1mice and the circulating levels of hSAA were increased in animals fed a high fat diet. Mouse Saa3 mRNA levels in adipose tissue and plasma levels of mSAA were higher in animals fed a high fat diet. There was a significant decrease in gonadal adipose tissue mRNA levels of mSaa3 in female hSAA1 mice fed a high fat diet compared to their wild type littermates. The same trend was seen in male hSAA1 mice fed a high fat diet.
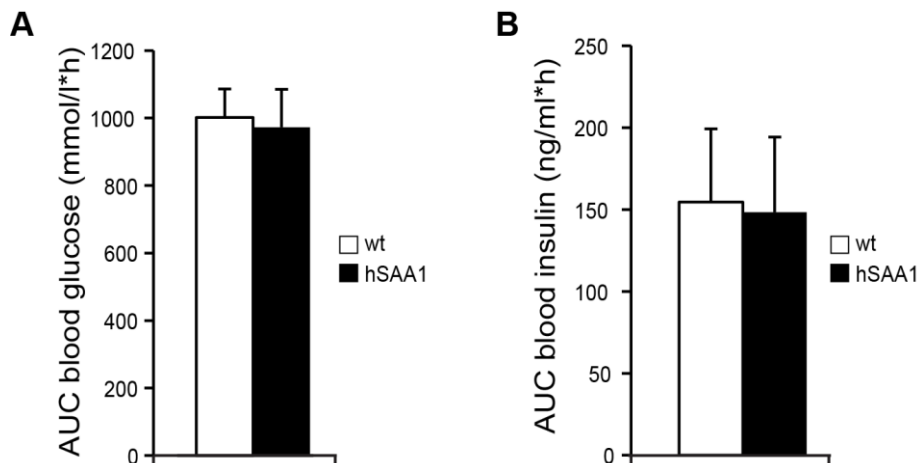

Figure 6. A. Blood glucose area under the curve (AUC) in male wild type mice (white bar) and hSAA1 mice (black bar) fed a high fat diet. B. Blood insulin area under curve (AUC) in wild type mice (white bar) and hSAA1 mice (black bar) fed with high fat diet.

Throughout the experiment, similar growth patterns were observed for both female and male hSAA1 and wild type mice fed the same diet. In line with these results, DEXA-analysis revealed a similar body composition at 18 weeks of age for hSAA1 and wild type mice in the same diet groups.

To estimate systemic insulin resistance, oral glucose tolerance tests were performed at 21 weeks of age in mice fed a high fat diet. Blood glucose and blood insulin area under the curve were similar in hSAA1 and wild type mice in both sexes (Figure 6). With the exception of significantly lower blood insulin levels 15 minutes after glucose administration in female hSAA1 mice, no difference was seen for blood glucose or blood insulin levels at 0, 15, 30, 60 and 120 minutes after glucose administration.

To study possible local effects of hSAA on insulin resistance in adipose tissue, mRNA levels of genes related to insulin sensitivity were analyzed (Figure 7). In male mice, adipose tissue mRNA levels of Irs1, Irs2, Glut4 and adiponectin were similar in hSAA1 and wild type controls in the same diet groups. This was true for both adipose tissue depots investigated. Male mice fed a high fat diet displayed a significant down regulation in the expression of all genes related to insulin sensitivity compared to mice fed normal chow. In female mice, gonadal and retroperitoneal mRNA levels of genes related to insulin sensitivity were similar regardless of genotype except for Glut4 in the gonadal depot which was decreased in wild type mice fed a high fat diet.

mRNA levels of the macrophage markers Cd68 and Emr1 were analyzed in retroperitoneal and gonadal adipose tissue depots (Figure 7). Both female and male mice displayed increased mRNA levels of macrophage markers when fed with high fat diet. However, no differences between hSAA1 and their wild type littermates fed with the same diet were found.

