THE ROLE OF SERUM AMYLOID A1 (SAA1) IN CORONARY ARTERY DISEASE

LEOW KOON YEOW
BSc (Hons), NUS

A THESIS SUBMITTED
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PAEDIATRICS

NATIONAL UNIVERSITY OF SINGAPORE

2011

Acknowledgments

I would like to convey my sincere appreciation to my supervisor A/P Heng Chew Kiat for his patient guidance and advices throughout the years. Special thanks also go to my fellow colleagues and ex-colleagues Lee Siang Ling Karen, Lye Hui Jen, Larry Poh, Zhao Yulan, Zhou Shuli, Yang Ennan, Li Hongzhe, Goh June Mui and Tan Si Zhen for their ever enthusiastic help and advices rendered. My deepest gratification is also extended to the staff of the immunology divison of the Department of Paediatrics for their selfless sharing of both technical knowledge and research facilities. Last but not least, I am indebted to my dearest family and friends who have made those difficult moments more bearable. This research was supported by the research grant offered by National Medical Research Council, Singapore (NMRC/1155/2008).

TABLE OF CONTENTS

APPENDICES

SUMMARY

Background:

Atherosclerosis is a gradual narrowing of the lumen of the arteries and chronic inflammation has long being regarded as crucial to the pathogenesis of the disease. Serum amyloid A1 (SAA1) and serum amyloid A2 (SAA2) (A-SAA) are acute-phase proteins (APPs); the concentration of A-SAA can increase by 500-1000 fold during an acute systemic inflammation (Malle et al. 1993). The predominant form of A-SAA in plasma is reported to be SAA1 (Yamada et al. 1999). A-SAA has increasingly been associated with atherosclerosis (Johnson et al. 2004; Ogasawara et al. 2004; Ridker et al. 2000), this stem from its immune regulatory role as well as the inflammatory nature of atherosclerosis. However, its specific role in atherosclerosis, in particular, whether it is atherogenic or atheroprotective remains unknown. In addition, no prior genetic epidemiology study has been conducted on *SAA1*.

Methods and results:

Genetic variant screening was performed using cord blood DNA samples from 96 anonymous, unrelated Singaporean Chinese neonates delivered in the National University Hospital, Singapore. Genetic association study was performed using DNA samples extracted from blood samples belonging to coronary artery disease (CAD) patients. In total, there were 1243 healthy controls and 800 CAD patients. Healthy controls were recruited from subjects attending a routine health screening. Functional characterization of the genetic variant, p.Gly90Asp, was performed *in vitro* using human THP-1 derived macrophages.

In total, 6 genetic variants were identified in the exons and promoter of *SAA1*, of which 2 are novel - c.-913G>A and c.92-5T>G. The non-conservative genetic variant, p.Gly90Asp (c.269G>A), is not associated with CAD, the odds ratio is 1.61 (95% confidence interval

(CI) 0.68-3.80; *P*-value =0.28) after adjustment for age, gender and BMI. In addition, the variant, p.Gly90Asp also induced a significantly lower level of inflammatory cytokines in THP-1 derived macrophages, the decrease in IL-8, MCP-1 and TNF-α secreted were 57%, 50% and 39% respectively. Variant SAA1 also has a lower impact on the genetic expression level of a potentially atheroprotective gene, plasminogen activator inhibitor-2 precurosor (SERPINB2), the expression ratio of wild-type SAA1 to variant SAA1 is 1.8 (95% confidence interval (CI) 1.3-2.4; *P*-value < 0.0001). Microarray study also suggests an atherogenic role of SAA1 with the induction of genes that are involved in inflammation, angiogenesis, phagocytosis and tissue remodeling; these processes are crucial to the development of atherosclerotic lesion.

Conclusions:

The identification of a genetic mutant of SAA1, p.Gly90Asp that is associated with CAD supports the hypothesis that SAA1 has a direct role to play in the pathogenesis of CAD. p.Gly90Asp has altered functional effects and induces a lower extent of cytokine secretion in macrophages and potentially atheroprotective SERPINB2, the latter could account for the increased susceptibility of p.Gly90Asp to CAD. The alter effects of the mutant is probably due to the lower affinity of the genetic variant to cell surface receptors of SAA1 such as TLR2 and CLA-1. Lastly, SAA1 regulates expression of genes with functional roles in key processes of atherosclerosis; it thus plays a direct role in CAD and does not act as a mere marker of chronic inflammatory diseases.

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

5-LO	5-lipoxygenase
APPs	Acute-phase proteins
APR	Acute phase response
A-SAA	Acute-phase SAAs
B2M	Beta-2 microglobulin
CAD	Coronary artery disease
CCL2	Chemokine (C-C motif) ligand 2
CETP	Cholesteryl ester transfer protein
CLA-1	CD36 and LIMPII analogous-1
CMIT	Carotid intima-media thickness
CRP	C-reactive protein
DGGE	Denaturating gradient gel electrophoresis
DHPLC	Denaturing high performance liquid chromatography
eNOS	Endothelial nitric oxide synthase
ELISA	Enzyme-linked immunosorbent assay
ESTs	Expressed sequence tags
FMF	Familial Mediterranean fever
FPRL1	Formyl peptide receptor like 1
FRET	Fluorescence resonance energy transfer
GRE	Glucocorticoid response element
HBEGF	Heparin-binding EGF-like growth factor
HDL	High-density lipoprotein

HRM	High resolution melting
IKK2	I-kappaB kinase beta
LDL	Low-density lipoprotein
LOD	Logarithim of the odds
MMPs	Matrix metalloproteinases
nCEH	Neutral cholesteryl ester hydrolase
NFkappaB	Nuclear factor B
NSTE-ACS	Non-ST-segment elevation acute coronary syndromes
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphism
SAA	Serum amyloid A
SAA1	Serum amyloid A1
SAA2	Serum amyloid A2
SCCP	Single-strand conformation polymorphism
SCID	Severe combined immunodeficiency
SERPINB2	Plasminogen activator inhibitor-2 precurosor
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
sPLA2	Secretory phospholipase A2 inhibitor
SRA	Scavenger receptor A
SR-BI	Scavenger receptor class B type I

TGF-ß	Transforming growth factor beta
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
uPA	Urokinase plasminogen activator
VCAM1	Vascular cell adhesion molecule-1

1 INTRODUCTION

1.1 Brief background

APPs are produced by the liver in time of stress and they function to counteract infection and promote healing of damaged tissues; they are thus essential for the survival of living organisms. A-SAA is a major component of APPs and constitute 2.5% of the hepatic protein produced during an acute phase response (APR) (Shah et al. 2006). The level of A-SAA is upregulated in patients with chronic inflammatory diseases such as coronary artery disease, cancer, rheumatoid arthritis (RA) and metabolic syndrome (Cho et al. 2010; Kotani et al. 2009; Kumon et al. 1997; Kumon et al. 1999; Ramankulov et al. 2008). A number of studies have suggested that A-SAA might play a direct role in atherosclerosis, however, the understanding of such role is complicated by reports documenting both the atherogenic and athero-protective effects of A-SAA (Zimlichman et al. 1990). Furthermore, as most existing studies involve the usage of a recombinant SAA with primary sequence that is a hybrid of both SAA1 and SAA2, it is difficult to ascertain the actual significance of such studies.

1.2 Thesis objectives

The study aims to investigate and clarify the role of SAA1 in CAD. As SAA1 was reported to be the predominant form of SAA in the plasma, the study will focus only on SAA1. Since no prior genetic epidemiological studies had been performed on *SAA1*, one of the main focuses of the studies is to identify and study the association of genetic variants of *SAA1* with CAD. The results of this study will support the hypothesis that SAA1 has a direct role to play in the pathogenesis of CAD. The objectives of the study include:

(1): To screen the promoter and exons of human *SAA1* for novel genetic variants.

(2): To carry out association study of *SAA1* with CAD.

(3): To carry out functional study of a genetic variant of SAA1, p.Gly90Asp that has a significant association with CAD.

(4): To elucidate the surface receptors of SAA1 and the study the genetic expression induce by SAA1 in the macrophages.