Levels of proinflammatory markers (CXCL1, IFN-γ, IL-10, IL12p70, IL-1β and TNF-α) were analyzed in male mice fed a high fat diet. With the exception of lower levels of CXCL1 in hSAA1 mice, similar levels of proinflammatory markers were seen in hSAA1 mice and wild type mice.
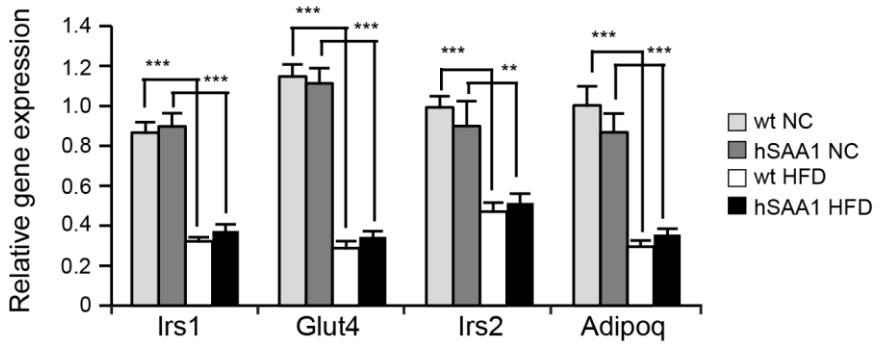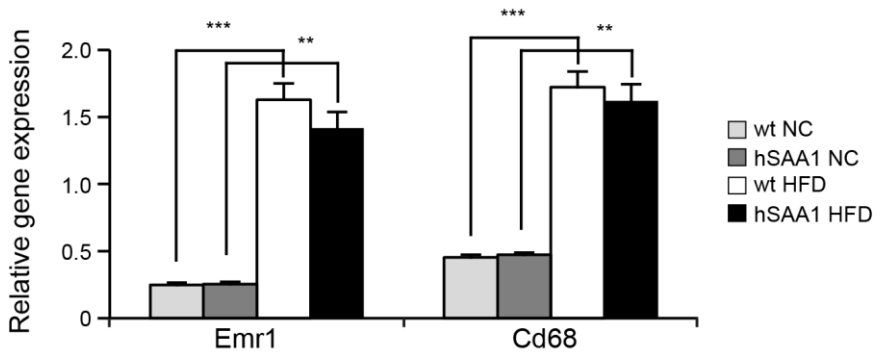
Figure 7. Gonadal adipose tissue mRNA level of A. insulin sensitivity-related genes and B. macrophage markers in hSAA1 mice and wild type mice (wt) fed normal chow (NC) or a high fat diet (HFD). The genes are represented by their gene symbol.

3.4 Paper IV

Adipose tissue-derived human serum amyloid A does not affect atherosclerotic lesion area in hSAA$^{+/-}$/ApoE$^{-/-}$ mice.

Both pro- and anti-atherogenic functions of SAA has been suggested and results from *in vivo* studies display divergent results regarding the role for SAA in atherogenesis. Hence, the role of SAA in atherogenesis is still not clear.

Male hSAA1$^{+/-}$ mice (n = 33) and hSAA$^{-/-}$ mice (n = 23) on an ApoE-deficient background were used in paper IV. The mice were fed normal chow for 35 weeks until the end of the experiment.

Human SAA1 was expressed in gonadal adipose tissue in hSAA1 mice but not detectable in wild type mice. Plasma levels of human SAA were in the same range as previously reported for hSAA1 mice fed with normal chow. mRNA levels of mouse Saa3 in gonadal adipose tissue displayed a trend towards down regulation in hSAA1 mice compared to wild type mice. The same trend was also seen for plasma levels of mouse SAA.

The hSAA1 mice and their wild type controls displayed similar plasma levels of cholesterol (13.2 ± 0.6 mmol/l and 14.0 ± 0.7 mmol/l, respectively) and triglycerides (1.5 ± 0.1 mmol/l and 1.6 ± 0.1 mmol/l, respectively).

Quantification of atherosclerotic lesion area was performed in *en face* prepared aortas where the atherosclerotic lesions were stained with Sudan IV. Computer-assisted image analyses revealed almost identical atherosclerotic lesion area in the total aorta in hSAA1 mice and wild type mice (3.09 ± 0.39 % and 3.08 ± 0.60 %, respectively, p = 0.306). Different trends in atherosclerotic lesion area were observed in the sectional analysis of the aorta but these were not statistically significant. The hSAA1 mice displayed a trend towards increased atherosclerotic lesion area in the aortic arch compared to wild type mice (10.62 ± 1.31 % and 8.11 ± 1.22 %, respectively, p = 0.254). The opposite trend was observed in the abdominal aorta where the hSAA1 mice displayed a decrease in atherosclerotic lesion area compared to wild type mice (1.43 ± 0.34 % and 3.52 ± 1.21 % respectively, p = 0.720). Similar atherosclerotic lesion area was observed in the thoracic aorta for hSAA1 mice and wild type mice (0.83 ± 0.15 % and 0.79 ± 0.18, respectively, p = 0.835).
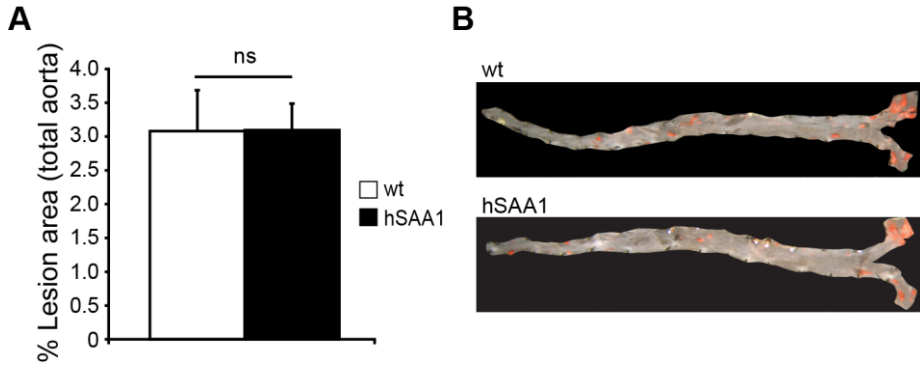
Figure 8. Quantification of atherosclerotic lesion area in en face prepared aortas from male hSAA1 mice (n = 33) and wild type mice (n = 23) on ApoE-deficient background. A. Lesion area with positive Sudan IV staining expressed as the percentage of total area of the whole aorta. Data are presented as mean ± SEM. ns = non significant with Mann-Whitney U-test. B. Photographs illustrating atherosclerotic lesions in wild type and hSAA1 mice.

4 DISCUSSION

4.1 Adipose tissue macrophages in metabolic disease

Previous studies have demonstrated an increased infiltration of macrophages in obese adipose tissue in both mouse and human [90,91,93,94]. The link between obesity and adipose tissue macrophages has been further supported as the number of macrophages in adipose tissue decrease with surgery-induced weight loss [93]. Both weight loss and treatment with thiazolidinediones reduce insulin resistance and macrophage infiltration in adipose tissue [93,196] which indicates that adipose tissue macrophages are involved in the development of insulin resistance.

We here report associations between expression of macrophage markers in adipose tissue and insulin sensitivity and serum lipid levels in a well characterized, large study population. Most importantly, this association remained, although weaker, when the analysis was adjusted for BMI. Furthermore, our data revealed increased gene expression of macrophage markers in adipose tissue from patients with type 2 diabetes mellitus compared to their BMI-matched non-diabetic controls. This indicates that adipose tissue macrophages could be actively involved in the development of metabolic disease, such as insulin sensitivity and serum levels of HDL-cholesterol and triglycerides.

Macrophages are present in many different tissues in the body and are a heterogeneous cell type. The macrophages have important functions in the innate immune system and can produce pro-inflammatory cytokines, mediate phagocytosis of dead or infected cells and are active in tissue remodeling. In 2003, two reports established that adipose tissue macrophages are important producers of proinflammatory cytokines [90,91]. *In vitro* studies of macrophage function have demonstrated that co-culture of murine macrophages and adipocytes blocks the action of insulin in adipocytes [197] and that TNF-alpha treatment reduces the expression of insulin sensitivity genes in adipocytes [198,199]. In addition, proinflammatory cytokines down regulate the expression of ABCA1 and ABCG1 that are active in the cholesterol transport from the cells to HDL [200]. These mechanisms could be part of the explanation why increased macrophage infiltration in adipose tissue leads to insulin resistance and dyslipidemia.