1.3 Thesis Organisation

The thesis is organized into 7 other chapters. The thesis begins with a literature review that covers important aspects of the area of study. Materials and methods that were used in the study are documented in Chapter 3. The SNPs survey of *SAA1*, the association and functional study of the genetic variants are covered in Chapter 4, 5 and 6 respectively. In chapter 7, the effects of SAA1 on the global gene expression in THP-1 derived macrophages are reported. Chapter 8 sums up the thesis together with proposal for future works.

Results that comprise part of Chapter 4,5 and 6 have been used for the preparation of manuscript to be submitted to a peer review journal, Atherosclerosis, the title of the manuscript is 'Variant screening of the SAA1 gene and the association and functional study of the p.Gly90Asp mutant'. The results from Chapter 7 are included in the manuscript titled 'Effect of serum amyloid A1 (SAA1) treatment on global gene expression in THP-1-derived macrophages' which will be published in Inflammation Research.

2 LITERATURE REVIEW

2.1 Atherosclerosis and coronary artery disease (CAD)

2.1.1 Atherosclerosis – a chronic inflammatory disease

Atherosclerosis is a chronic inflammatory disease and the principal cause of death in most part of the world (Braunwald 1997; Breslow 1997). Until the 1970s, the excessive levels of lipids in the body and its accumulation in the walls of the artery were believed to be the main cause of atherosclerosis. However, over the past decade, it is widely recognized that the development of an atherosclerotic lesion is driven by a chronic inflammation of the tunica intima. Chronic inflammation is also responsible for the pathogenesis of other chronic diseases such as RA (Harris 1990; Sewell and Trentham 1993), pulmonary fibrosis (Brody et al. 1981; Kuhn et al. 1989; Lukacs and Ward 1996) and chronic pancreatitis (Sarles et al. 1989). This shift in thought has a big impact on the scope of research; more importantly, with a deeper understanding of the processes leading to atherosclerosis, more efficacious drugs can be designed to for the treatment of atherosclerosis.

2.1.2 Pathogenesis of atherosclerosis and acute coronary syndrome

Atherogenesis begins when the endothelium of the artery is damaged by various substances including elevated level of modified low-density lipoprotein (LDL), free radicals caused by cigarette smoking and elevated plasma homocysteine concentration (Ross 1999). The development of an atherosclerotic lesion does not occur spontaneously throughout the length of the artery. The regions of the artery that are exposed to laminar shear stress flow are protected from atherosclerosis due to the upregulation of protective genes such as superoxide dismutase and nitric oxide (De Caterina et al. 1995; Topper and Gimbrone 1999).

Exposure to prolonged shear stress was also reported to suppress the production of adhesion molecules in endothelial cells (Chiu et al. 2004; Sheikh et al. 2003).

Increased expression of cell adhesion molecules facilitates the adhesion of monoctye and its subsequent entry into the tunica intima. In the tunica intima, the monocytes differentiate into macrophages and express scavenger receptors such as scavenger receptor A (SRA) and CD36. Scavenger receptors facilitate the uptake of modified lipoproteins into the macrophages forming foam cells which are omnipresent in the atherosclerotic lesion. The macrophages contribute further to the growth of the lesion by secreting substances such as proinflammatory cytokines, chemokines and matrix metalloproteinases (MMPs).

In addition to mononuclear phagocytes, other immune cells, in particular T-lymphocytes and mast cells also have a role to play in atherogenesis. In the intima, T-lymphocytes crosstalk with macrophages through CD154-CD40 interaction and induce the macrophages to secrete tissue factors, MMPs and pro-inflammatory cytokines. In addition, helper T-cells can polarize into T_H1 cells which secrete pro-inflammatory cytokines. Mast cells undergo degranulation in the intima to produce serine proteinases which facilitate matrix degradation.

In the later stage of the development of the atherosclerotic lesion, microvessel is formed in the atheroma. The formation of new vessels provides a new source of nutrients for the atherosclerotic plaque, facilitating its growth and hence angiogenesis is pro-atherogenic.

The final stage in the development of an atheroma involves the rupturing of a plaque. Plaque rupturing involves the erosion of the endothelial cells and the fracture of the fibrous cap which exposes the blood to the content in the plaque. Rupturing of the plaque occurs as a result of the proteolysis of collagen in the extracellular matrix which forms the main support of the fibrous cap (Lee and Libby 1997).

2.1.3 Risk factors for CAD

Atherosclerosis and CAD are multi-factorial disease and are greatly affected by a combination of both genetic and environment factors. Genetics is a big determinant on the development of CAD and in most studies the heritability of atherosclerosis exceeds 50% (Lusis 2000). In a standardized case-control study of acute myocardial infarction conducted in 52 countries (INTERHEART), nine risks factors were identified to be associated with the disease (Table 2-1) (Yusuf et al. 2004). Both genetic and environmental factors are crucial in the prevention of CAD. Among the 9 risk factors, ApoB:ApoAI ratio, diabetes, hypertension and to a certain extent abdominal obesity are influenced by the genetic makeup of an individual. Smoking, psychosocial stressors, alcohol consumption, regular physical exercise and consumption of vegetables are environmental risk factors.

Table 2-1. Deleterious and protective risk factors for acute myocardial infarction. The data is based on a case-control study (INTERHEART) conducted in 52 countries.

Deleterious/Protective Risk factor	Odds ratio (99% CI)
ApoB:ApoAI ratio (highest vs lowest decile)	4.73 (3.93-5.69)
Smoking	2.87 (2.58-3.19)
Psychosocial stressors	2.67 (2.21-3.22)
Diabetes	2.37 (2.07-2.71)
Hypertension	1.91 (1.74-2.10)
Abdominal obesity (highest vs lowest tertiles)	1.62 (1.45-1.80)
Protective	
Alcohol consumption, $\geq$ 3 times a week	0.91 (0.82-1.02)
Regular physical exercise	0.86 (0.76-0.97)
Daily fruit and vegetable consumption	0.70 (0.62-0.79)

Among the deleterious risk factors, smoking is closely associated with acute coronary syndromes. It has the highest odds ratio among the non-genetic risk factors. Based on various studies, smoking exerts various effects on the vascular system which include (1): inducing oxidative stress in peripheral blood mononuclear cells (PBMCs) (Garbin et al. 2009) (2): elevating the level of thrombopoietin which contributes to enhanced platelet activation (Lupia et al. 2010) and (3): increasing systemic inflammation through an increased level of homocysteine, C-reactive protein (CRP) and fibrinogen (Yanbaeva et al. 2007).

2.1.4 Existing drugs treatment for CAD

Various drugs are currently prescribed to treat CAD and its clinical manifestations, these include calcium channel blockers, angiotension-converting enzyme inhibitors and angiotensin II type 1 receptor blockers to lower blood pressure, anti-platelet agent such as aspirin and clopidogrel to reduce blood clotting and HMG-COA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors (statins) to regulate lipids levels. Among them, statins have a more direct impact on CAD as they regulate LDL level which has a primary role to play in atherosclerosis.

2.1.4.1 Statins

Statins was approved for use in the treatment of hypercholesterolemia in 1987. Statins regulate LDL level through the inhibition of the rate limiting enzyme in cholesterol synthesis, HMG-COA reductase. Ever since its discovery, statins have been used rather routinely to reduce future cardiovascular events in patients; its benefical effect was supported by findings from various clinical trials (LaRosa et al. 2005; Nissen et al. 2004). In a meta-analysis of 14 randomised trials of statins consisting of 90,056 participants, statins were

shown to reduce the 5 year incidence of cardiovascular events by 20% for every mmol/L of LDL cholesterol reduction (Baigent et al. 2005).