The exact role for macrophages in adipose tissue is hard to study *in vivo*. Due to technical limitations, it is not possible to knockout one cell type in a specific tissue. Some conclusions may be drawn from whole-body knockout models where genes in inflammatory cascades are deleted. Myeloid specific IKK-β knockout mice, where the myeloid cells (e. g. macrophages, dendritic cells) do not have the ability to respond to inflammatory stimuli, are more insulin sensitive [201,202]. However, it is possible that other myeloid immune cells in other tissues contribute to the improved insulin sensitivity. Consequently, indirect studies of macrophage infiltration and subsequent alterations in metabolic dysfunctions after different interventions such as knockout mouse models or transgenic mouse models may be as far as we can go to investigate the function of adipose tissue macrophages *in vivo*. Decreased infiltration of macrophages into adipose tissue and, subsequently, improved insulin sensitivity was demonstrated in a knockout-model for the macrophage chemoattractant MCP-1 receptor, Ccr2 [203]. Conversely, over-expression of MCP-1 in the adipose tissue of mice increased macrophage infiltration into adipose tissue and rendered mice more insulin resistant [101,102].

Different subgroups of macrophages have been identified in the adipose tissue. In the adipose tissue of lean individuals, the macrophages are found interspersed in the adipose tissue while the macrophages in obese individuals form crown-like structures around adipocytes [93,95]. In contrast to interspersed macrophages, macrophages in crown-like structures contain lipid droplets and resemble the foam cells that are present in atherosclerotic lesions [204]. Thus, immunohistological examinations suggest different functions of macrophages in lean and obese individuals.

Macrophages are often classified as proinflammatory macrophages "M1" or anti-inflammatory macrophages "M2" depending on which surface markers they express *in vitro*. Data from mice suggest that obesity induces a phenotypic switch from anti-inflammatory M2 to pro-inflammatory M1 macrophages [204]. *In vivo* polarization of adipose tissue macrophages towards a M1 phenotype has been achieved by macrophage-specific deletion of the PPAR-γ gene in mice. The M1 polarization made mice more insulin resistant, suggesting that M1 macrophages are involved in obesity-related insulin resistance [205]. However, other studies have shown that the expression of M2 markers that correlates with BMI, indicating that it is actually the M2 macrophages that are increased in obesity [206]. Furthermore, one study has shown that macrophages are able to express both M1 and M2 markers [207]. These data indicate that adipose tissue macrophages are not homogenous in phenotype and may exhibit both

proinflammatory and anti-inflammatory functions. Hence, using the M1/M2 classification may be an oversimplification that is more relevant *in vitro* than *in vivo*. For this reason, we have used many different macrophages markers when investigating the expression in adipose tissue and we have decided not to classify the markers into M1 and M2 categories.

Our study is a correlative study of adipose tissue macrophage infiltration, estimated by the expression of macrophage markers, in relation to anthropometric and metabolic parameters. This means that no causality can be determined which is a limitation in our study. Another limitation is that the expression of macrophage markers was only studied in the adipose tissue and may therefore not investigate the whole complexity of obesity-related inflammation. Inflammation is an interplay between different cells and cytokines. Recent studies have shown that other inflammatory cells are present in the adipose tissue and these may have important roles in metabolic disease [100,208-213].

In conclusion, our study represents a systematic examination of the association between gene expression of macrophage markers in adipose tissue and metabolic dysfunction. The obesity-independent links between macrophages in adipose tissue and insulin sensitivity and serum levels of lipids suggests that the increase of macrophages in adipose tissue of obese individuals is actively linked to, rather than only a marker of, obesity. Our data, together with other recent studies, suggest that adipose tissue macrophages play a role in the development of metabolic disease in humans.

4.2 Establishment of a transgenic mouse model with adipose tissue-specific expression of hSAA1

In paper II, the establishment and initial characterization of a transgenic mouse model with expression of human SAA1 in the adipose tissue were reported.

The mouse is one of the most common experimental animal models used in medical research today. There are many different mouse strains for medical research of which some develop complex diseases such as obesity, type 2 diabetes mellitus and cancer [214]. Many strains are a result of constant inbreeding so that the individuals in one strain have as identical genetic background as possible. The even genetic background makes the inbred mouse strains suitable for genetic modifications to study the function of a

specific gene [215-218]. We established our hSAA1^{+/-} transgenic mouse model on a C57Bl/6 background. The whole genome of the C57Bl/6 strain has been surveyed which makes it suitable for genetic modifications. The strain is the most commonly used mouse strain for studies on obesity, cardiovascular disease and transgenic research.

Our hSAA1^{+/-} transgenic mice display specific expression of human SAA1 in the adipose tissue under the aP2 promoter. mRNA levels of the human SAA1 is detected in the different adipose tissue depots and only display low levels of hSAA1 expression in other tissues investigated. This is of importance since much data indicate that the adipose tissue is a main producer of SAA in obese humans during non-acute phase [128-131].