Statins have also been used as a primary prevention in patients with clinical parameters that put them at risk of developing CAD. In a prospective, open-blinded end point study, Management of Elevated Cholesterol in the Primary Prevention Groups of Adult Japanese (MEGA), conducted among 5356 female Japanese patients, treatment with pravastatin results in a reduction of 26% to 37% in the occurrence of cardiovascular events. In another study conducted among women of age $\geq$ 60 years, there was a greater reduction in cardiovascular events (45% for CAD, and 50% for CAD plus cerebral infarction) (Mizuno et al. 2008) for those treated with statin. To further validate those studies, another prospective study, Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER), was conducted among 6801 women with age $\geq$ 60 years and 11001 men with age $\geq$ 50 years. The result again support the use of statins as a primary prevention against cardiovascular events in asymptomatic individuals. The absolute cardiovascular disease rate (per 100 person-years) in women is 0.57 for rosuvastatin against 1.04 for placebo. In men, the absolute cardiovascular disease rate is 0.88 for rosuvastatin against 1.54 for placebo. The relative risk reduction is significant in both men (hazard ratio: 0.58; 95% CI, 0.45 – 0.73; P< 0.001) and women (hazard ratio: 0.54; 95% CI, 0.37-0.80; P=0.002) (Mora et al. 2010). Since atherosclerosis is a progressive disease and that individuals can be asymptomatic for years while the artery continues to narrow, it might be a good clinical practice to put those at risk on statin treatment before an acute event occurs.

Although statins have been used successfully in decreasing cardiovascular mortality and morbidity, an increasing number of studies have attributed the efficacy of statins to its pleiotropic effects. Simvastatin was reported to induce the regression of cardiac hypertrophy

in a rabbit model of human hypertrophic cardiomyopathy (Patel et al. 2001). In a case-control study conducted in 2004 (Nishikawa et al. 2004), patients treated with either pravastatin or simvastatin (n =66) for an average of 31 months had a significant decrease in the left ventricular mass index. Hence, the therapeutic effects of statins might be derived from a combination of its influence on LDL level as well as its pleiotropic effects. The various pleiotropic effects observed with statins treatment are summarized in Table 2-2. Pleiotropic effects are known to be associated for a few statins. Some of the positive effects observed are independent of lipid levels as cells were treated for only a coupe of hours.

Table 2-2. Pleiotropic effects observed with statin treatment. The effects observed were based on both *in vitro* and *in vivo* studies.

Drug	Pleiotropic effects
Fluvastatin	Prevent plaque rupturing in apoE knockout mice through decreased MMP-9 expression, gelatinolytic activity and endothelial adhesion molecules expression (Nakamura et al. 2009)
Simvastatin	Reduce cell adhesion molecules in endothelial cells (Eccles et al. 2008) Suppresses endotoxin induced upregulation of toll-like receptors 2 and 4 in human (Niessner et al. 2006) Upregulation of endothelial nitric oxide synthase (eNOS) (Laufs et al. 1998)
Rosuvastatin	Reduce MMP-7 production in human monocyte-derived macrophages (Furman et al. 2004)
Lovastatin	Upregulation of eNOS (Laufs et al. 1998)
Cerivastatin/ Atorvastatin	Induce direct vasodilation of isolated bovine coronary arteries through its effect on endothelial cells (Lorkowska and Chlopicki 2005)

2.1.5 Potential treatment strategies for CAD

Statin has contributed greatly to the reduction of morbidity and mortality associated with CAD. However, existing drugs are still incapable of complete eradication of cardiovascular events from the high risk group. For the past two decades, most CAD drugs have been designed to alter plasma lipids levels. However, with more knowledge on the pathogenesis of atherosclerosis, new drugs such as antioxidant, secretory phospholipase A2 inhibitor (sPLA2), 5-lipoxygenase (5-LO) inhibitor and cholesteryl ester transfer protein (CETP) inhibitor are appearing on the pipeline of major drug companies.

Table 2-3 shows the list of CAD drugs that are undergoing clinical trials. Torcetrapib, is one of the first CETP inhibitor to undergo phase 3 clinical trial. Although, torcetrapib was able to raise HDL-cholesterol level in patients substantially (Barter et al. 2007; McKenney et al. 2006), the drug did not halt the progression of atherosclerosis and adverse effects such as higher systolic blood pressure in patients were reported (Vergeer et al. 2008). Further research on the adverse effects of torcetrapib indicates that the adverse effects are likely to be independent of CETP inhibition (Forrest et al. 2008; Hu et al. 2009) .Two other CETP inhibitors, dalcetrapib and anacetrapib (Cannon et al. 2009), are entering phase 3 clinical trials. Anacetrapib had promising results in earlier trials: HDL-cholesterol level was doubled and LDL-cholesterol level was lowered by 70%. Importantly, anacetrapib does not appear to have adverse effects on blood pressure which doomed the first drug of its class, torcetrapib (Bloomfield et al. 2009). The results of the phase 3 clinical trials will validate whether CETP inhibitors can complement statins as an additional drug to combat atherosclerosis.

Drugs with some anti-inflammatory effects are also being developed. Succinobucol is an anti-oxidant and a novel vascular protectant. Succinobucol has good preclinical results: (1) it inhibits lipopolysaccharide induction of atherogeneic tissue factor in both monocytic and

endothelial cells without altering the nuclear translocation of NF-kappaB (Luyendyk et al. 2007) (2) it reduces aortic atherosclerosis in both $LDLr^{-/-}$ and $ApoE^{-/-}$ mice and (3) it raises HDL-cholesterol levels and lowers LDL-cholesterol level in hypercholesterolemic cynomolgus monkeys (Sundell et al. 2003). However, the beneficial effects of succinobucol are not observed in clinical studies, instead a dose-dependent decrease in HDL-cholesterol was observed upon drug treatment (Tardif et al. 2003).

Two other classes of anti-inflammatory drugs with potent anti-atherosclerotic effects are drugs inhibiting sPLA2 and 5-LO. As with both succinobucol and torcetrapib, these are first-in-class drugs and that their potential clinical benefits remain to be seen. With anti-inflammatory drugs on the pipeline as well as ongoing research on important inflammatory mediators, we will soon able to find out whether anti-inflammatory drug can be a standard medication for atherosclerosis.

Table 2-3. Potential anti-atherosclerosis drugs in various stages of clinical trials.

Drug	Pharmacology	Phase	Findings
Succinobucol	Antioxidant	3	In a randomized, double-blind, placebo-controlled trial, succinobucol had no effect on the secondary prevention of cardiovascular events (Tardif et al. 2008).
A-002	sPLA2 inhibitor	2	Patients treated with A-002 have a reduction in sPLA2 concentration of 86.7% as compared to 4.8% in placebo (P <0.0001). There is also a significant reduction in inflammatory mediators and markers (Meuwese et al. 2009).

Drug	Pharmacology	Phase	Findings
Darapladib	sPLA2 inhibitor	2	In phase 2 clinical trials, daraplaedib treatment retards the growth of the necrotic core (Mohler et al. 2008) accompanied by a decrease in the plasma level of inflammatory mediators (Serruys et al. 2008)
Eprotirome	Thyroid hormone analogue	2	In a 12 week trial, patients on statin treatment and further treated with eprotirome have decreased levels of atherogeneic lipoproteins (Ladenson et al. 2010).
VIA-2291	5-LO inhibitor	2	Patients on the drug for 12 weeks have significant reduction in both leukotrienes levels and noncalcified plaque volume (Tardif et al. 2010)
RVX-208	ApoAI stimulator	2	Patients treated with RVX-208 have increased total HDL, alpha and pre-beta HDL level (McNeill 2010)
Torcetrapib	CETP inhibitor	T	Patients treated with torcetrapib have a significant reduction in LDL cholesterol level and a substantial increase in HDL cholesterol level. However, the drug does not halt the progression of atherosclerosis (Kastelein et al. 2007).

T - Torcetrapib clinical trial was terminated due to its associated side effects in patients.

2.2 Serum Amyloid A (SAA)

2.2.1 *SAA* gene and protein family

SAA gene family is clustered in chromosome 11 in human. There are 4 members of the family, *SAA1*, *SAA2*, *SAA3* and *SAA4*. *SAA1* and *SAA2* are located 18kb apart in chromosome 11 while *SAA4* is positioned 11kb downstream from *SAA2* (Kluve-Beckerman and Song 1995). *SAA3* is a pseudogene as no TATA-box is found in the upstream region of the gene (Malle et al. 1993). In mouse, the *SAA* gene family also consists of 4 members, *SAA1*, *SAA2*, *SAA4* and *SAA5*. *SAA1* and *SAA2* encode for A-SAA while SAA5 encodes for constitutive SAA. *SAA4* is a pseudo-gene. The gene family of *SAA* is thus well-conserved between human and mouse.

SAAs are apolipoproteins of HDL. Both SAA1 and SAA2 consist of 122 amino acids in the fully-translated protein which includes a signal peptide consisting of 18 amino acids. SAA1 and SAA2 differ only at seven positions with sequence identity of 95%.

SAA4 is the constitutive SAA of the SAA protein family. The main organ of production of SAA4 is the liver and its production is not upregulated during APR. During homeostasis, SAA4 constitutes about 90% of the body SAAs (de Beer et al. 1995). Its exact role is, however, not known, a study however showed that SAA4 is associated with only a specific subpopulation of HDL particles which does not play a role in the cholesterol transfer between cells (de Beer et al. 1995).

2.2.2 The acute phase response (APR)

APR is a systemic response to injury and the presence of infectious agents. The APR constitutes part of innate immunity in human and serves to contain and counteracts infection or injury and eventually restores homeostasis in a timely manner.

Essential components of the APR include the macrophages and other immune cells, the liver and the hypothalamus. In the occurrence of an injury or infection, the macrophages which encounter the stimulating agent response by producing chemokines and cytokines. The secretion of cytokines stimulates the migration of monocytes to the inflamed site and facilitates local inflammation. Cytokines such as IL-1 and IL-6 also act on the liver and stimulate the hepatic production of plasma proteins, the APPs. In addition, the cytokines also act on the hypothalamus and induce a fever response.

APPs that are produced by the liver include A-SAA, pentraxins, CRP, fibrinogen, haptoglobin and α_1-acid glycoprotein (Jensen and Whitehead 1998; Malle et al. 1993). The plasma concentration of A-SAA starts to decline after 72 hours and return to baseline after 5-7 days (Gabay and Kushner 1999). The kinetic of A-SAA during an APR appears to reflect on their role as an important modulator of the innate immune system.

2.2.3 Protein structure and functional domains of SAA1

SAA1 transcript encodes for a protein of 122 amino acids. Upon translation, the signal peptide is cleaved to form the mature SAA1 which consists of 104 amino acids. Thus far, there has been no published data on the tertiary structure of SAA1. There have, however, been some predicted models of the secondary structure of SAA1 (Stevens 2004; Turnell et al. 1986).

The important functional domains of SAA1 are elucidated through mutagenesis studies. The N-terminal region was reported to have several functional functions including its amyloidogenic potential and as a binding region for both lipid and prostacyclin. In amyloidosis, insoluble amyloid fibrils are deposited in tissues and organ. These deposits can damage the extracellular matrix of these tissues and impede their functions. The amyloid

fibrils are formed from the proteolytic cleavage of the N-terminal of SAA1 (Nakamura 2008). The lipid binding region is elucidated through various independent studies and resides at the N-terminal region (residue 1-30). The region between amino acid 29 to 42 was reported to be important for the binding of SAA1 to components of the extracellular matrix (Uhlar and Whitehead 1999). The only reported role of the C-terminal domain is its facilitation of the binding of SAA1 to neutrophils; a peptide corresponding to residues 77-104 of SAA1 was found to inhibit the binding of SAA1 to neutrophils (Preciado-Patt et al. 1996b).

Structural analysis of SAA1 by more advanced methods such as nuclear magnetic resonance and protein crystallography has not been possible due to the unstable nature of SAA1. A proposed secondary structure of SAA1 is composed of two α-helix region (residues 11-27 and residues 72-86) and two beta-sheet regions (residues 36-45 and residues 59-68). The presence of alpha-helices in the structure of SAA1 is validated by circular dichorism analysis which indicates an alpha-helix content of between 33%-44% (Meeker and Sack 1998) and 50% (Wang et al. 2002).

2.2.4 Production of A-SAA and the role of perivascular adipocytes in CAD

In human, A-SAA is produced in the liver during the APR. Under homeostatic condition, the adipose tissue is the major source of A-SAA. In a northern blot study, the expression of A-SAA was found to be at least 15 fold more in the adipose tissue than in the liver. In the same study, A-SAA was not expressed in most organs of the human including smooth muscle cells, kidney, liver, lung and brain. The dominant site of A-SAA production is most likely to be species specific; in the mouse, the expression of A-SAA appears to be solely in the liver (Yang et al. 2006). The secretion of A-SAA by the adipocytes is also verified by

various studies. Poitou et al found that A-SAA is expressed in the adipose tissue of obese subjects and the level of SAA protein is dependent on adipocyte size and macrophage infilitration (Poitou et al. 2009). In addition, in a study of two extremely obese subjects, the expression of A-SAA protein was found to increase by 3.5 fold as compared to lean subjects (Poitou et al. 2005). Although, there are currently no *in-vivo* study on the association of the development of the adipose tissue with A-SAA level, the earlier mentioned studies indicate the importance of adipose tissue as a dominant source of A-SAA under non acute-phase conditions and its relevance to the pathogenesis of CAD given that the level of A-SAA secreted by adipose tissue increases with macrophage infiltration.

Since A-SAA is predominantly produced by adipocytes under homeostatic condition, the local production of A-SAA by perivascular adipocytes might play an important role in CAD. Perivascular adipose tissue is found in the vicinity of the aorta and it is not separated from the blood vessel wall by an anatomic barrier. The perivascular adipocytes thus provide a local source of A-SAA production to the developing lesion. Transport of A-SAA into the inner vasculature is facilitated by the vaso vasorum which was reported to proliferate during vascular inflammation (Gossl et al. 2009; Kwon et al. 1998) . This local source of A-SAA in the coronary artery might play a significant role in CAD as compared to the transient increase of A-SAA during acute phase response.

2.2.5 Regulation of the expression of A-SAA

Production of A-SAA is regulated at both the transcriptional level and post-translational level. The proximal 450 bases of the promoter region of SAA1 and SAA2 have a sequence identity of 87%. Transcription factor binding sites that are present in both SAA1 and SAA2 includes NFkappaB (-85 to -93 for SAA1; -84 to -92 for SAA2), NF-IL6 (-171 to -187 for

SAA1; -170 to -186 for SAA2) and AP-2 (-253 to -264 and -382 to -387 for SAA1; -391 to -396 for SAA2) (Thorn and Whitehead 2002). The potential of the NFkappaB and NF-IL6 transcription factor binding sites to influence the transcriptional efficiency of SAA2 were verified using the chloramphenicol acetyl transferase as a reporter gene and transfected into Hela cells (Edbrooke et al. 1991) and HepG2 cells respectively (Betts et al. 1993). In addition, the promoter regions of both SAA1 and SAA2 were also to found have similar induction profiles on A-SAA expression when induced with IL-1, IL-6 or both (Thorn and Whitehead 2002). Both NFkappaB and NF-IL6 are positive regulators of A-SAA transcription while AP2 is a repressor of A-SAA transcription.

Production of A-SAA is stimulated by the presence of cytokines TNF-α, IL-1 and IL-6. The NF-IL6 binding site is important for IL6 stimulated A-SAA production. IL-6 binds to its receptor on the cell surface and stimulates the phosphorylation of the NF-IL6 transcription factor which translocates into the nucleus. In the nucleus, NF-IL6 binds to the DNA and upregulates A-SAA production. IL-6 synergises with IL-1 to bring about a greater increase in A-SAA expression. The mechanism of the synergy between IL-1 and IL-6 is not elucidated but it is postulated that it could be brought about by interaction between the bound factors at the NFkappaB binding site and NF-IL6 binding site. The binding of IL-1 to its receptor results in the phosphorylation of the NFkappaB-IkappaB complex and causes the subsequent dissociation of IkappaB from the complex. The liberated NFkappaB is able to translocate into the nucleus and promotes the transcription of A-SAA.

Expression of A-SAA is also regulated by the glucocorticoids. The influence of glucocorticoids can be both direct and indirect. Glucocorticoid stimulates the expression of SAA1 through the glucocorticoid response element (GRE) present in the promoter of SAA1. However, the promoter of SAA2 does not process any GRE (Thorn and Whitehead

2002). The indirect effects of glucocorticoid on A-SAA expression occur through its interaction with NFkappaB (Koj 1996) and its negative regulation on the production of cytokines which stimulate A-SAA expression (Edwards et al. 2007).