In the acute-phase, the levels of SAA can rise 1000-fold and then decrease to normal levels a few days after the inflammatory stimuli have been removed [119]. However, in obesity the increase of SAA is moderate and chronic and the moderately elevated levels of SAA in obesity are linked to both insulin resistance and cardiovascular disease [84,86,89,132,133]. The hSAA1 mice fed with normal chow or high fat diet display levels of human SAA comparable to lean and obese humans. Hence, the hSAA1 mouse is a new model suited to investigate the effects of the chronically elevated levels of hSAA derived from adipose tissue.

Mice also display elevated levels of SAA in the circulation in the obese state [148,163]. However, the origin of SAA is different. The homologues of SAA1/SAA2 (Saa1/Saa2) in mice only display a limited gene expression in adipose tissue and the expression is not affected by diet-induced obesity [163]. Instead, a third acute-phase form, Saa3, is expressed in the adipose tissue and the expression is increased by high fat diet [163,219]. However, Saa3 gene expression in adipose tissue does not contribute to the circulating levels of SAA [219] indicating that the moderately elevated levels of SAA found in obese mice have a hepatic origin. Thus, adipose tissue-specific expression of hSAA1 is important when further investigating local effects of SAA in adipose tissue.

The hSAA was found in the HDL-containing FPLC-fraction in hSAA1 mice. This indicates that the human SAA has a functional binding site for HDL and that it associates with HDL in the circulation. The binding of SAA to HDL may also be of importance for SAA function. Many *in vitro* studies have used a lipid-free form of SAA. The use of lipid-free SAA in experimental set-up has been questioned [220] since it does not exhibit the same functions as SAA associated to HDL [159,166,221,222]. Hence, results from previous

studies using lipid-free SAA can lead to misinterpretation of SAA function in the circulation where the majority of SAA is associated with HDL. However, it is possible that HDL-free SAA exhibits functions in its local production site before it reaches the circulation and associates with HDL. This further highlights the importance of a tissue specific production of SAA.

In our hSAA1 transgenic mouse model we have used transgenic expression of the human SAA1 gene in adipose tissue. Many *in vitro* studies of SAA function have used a recombinant form of human SAA, which is a combination of the SAA1 and SAA2 isoforms. We and others have shown that the recombinant protein does not share the same function as the endogenous SAA [174,175,223]. The recombinant protein exhibit proinflammatory functions as it activates neutrophils *in vitro*. In contrast, purified endogenous SAA did not have this effect [174,175]. Hence, the proinflammatory function that has been ascribed SAA may not be physiologically relevant, highlighting the need for *in vivo* studies to clarify the physical roles of SAA.

In our transgenic mouse model, a human gene is inserted in the mouse genome. This does not automatically result in a functional protein or that the human protein binds to mouse receptors and exhibit a function. In our hSAA1 mice the human SAA protein was detectable by an ELISA indicating correct protein folding of the human protein. Furthermore, the endogenous Saa3 gene expression in adipose tissue was reduced in hSAA1 mice compared to their wild type littermates. This indicates that the human protein may exhibit a negative feedback on Saa3 expression in mouse adipose tissue. Other studies have shown that adenoviral overexpression of human SAA1 in mice results in an increased vascular proteoglycan synthesis [152]. In addition, domains of the human SAA protein are functional in mouse J774 cells (monocyte cell line) by increasing acyl CoA cholesterol acyltransferase activity increase in the same range as the mouse SAA [224]. These findings further support that human SAA is functional in mice.

Taken together, the links between circulating levels of SAA and cardiovascular disease [84,86,89,132,133,225] and the many *in vitro* studies suggesting a mechanism for SAA in metabolic disease [145,154,162-164,226] place adipose tissue-derived SAA in focus as a possible player in the development of atherosclerosis and insulin resistance. With the establishment of our transgenic hSAA1^{+/-} mouse model with specific expression of hSAA1 in adipose tissue, we have created an *in vivo* model suited to study the moderately chronic increase of SAA that is associated with metabolic disease.