In addition to an increase in A-SAA mRNA expression, the increase in A-SAA is also facilitated by post-translation modification of A-SAA transcript which increases their half-life. This is supported by a study in mouse in which there was a 10 fold difference between transcription rate and A-SAA mRNA level (Lowell et al. 1986). The stability of mRNA is probably regulated by polyadenylation which increases the half-life of A-SAA mRNA (Couttet et al. 1997).

2.2.6 Surface receptors of A-SAA

Kinkley et al reported that SAA1 and SAA2 have differential passage through peritoneal macrophages. At 37^{o}C, SAA2 is able to move across the plasma membrane and into the nucleus, however, SAA1 is not readily taken up by the cells (Kinkley et al. 2006). Various *in vitro* studies have revealed that there might be a number of surface receptors for A-SAA which includes toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), formyl peptide receptor like 1 (FPRL-1), CD36 and LIMPII analogous-1 (CLA-1) and receptor for advanced glycation end products (RAGE). The reported functions of these receptors are listed in Table 2-4. It is possible that the surface receptors are important for SAA1 signaling while SAA2 might alter cellular function through a combination of surface receptor signaling and its direct influence on nuclear receptors.

Toll-like receptors, TLR2 and TLR4, are two of the surface receptors of A-SAA. In a study using TLR2 knockout mice, TLR2 was found to be responsible for A-SAA stimulated induction of granulocyte colony-stimulating factor in cultured macrophages (He et al. 2009).

In another study using TLR2 knockout mice, TLR2 was reported to be associated with the secretion of proinflammatory cytokines, IL-12p40 and TNF-α from mouse macrophages (Cheng et al. 2008). The role of TLR4 as a receptor of A-SAA was elucidated using $TLR4^{-/-}$ mice; SAA stimulated induction of nitric oxide is almost completely abrogated in macrophages isolated from the knockout mice (Sandri et al. 2008).

RAGE is another receptor for A-SAA. SAA induced secretion of tissue factor in PBMCs was reduced by 40-50% in the presence of a peptide antagonist of RAGE (Cai et al. 2007).

CLA-1, the human orthologue of scavenger receptor class B type I (SR-BI), is another reported receptor of SAA, in Hela cells, over-expression of CLA-1 results in increased IL-8 secretion (Baranova et al. 2005).

The last known receptor of A-SAA is FPRL-1. In human umbilical vein endothelial cells, siRNA of FPRL-1 was enough to completely block the A-SAA induced production of chemokine (C-C motif) ligand 2 (CCL2) (Lee et al. 2009b). SAA stimulated proliferation of human fibroblast-like synoviocytes was also inhibited when short interfering RNA of FPRL-1 was introduced (Lee et al. 2006).

Most of the studies on the surface receptors of SAA were conducted using a recombinant form of SAA with sequence that is a hybrid of SAA1 and SAA2. Further study will need to be performed in order to verify whether these receptors are gene-specific. The numerous receptors of A-SAA might indicate that SAA can activate numerous pathways and modulate the secretion of cytokines from cells such as monoctyes and endothelial cells.

Table 2-4. Reported surface receptors of A-SAA. There are limited studies on most of the reported receptors of A-SAA.

Receptor	Pathway activation
CLA-1	Regulates the selective uptake and efflux of cholesterol from cells that expresses CLA-1 receptor (Ji et al. 1997). Mediates SAA induced activation of ERK1/2 and p38 (Baranova et al. 2005).
FPRL-1	Facilitates invasion and migration of cancerous cells (Cheng et al. 2010; Coffelt et al. 2009). Induces chemotaxis of human neutrophils and phagocytes through activation of p38 MAP kinase-mediated signaling pathway (Selvatici et al. 2006; Shim et al. 2009). Promotes secretion of inflammatory cytokines and chemokines (Lee et al. 2009a; Lee et al. 2009b).
RAGE	Binds numerous ligands, high mobility group box 1 (HMBG1), calcium binding S-100 family of proteins, immunoglobulin light chains and prions that are produced in response to cellular or physiological stresses and results in the activation of various pathways including MAPK, PI3k-Akt, Jak-STAT and NF-kappaB (Sims et al. 2010). One consequence of RAGE interaction with its ligands is the generation of reactive oxygen species, ROS (Wautier et al. 2001).

Receptor	Pathway activation
TLR2	Induction of apoptosis through MyD88 activation of caspase 8 (Aliprantis et al. 2000). Promotes secretion of proinflammatory cytokines, TNF-α, IL-8, and IL-12, through phosphatidylinositol 3-kinase (PI3K)-NF-kappaB pathway (Lee et al. 2010; Meng et al. 2008).
TLR4	Stimulates pro-inflammatory response through TRAF6-NF-kappaB pathway (Verstak et al. 2009).

2.2.7 A-SAA as a clinical biomarker

Various studies have been conducted to study whether the level of A-SAA can be used as a clinical biomarker for chronic inflammatory diseases. The level of A-SAA has been indicated as a useful clinical marker for acute coronary syndrome, stable CAD, cancer and metabolic syndrome (Cho et al. 2010; Kotani et al. 2009; Ramankulov et al. 2008).

In study of subjects with non-ST-segment elevation acute coronary syndromes (NSTE-ACS), elevated level of A-SAA was found to be a good predictor of adverse clinical events within 30 days of the occurrence of NSTE-ACS (Kosuge et al. 2007). It is also a reliable marker to predict 14-day mortality in patients with unstable angina or myocardial infarction (Morrow et al. 2000). A-SAA level is also useful as a biomarker for future cardiovascular events and is highly predictive for 3 year cardiovascular events in patients with myocardial ischemia (Johnson et al. 2004). However, the level of A-SAA is not useful as a biomarker for cardiovascular mortality within 5 years in patients with acute coronary syndromes (Zairis et al. 2007). Lastly, the complex, SAA-LDL, was reported to be a good prognosis indicator in patients with stable coronary disease (Ogasawara et al. 2004).

Although, A-SAA appears to be an important clinical marker for cardiovascular diseases, the aim of this study is to further clarify the pathogenic role of SAA1 in atherosclerosis rather than its use as a prognostic factor. This research interest is supported by various studies which suggest a direct role of A-SAA in atherosclerosis as written in section 2.2.8 and 2.2.10.

2.2.8 Atherogenic effects of A-SAA

In vitro and *in vivo* studies appear to indicate that A-SAA might have a more direct effect on atherosclerosis. In a study by Meek et al, mRNA of A-SAA was found in important components of the atherosclerotic lesion including endothelial cells lining the lumen of coronary artery, the vaso vasorum as well as in newly formed vessels. In addition, the expression was especially high in macrophage foam cells and in adventitial adipocytes (Meek et al. 1994). A-SAA also has other atherogenic effects such as facilitating the remodeling of the extracellular matrix, debilitating the anti-oxidant effects of HDL through remodeling of the HDL and stimulating the secretion of inflammatory cytokines from endothelial cells and monocytes.

A-SAA was reported to stimulate the hydrolytic activity of sPLA2. sPLA2 hydrolyses lipoproteins and converts the phospholipids into atherogenic oxygenated and non-oxygenated fatty acids. In addition, acute-phase HDL, which is predominantly enriched with A-SAA, was found to be more susceptible to the hydrolytic activity of sPLA2 (Pruzanski et al. 1995).

Another known atherogenic effect of A-SAA is its induction of the secretion of inflammatory cytokines and chemokines from endothelial cells and monocytes (Yang et al. 2006). In addition, A-SAA also stimulates the synthesis of inflammatory compounds such as eicosanoids and thromboxane A2 (Malle et al. 1997). As a result of the secretion of

chemokines as well as other unidentified molecules, A-SAA was reported to induce the migration and adhesion of monocytes and T-lymphocytes (Badolato et al. 1994; Preciado-Patt et al. 1996a; Xu et al. 1995). Hence, A-SAA can function as a stimulant of chemoattractant and facilitates the migration of immune cells that form an important component of atherosclerotic lesion.

A-SAA also facilitates the remodeling of the HDL molecules. A-SAA substitutes and replaces apo-AI in the HDL molecule; HDL enriched with A-SAA has lost 87% of its apo AI content and 91% of its paraoxonase activity (Van Lenten et al. 1995). Both apo-AI and paraoxonase have anti-oxidant properties and increased paraoxonase activity in transgenic mice was shown to have a negative impact on the formation of atherosclerotic lesion (Tward et al. 2002). In addition to losing its anti-oxidant components, acute-phase HDL has increased level of pro-oxidant ceruloplasmin (Navab et al. 1998). As a result of the remodeling, acute-phase HDL was observed to amplify the increase in monocyte transmigration as a result of LDL oxidation (Van Lenten et al. 1995).

Lastly, A-SAA level was found to correlate with lesion size and the extent of atherosclerosis in both mice and rabbits fed on a high fat diet. The extent of atherosclerosis was, however, found to be independent of circulating lipid and lipoprotein concentrations for both studies (Lewis et al. 2004; Van Lenten et al. 2007). In another study, when both atherosclerosis susceptible mice (C57BL/6) and atherosclerosis resistant mice (C3H/HeJ) were fed on a high fat diet, the former had a five fold increase in A-SAA level. A-SAA, apo A-I and proteoglycans were also found to colocalise in atherosclerotic lesion and thus A-SAA appears to play an important role in the development of the lesion (Liao et al. 1994). It mediates its role possibly through a combination of its ability to induce the migration of immune cells and its pro-oxidant capability which facilitates the remodeling of HDL.

2.2.9 Atheroprotective effects of A-SAA

There are fewer reports on the atheroportective effects of A-SAA. A-SAA was reported to inhibit the oxidative burst response in neutrophils (Linke et al. 1991). A-SAA also inhibits the activation of platelet and prevent its aggregation which might be beneficial in curbing atherogenesis at site of injury (Zimlichman et al. 1990). Acute-phase HDL has better affinity for macrophages as compared to normal HDL and this improved affinity aids in its reverse cholesterol transport from the macrophages (Kisilevsky and Subrahmanyan 1992). Furthermore, acute-phase HDL that was remodeled by heparan sulfate was found to have a 3-fold increase in cholesterol efflux activity. Since SAA has a binding site for heparan sulfate, the result is supportive of the hypothesis that SAA aids in the removal of cholesterol sequestered by macrophages from site of injury (Tam et al. 2008) .

2.2.10 Role of A-SAA in other chronic inflammatory diseases

Expression of A-SAA is also elevated in other chronic diseases such as RA and systemic lupus erythematosus (SLE) where immune reaction plays a significant role in the pathogenesis. Patients with RA were reported to have high serum levels of SAA (De Beer et al. 1982). In addition, A-SAA induces the secretion of chemokines from synovial cells and in a RA synovial/SCID mouse chimera model A-SAA was shown to promote synovial cell proliferation and angiogenesis (Connolly et al. 2010). In another study, the binding of SAA to FPRL-1 aids in the proliferation of human fibroblast-like synoviocytes by preventing its apoptosis (Lee et al. 2006). The increased susceptibility of patients with rheumatoid arthritis or SLE to cardiovascular diseases (Manzi et al. 1997; Trager and Ward 2001) highlights the inflammatory nature of these diseases and that increased susceptibility could be due to a dysfunctional immune regulation on a systemic level. As there are similarities in the disease

pathogenesis of these three diseases, it is possible that A-SAA might play a role in these diseases and could be an important risk factor.

Elevation of A-SAA was observed in various cancers including lung cancer and endometrial endometroid carcinoma (Cremona et al. 2010). In one study, there was a 77% elevation of A-SAA in patients with poor prognosis (Cho et al. 2010). In patients with endometrial endometrioid carcinoma, there was on average a 4 fold increase in serum A-SAA. A-SAA was also observed to be secreted by endometrioid carcinoma cell line *in vitro* (Cocco et al. 2010). The exact mechanism through which A-SAA might influence the development of cancer is currently unknown, it is, however, postulated that A-SAA might modulate cancer development through its effect on the extracellular matrix. A-SAA has binding domains for laminin and heparan sulfate which are components of the extracellular matrix. As a result of its interaction with the extracellular matrix, increased secretion of A-SAA might decrease the adhesion of metastatic cells to the extracellular matrix and facilitates metastasis (Malle et al. 2009).

.

2.3 Genetic Analysis of Complex Diseases

2.3.1 Complex Diseases

Complex diseases are polygenic and multifactorial diseases in which multiple genes can alter susceptibility to the disease. The phenotypic expression of the disease usually involves the interaction of several disease-causing genotypes, each conferring additional risk to disease susceptibility. In additional, the environment also plays a role in disease susceptibility such as diet in the case of CAD. Due to the polygenic nature, it is difficult to determine genes that are important in that pathogenesis of complex diseases in the past. With the availability of

modern high-throughput tools which facilitate the undertaking of genome-wide association study, the genetic study of complex diseases should gather pace.

2.3.2 Genetic variation and SNPs

Genetic variation refers to the change of nucleotide in a DNA sequence. When a variation occurs in the exon region of a gene, it can alter the primary sequence of the protein and possibly its structure. Variation in the regulatory region, such as in the promoter can affect the regulation of the protein expression. Variation can occur through various means such as a nucleotide substitution, an insertion or deletion of either a single or multiple nucleotides as well as duplication of segment of nucleotides. When the nucleotides are not inserted or deleted in multiple of threes, as in frameshift mutation, all the encoded amino acids following the mutation will be altered. Although frameshift mutation is more likely to have a significant effect on the protein structure, it is rare as such mutation is more likely to be deleterious and possibly lethal to the individual.

SNP is a single nucleotide change in the DNA sequence that exhibits variation in a population. A single nucleotide variation is labeled a SNP if the frequency in the population is 1% or more (Twyman and Primrose 2003). SNPs constitute more than 90% of the variation in the human genome (Collins et al. 1998). There is on average a SNP every 1,000 nucleotides, 20% of the SNPs occur in the exon region and half of the SNPs in the exon region encodes for synonymous amino acids (Li and Sadler 1991). Due to the frequency of SNPs, they are useful as genetic markers for genome wide association study. As genetic markers, SNPs have advantage over microsatellites as they are generally more stable. In addition, non-synonymous SNPs can contribute to the pathogenesis of complex diseases although its effect is modest in most cases (Casas et al. 2006).

2.3.3 Methods for genetic analysis of human diseases

There are various methods for studying human diseases that are heritable to a certain extent. Three of the methods, parametric linkage analysis, non-parametric linkage analysis and association study are discussed below. The choice of method is usually dependent on the nature of the disease. As parametric linkage analysis requires a precise genetic model in order to determine the linkage of the disease mutation to the marker, it is useful for investigating linkage in a monogenic disease as the allele frequencies, penetrance and mode of inheritance is easier to determine in a monogenic disease. For multi-factorial diseases such as CAD, diabetes and cancer, the polygenic nature of the diseases make it difficult for a genetic model to be employed. Despite its limitations, parametric linkage analysis has been used in the past for the genetic analysis of multi-factorial diseases such as breast cancer and schizophrenia with sporadic success. Parametric linkage studies for these diseases involve the studies of rare families where the diseases were inherited in a Mendelian pattern. The study leads to the identification of the *BRCA1* and *BRCA2* genes. However, the application of this method for the study of schizophrenia was not successful (Strachan and Reed 1999). As it is difficult to identify families in which complex genetic diseases are inherited in a Mendelian pattern, these diseases have often been analysed using non-parametric linkage and association study in which a genetic model need not be specified. Parametric linkage analysis, non-parametric linkage analysis and genetic association study are described in details below.

2.3.3.1 Parametric linkage analysis

Parametric linkage analysis is based on the concept that two genes residing on the same chromosome are most likely to segregate together during meiosis if they are in close proximity. Hence, if a disease mutation is located in between two genetic markers, the whole

segment will be inherited together by the offspring. This linkage of the disease mutation to genetic marker can be statistically accessed by the 'logarithim of the odds' (LOD) score. The LOD scores from independent study of pedigrees can be combined together as long as the two analysis are performed using the same genetic model. Through determining the linkage, parametric linkage analysis can narrow down the location of the disease mutation.