4.3 Adipose tissue-derived human SAA1 in relation to insulin resistance and obesity-related inflammation

In paper III, we have investigated whether adipose tissue-derived hSAA1 could be linked to obesity, increased macrophage infiltration in adipose tissue or insulin resistance. An increase in macrophage infiltration in adipose tissue with subsequent decrease in local and systemic insulin sensitivity has previously been shown in a mouse model with adipose tissue-specific expression of MCP-1 [101,102]. The chemoattractant function of recombinant hSAA1/2 and the association between serum levels of SAA and insulin resistance [84,89,133,159,160,163,164] indicated that adipose tissue-derived SAA could have similar effects.

The hSAA1 mice fed with high fat diet display no increase in blood glucose or blood insulin levels during an oral glucose tolerance test. This was somewhat unexpected since previous studies have shown an association between circulating levels of SAA and insulin resistance in both human and mouse studies [84,89,133]. In addition, circulating levels of SAA decrease in parallel with an improvement in glycemic status when patients are treated with PPAR-γ agonists [133]. However, our results indicate that the moderately elevated serum levels of hSAA1 do not affect systemic insulin sensitivity in mice.

Even though the adipose tissue can affect systemic insulin resistance, our results from the oral glucose tolerance test do still not rule out the possibility that adipose tissue production of human SAA1 may affect local insulin sensitivity. *In vitro* studies of SAA function have shown that recombinant human SAA1/2 down regulates genes involved in insulin sensitivity [162-164]. As expected, genes related to insulin signaling and glucose homeostasis (Glut4, Irs1, Irs2, and Adipoq) displayed a significant reduction in both gonadal and retroperitoneal adipose tissue depots in male mice fed a high fat diet compared with those fed normal chow. The same trend was seen in the retroperitoneal depot in female mice. However, no decrease in the expression of insulin sensitivity genes was seen in hSAA1 mice compared to wild type mice in the same diet groups. Hence, in our mouse model, adipose tissue-derived hSAA1 does not seem to affect local insulin sensitivity in adipose tissue.

The mRNA levels of the macrophage markers Emr1 and Cd68 in adipose tissue was similar in hSAA1 mice and wild type controls. The human

recombinant hSAA1/2 is a chemoattractant for neutrophils and monocytes [159,160] but the chemoattractant effect of SAA is inhibited by the association of SAA to HDL [159]. In the circulation of hSAA1 mice, the human SAA is found associated with HDL. Possibly, the human SAA could have chemoattractant effects in the adipose tissue before it associates with HDL. However, our results do not indicate that this is the case. In the adipose tissue, SAA could also possibly interact with macrophage recruitment via up regulation of CCL2, a known macrophage chemoattractant [163,226] or induction of cell adhesion molecules in endothelial cells [157]. However, we found no indication that the adipose tissue-derived hSAA1 increases macrophage infiltration in adipose tissue.

The low-grade inflammation in obesity includes moderately increased levels of proinflammatory markers e. g. IL-6, TNF-α and CRP [84,88,227,228]. Recombinant human SAA1/2 stimulates cytokine and chemokine expression in both macrophage and endothelial cell lines [156,157,169]. It is reasonable to believe that the moderately elevated levels of SAA in our mouse model could stimulate the production of proinflammatory markers. However, male hSAA1 mice fed a high fat diet displayed no increase in the circulating levels of proinflammatory cytokines (IFN-γ, IL10, IL12p70, IL-1β, IL-6, TNF-α, and CXCL1). The recent findings that recombinant hSAA1/2 induce proinflammatory cytokine production *in vitro* should be interpreted carefully, since we previously have shown that the recombinant hSAA1/2 exhibits proinflammatory functions that are not shared by the endogenous protein [174,175]. Results from our hSAA1 mice provide further support that the human SAA1 may not have proinflammatory properties.

In conclusion, we show that adipose tissue-derived hSAA1 does not affect systemic glucose tolerance or local insulin sensitivity; nor does it increase obesity-related inflammation in hSAA1 mice.

4.4 Adipose tissue–derived human SAA1 and the development of atherosclerosis

There are several links between SAA and atherosclerosis. Both mRNA encoding SAA and the SAA protein are present in the human and mouse atherosclerotic plaque [147-149]. The circulating levels of SAA have been suggested to be predictor for cardiovascular disease [86] and the six–month mortality in patients with acute myocardial infarction [225]. The role of SAA in atherogenesis is much disputed and studies have provided evidence for both proatherogenic and anti-atherogenic roles of SAA (See section 1.6.2).

However, there are a limited number of *in vivo* studies investigating the role of SAA in atherosclerosis development.