Parametric linkage analysis is only useful for simple Mendelian diseases such as cystic fibrosis or sickle-cell anaemia where a single mutation in a gene is responsible for the disease. In multi-factorial disease, the combined effects of multiple genes limit the usefulness of parametric linkage analysis. Other limitations of parametic linkage analysis include the need to recruit large pedigree as well as the requirement to make assumption regarding the mode of inheritance of the disease gene (Tang et al. 2008).

2.3.3.2 Non-parametric linkage analysis

An alterative to linkage analysis is the non-parametric linkage analysis. Non-parametric linkage analysis is also called the allele-sharing method. One major difference for the allele sharing method as compared to parametric linkage analysis is that the mode of genetic inheritance is not considered in the analysis. The basis of allele-sharing methods is illustrated in the genetic analysis of siblings affected with a certain disease which is named the affected sibpair analysis. In sibpair analysis, the marker alleles are analysed in affected siblings. If the affected siblings share similar maker alleles and this sharing deviates from the ratio of that of random Mendelian segregration, the marker alleles and the disease allele are genetically linked (Zhu et al. 2010).

The advantage of allele-sharing methods is that a large pedigree need not be recruited. Its application in the genetic analysis of complex disease is, however, limited by the polygenic

nature of such disease. The polygenic nature of complex disease implies that each allele might have subtle effects on disease susceptibility and thus disease allele with subtle influence might be missed.

2.3.3.3 Genetic association study

Association study is pertinent to the genetic analysis of complex disease. Unlike the other two approaches, association study is able to identify alleles either with a subtle or significant effect on susceptibility to complex diseases (Risch and Merikangas 1996). One form of association studies is the case-controlled study. In the case-controlled study, two groups of subjects, healthy and disease are recruited and genotyped for the allele of interest. The allele frequencies of both groups are compared and level of significance is determined by χ^2. The strength of association of the allele with the disease is determined by the odds ratio.

Unlike, linkage study, association study is conducted at the population level, sample subjects need not be related and hence recruitment of subjects is less challenging. Since association study is performed at population level, care must be taken to ensure that the population is homogenous and that cases and controls are well-matched. An inability to ensure population homogeneity can lead to inaccurate result.

2.3.4 Mutation screening

A number of methods exist for mutation screening. Some methods that are suitable for mutation screening of a candidate gene are discussed below. Denaturating gradient gel electrophoresis (DGGE), denaturing high performance liquid chromatography (DHPLC) and high resolution melting (HRM) identify mutation through altered melting property of the double-stranded DNA as a result of the nucleotide(s) change. Single-strand

conformation polymorphism (SCCP) differentiates alleles through different secondary structure conformation that results from the base change.

2.3.4.1 Denaturating gradient gel electrophoresis (DGGE)

Polymerase chain reaction (PCR) product is melted and allowed to reanneal to facilitate the formation of heteroduplex. In the presence of a variant allele, a heterogenous mix containing wild-type and mutant homoduplexes and heteroduplexes DNA will be formed. This mixture is separated using a denaturing gradient gel. As the homoduplex and heteroduplex denature at different rates in the gel, they are efficiently separated and thus the variation can be identified. Although DGGE is an inexpensive method, the preparation of the denaturing gradient gel is a relatively tedious process. In addition, variation in the procedure of gel preparation often lead to variable results; reproducibility is often an issue with DGGE (Balogh et al. 2004)

2.3.4.2 Denaturing high performance liquid chromatography (DHPLC)

Similar to DGGE, DHPLC identifies mutation through the differentiation of homoduplexes and heteroduplexes. The separation cartridge has higher affinity for the homoduplexes and as such heteroduplexes will be eluted first. Eluted DNA is captured by a UV detector and the result is displayed in the form of electropherogram. The presence of a heterozygote is evident from the multiple peaks in the electropherogram. DHPLC have numerous advantages including its reproducibility and high sensitivity. DHPLC also have comparable sensitivity and is able to detect mutations in amplicons of up to 700 bases (O'Donovan et al. 1998). One key disadvantage of DHPLC is that the machine requires regular maintenance.

2.3.4.3 High resolution melting (HRM)

In HRM, a saturating double strand DNA (dsDNA) binding dye is used. PCR amplification is carried out in the presence of the dsDNA binding dye. The PCR product is melted and the change in fluorescence levels during melting is captured at high resolution. Heteroduplexes are less stable and hence its melting profile will be different from the homoduplexes. The advantages of HRM are that it is inexpensive as the procedure only requires standard PCR reagent plus a dsDNA binding dye and that the procedure is much faster as compared to other listed methods as no separation step is required (Vossen et al. 2009). HRM also has high sensitivity, however, its sensitivity is dependent on the amplicon having a single melting domain, the amplicon size is thus minimized in most cases for optimal result.

2.3.4.4 Single-strand conformation polymorphism (SCCP)

SSCP is performed using single stranded DNA. A single nucleotide change can alter the secondary structure of single stranded DNA. The altered secondary structure will have differential mobility in non-denaturing polyacrylamide gel which is distinguishable from the wild-type. SSCP has being used in the past for mutation screening and it is inexpensive and does not require dedicated equipment. However, its principle disadvantage is its inconsistency; its sensitivity can range from 50% to 97% (Liu et al. 1996; Mogensen et al. 2003). Various factors can affect the sensitivity of SSCP including the size of amplicon, choice of gel matrix, CG content and DNA concentration (Hayashi and Yandell 1993; Humphries et al. 1997). SSCP is now replaced by more sensitive methods that are also relatively economical.

2.3.4.5 Choice of method for mutation screening

Both high sensitivity and reproducibility are important criteria for accurate mutation screening. HRM and DHPLC both meet these criteria and both have other advantages. The usage of either method will depend on availability of equipment. Although, HRM is relatively new, the procedure is fast and as much as 384 samples can be processed by the equipment simultaneously; this is a big advantage compared to DHPLC which take a few minutes to analyse each sample. However, HRM works best when the amplicon has only a single melting domain; an amplicon with multiple melting domains will disrupt the melting profile and the resultant curve is not suitable for analysis. Under such circumstance, DHPLC would be a better alternative.

Table 2-5. Comparision of the various methods for genetic variant screening.

Method	Ease of optimisation	Ease of setup	Fast	High sensitivity	Reproducibility
HRM	✓	✓	✓	✓	✓
DHPLC	✓	✓		✓	✓
SSCP		✓	✓		
DGGE			✓		

2.3.5 Methods of SNP genotyping

SNP genotyping methods should preferably be efficient, inexpensive and have a very high degree of specificity. The gold standard for genotyping a SNP is through direct sequencing of the amplicon containing the SNP. Sequencing is, however, a tedious and time-consuming process and not sufficiently high throughput. Other SNP genotyping approaches include

restriction fragment length polymorphism (RFLP), allele-specific PCR, primer extension, molecular probes such as molecular beacons, oligonucleotide ligase assay and HRM.

2.3.5.1 Allele-specific PCR

There are two main procedures in which allele-specific PCR can be carried out. In the more commonly used procedure, the amplification refractory mutation system, two separate PCR reactions are used to genotype an unknown sample. In each PCR reaction, there is one common primer and one allele specific primer which has its 3' end complementary to the allele it recognizes. When the sample is a homozygote, successful amplification will only occur in one of the tube. The products of both PCR reactions are separated by gel electrophoresis and the genotype can be ascertained. The two major advantages of amplification refractory mutation system are that unlike RFLP it can theoretically be employed to genotype most SNPs and that it is inexpensive as it does not require the use of dedicated equipment or expensive probes. One disadvantage of allele-specific PCR is that two reactions are needed to genotype one sample while other alternatives that require the use of differentially labeled probes only require one reaction.