Mice do not normally develop atherosclerosis and the feeding of cholesterol-rich diets only leads to the early atherosclerotic lesions, "fatty streaks", in C57BL/6 mice [229]. Thus, to study atherosclerotic development special mouse strains that develop atherosclerosis are needed. In paper IV, hSAA1 mice were crossbred with ApoE$^{-/-}$ mice to generate hSAA1$^{+/-}$/ApoE$^{-/-}$ mice and hSAA1$^{-/-}$/ApoE$^{-/-}$ mice. The ApoE$^{-/-}$ mouse model is, together with the Ldlr$^{-/-}$ mouse, the most common mouse model for atherosclerotic research [230-232]. The genetic deficiency of the apolipoprotein E or the LDL-receptor leads to hypercholesterolemia and development of atherosclerotic lesions. The atherosclerotic development can be accelerated in these models by feeding the mice a cholesterol-rich diet. However, the ApoE$^{-/-}$ mice are hypercholesterolemic and develop atherosclerotic lesions even when fed a normal chow diet [229,233]. In paper IV, the mice were fed with normal chow for 35 weeks to minimize the risk that the severe hypercholesterolemia present in ApoE$^{-/-}$ mice fed a high fat diet would mask possible differences in atherosclerotic development.

Our data from the hSAA1 mice reveal no differences in atherosclerotic lesion area in any section of the aorta in hSAA1 mice compared to their wild type littermates. This is in line with a previous study showing that over-expression of human SAA1 using adenovirus in ApoE mice fed normal chow had no effect on atherosclerosis development [152]. Other studies investigating the effect of SAA in atherogenesis display divergent results. Lenti-viral expression of mouse SAA1 in ApoE$^{-/-}$ mice fed normal chow for 14 weeks accelerated the atherosclerotic lesion progress whereas no difference was seen in ApoE$^{-/-}$ mice fed high fat diet [234]. In contrast, liposomal administration of mouse SAA2 peptides reverses and prevents atherosclerotic lesion development in ApoE$^{-/-}$ mice [224].

The divergent results from *in vivo* studies of SAA function in atherogenesis may depend on different isoforms, source and expression of SAA. The use of lentiviral expression of mouse SAA1 resulted in an increase of atherosclerotic lesion area in ApoE $^{-/-}$ mice fed with normal chow [234]. The levels of mouse SAA in these mice were in the same range as the human SAA in our hSAA1 mouse fed a high fat diet. If these levels are comparable is not clear. However, the overexpression site of mSAA1 was not indicated or investigated which makes it impossible to draw any conclusions on the impact of SAA production site of the development of atherosclerosis.

Furthermore, our study does not leave out the opportunity that a possible local production of SAA in the vessel wall affects atherogenesis.

Atherosclerosis is an inflammatory condition with a local inflammation in the vessel wall. It is possible that the moderately increased levels of SAA in the circulation is induced by the local inflammatory process and acts as a marker for atherosclerosis. However, results presented here do not support a causal role for the adipose tissue-derived SAA in atherosclerosis development.

5 CONCLUSIONS

In this thesis, adipose tissue-derived SAA and adipose tissue macrophages have been investigated as possible factors behind obesity-related disorders. The main findings of this thesis are summarized in figure 9.

We have shown that macrophage infiltration in human adipose tissue, estimated from mRNA levels of macrophage markers, is increased in obesity and type 2 diabetes mellitus. The macrophage gene expression in adipose tissue is linked to insulin sensitivity and serum lipid levels and this link remained significant after adjustment for BMI. Hence, our data suggest that adipose tissue macrophages may play a role in the development of insulin resistance and dyslipidemia and that macrophage infiltration should be considered as a marker of metabolic disease.

We have established a new hSAA1$^{+/-}$ transgenic mouse model to investigate the effects of adipose tissue-derived hSAA1 on metabolic disease. Firstly, the hSAA1 mice have a specific expression of human SAA1 in the adipose tissue and the human SAA protein is found in the circulation at levels similar to those found in lean and obese humans. Secondly, the human SAA is found associated with HDL in the circulation which previously has been shown to be important for SAA function. Hence, the hSAA1 mice display important features for investigating the function of adipose tissue-derived human SAA *in vivo*.

During an oral glucose tolerance test, hSAA1 mice displayed similar levels of blood glucose and blood insulin as their wild type controls. In addition, no decrease in mRNA levels of genes related to insulin sensitivity was found in adipose tissue. The mRNA levels of macrophage markers were similar in hSAA1 mice and wild type controls. Circulating levels of proinflammatory markers were not increased in hSAA1 mice. This taken together, we found no evidence that adipose tissue-derived human SAA1 acts as a systemic or local regulator of insulin sensitivity in mice. Our data do not support a role for adipose tissue-derived human SAA1 in macrophage recruitment into adipose tissue or that hSAA could be an important inducer of circulating proinflammatory markers in mice.

Analyses of *en face* prepared aortas displayed similar atherosclerotic lesion areas in hSAA1$^{+/-}$ /ApoE$^{-/-}$ mice and hSAA1$^{-/-}$/ApoE$^{-/-}$ mice. Thus, our data suggests that adipose tissue-derived human SAA1 does not have a major impact on atherogenesis in mice.
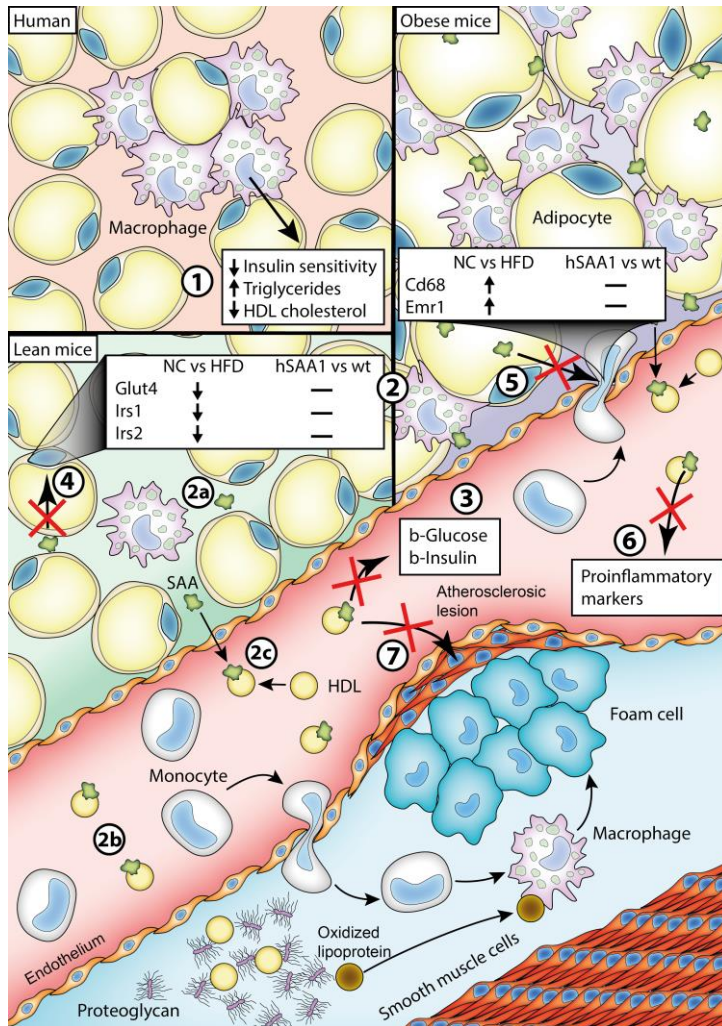
Figure 9. Main findings in this thesis. 1. Adipose tissue gene expression of macrophage markers is associated with insulin sensitivity and serum lipid levels independent of obesity in human. 2. Findings from the initial characterization of our hSAA1 transgenic mouse model specifically expressing human SAA1 in the adipose tissue. The mouse model mimics the human non-acute phase with: a. Specific hSAA1 expression in the adipose tissue. b. Levels of hSAA in the circulation which resembles those seen in lean and obese human. c. The human SAA is found associated to HDL. 3. The hSAA1 mice do not display alterations in systemic glucose tolerance. 4. The adipose tissue-derived hSAA1 does not down regulate insulin sensitivity genes in adipose tissue. 5. The adipose tissue-derived hSAA1 does not affect mRNA levels of macrophage markers in the adipose tissue. 6. Levels of proinflammatory markers are not increased in hSAA1 mice. 7. Adipose tissue-derived hSAA does not affect atherosclerotic lesion area in hSAA1 mice. *Picture by J. Carlsten.*

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