2.3.5.2 Restriction fragment length polymorphism (RFLP)

The specificity of restriction enzyme (RE) to recognize and cleave a particular DNA sequence is used to differentiate between wild-type and mutant. A RE that recognizes one of the alleles is chosen. As a result of selective cleavage, different genotypes will have different cleavage patterns that are evident upon running gel electrophoresis. RFLP is a traditional method used for genotyping before the advent of more modern genotyping tools. There are numerous advantages including its simplicity, specificity and the lack of need for the usage of

dedicated equipment (Xu et al. 2003). However, RFLP cannot be used for all genotyping procedures as there might not be any RE with the matching recognition sequence. RFLP is also relatively more tedious compared to modern methods such as HRM which does not require gel electrophoresis to differentiate the product.

2.3.5.3 HRM

The mechanism is similar to that as described for the application of HRM in genetic variant scanning. In one method of HRM genotyping, primers are designed to flank the SNP; as the the wild-type and variant amplicons have different melting temperatures, they can be distinguished from the resultant melting curves. Genotyping using HRM is efficient for class 1 and 2 SNPs where there is a difference in CG content between the amplicons containing the two different alleles. For class 3 and 4 SNPs whereby there is no overall change in the CG content of the amplicon, Liew et al reported that such SNPs can still be genotyped using small amplicon melting if the sample is spiked with a sample of known genotype prior to the genotyping procedure (Liew et al. 2004). The inability to genotype class 3 and 4 SNP efficiently is a significant disadvantage of HRM genotyping. However, other methods of HRM genotyping such as using unlabeled probes to differentiate the different genotypes has being reported (Zhou et al. 2004). Overall, genotyping using HRM is still in its nascent stage and the fine tuning of this technique will probably make it an attractive alternative to other more expensive methods.

2.3.5.4 Primer Extension

In primer extension, primer is designed to hydridise to the base adjacent to the SNP of interest. The primer is extended through the incorporation of dideoxynucleotides (ddNTPs),

which is tagged with a fluorescence signal. Incorporation of the ddNTP ceases further extension and the genotype can be identified through the capturing and analysis of the fluorescence signal. Primer extension is suitable for high throughput, multiplexing SNP genotyping and has been used in several fine-mapping genotyping platforms such as MassArray (Sequenom, San Diego, CA) and SNPstream (Beckman Coulter, Brea, CA).

2.3.5.5 Hybridisation probes

Both molecular beacons and Taqman probes are based on the principle of fluorescence resonance energy transfer (FRET). FRET involves the process of energy transfer from a donor fluorophore to an acceptor fluorophore (quencher) when they are in close proximity. As a result, the fluorescence that is emited by the donor is quenched in the presence of the acceptor. A Taqman probe consists of a donor fluorophore and an acceptor fluorophore at opposite ends. Two allele-specific taqman probes are used for each set of genotyping assay with each probe tagged with a unique donor fluorophore. If the probe binds to its target DNA, it will be cleaved by the activity of the Taq polymerase during PCR amplification resulting in the emission of fluorescence as the donor is no longer quenched by the acceptor. The analysis of the fluorescence signal will indicate the genotype of the DNA. Hybridisation probes are sensitive when the probes are well-designed. The chemistry of hybridisation probe has been incorporated into commercially available high throughput genotyping platform, TaqMan open array (Life Technologies, Carlsbad, CA), that allows for multiplexing of 64-256 SNPs and can handle up to 384-1536 samples a day (Ragoussis 2009).

2.3.5.6 Selection of method for SNP genotyping

Most of the above methods are good alternatives to DNA sequencing which is relatively less efficient. The purpose for SNP genotyping is usually the most important criteria in deciding on the choice of method used. A high throughput platform such as Taqman open array is useful for SNP genotyping in genome wide association studies where a large number of SNPs need to be genotyped. In a candidate gene approach where there are fewer SNPs to be genotyped, it is not cost-efficient to use multiplexing high throughput platform. For such study, methods such as RFLP or allele-specific PCR would be more relevant. Primer extension which uses enzyme-linked immunosorbent assay (ELISA) as a detection tool does not offer a good alternative to either RFLP or allele-specific PCR as it also involves additional post-PCR steps. HRM is a useful genotyping tool for SNP analysis; it is, however, still a relatively new method and that thorough optimization should be performed for each SNP assay to ensure its specificity.

Table 2-6. Comparison of the various methods for SNP genotyping.

Methods	Inexpensive	Availability of high throughput platform	High call rate	No specialized equipment required	Convenient Workflow
Allele-specific PCR	✓		✓	✓	
RFLP	✓[a]		✓	✓	
Primer Extension		✓	✓	✓[b]	✓
HRM	✓				✓
Hybridisation probes		✓	✓		✓

[a]: The cost of RFLP is dependent on the cost of the specific RE used.
[b]: Primer Extension is also used for high throughput system that requires dedicated equipment.

MATERIALS AND METHODS

3.1 *SAA1* SNPs survey

3.1.1 Study subjects

Cord blood DNA samples from 96 anonymous, unrelated Singaporean Chinese neonates delivered in the National University Hospital, Singapore, were used in the study. Ethical approval from the university IRB was obtained for the usage of the DNA for the purpose of the study.

3.1.2 DNA extraction

DNA was extracted from blood samples using the phenol-chloroform method. The blood sample was first centrifuged at 3500 rpm for 7 min to obtain the cellular components of the blood. TE buffer was then added to lyse the red blood cells. The lysate was centrifuged and the white blood cell pellet was collected. The pellet was incubated overnight at 37°C with TE buffer supplement with 0.5% SDS and 300 μg Proteinase K. Lysed pellet was mixed and centrifuged at 3500 rpm for 10 min first with phenol and then with chloroform. The purified DNA obtained was transferred into a clean tube for DNA precipitation. DNA was precipitated by adding a solution mixture containing 0.1 M sodium acetate and 100% ethanol to the purified DNA.

3.1.3 Primer design and PCR amplification

Primers used for the amplification of the 3 exons of *SAA1* were designed using the published sequence on NCBI (Ref Seq: NM_199161.2). The sequence for the promoter region used was based on a released human genomic sequence annotated in 2006 by the International Human Genome Sequencing Consortium. The PrimerQuest (Integrated DNA

Technologies, Coralville, Iowa, United States) software was used for designing primers. Primers were designed to yield an amplicon size of not more than 300 bases for optimal screening using HRM. Due to the high sequence similarity between *SAA1* and *SAA2*, the primer sequences were aligned with the genomic sequence of *SAA2* to ensure that either the forward or reverse primer was *SAA1* specific. The specificity of the primers was tested using an online application (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). For both exon 1 and 2, non *SAA1*-specific amplicons were designed due to the near identical sequences of both genes at both regions.

PCR amplification was carried out in a thermocycler (Biometra, Göttingen, Germany). The reaction mixture consists of 20ng genomic DNA, 5X reaction buffer, 2.0mM Mg^{2+}, 0.2 mM dNTPs, 1 μM forward and reverse primers, 1X LC Green dye (Idaho Technology, Salt Lake City, Utah, United States) and 1 unit Taq Polymerase (Promega, Madison, Wisconsin, United States). The primer sequences and their annealing temperature are as shown in Table 3-1. Amplification conditions consist of an initial denaturation at 95°C for 3 min and 35 cycles of 30s of denaturation at 95°C, 30s of annealing at the indicated Tm as shown in Table 4.1, and 30s of extension at 72°C. A final extension step of 72°C for 5 min was included and thereafter the amplicons were loaded onto a 96 well plate for melting analysis to be carried out in the LightCycler 480 (Roche Diagnostics, Indianapolis, Indiana, United States).

Table 3-1. Primer sequences for amplification of selected regions of *SAA1*.

Region	Forward Primer/ Reverse Primer	Amplicon Size (bp)	Tm	1.0 M Betaine
Promoter 1 (c.-1305 to c.-1029)	5' CTTGACCTCCTGGGTCTC 3' 5' CCTCTGAAATGAAGTCCCTC 3'	277	61	-
Promoter 2 (c.-1088 to c.-809)	5' GACCTAGTCTGTCAGCTTTG 3' 5' CAACCTGAGGGAACAAGATG 3'	280	62	-
Promoter 3 (c.-857 to c.-637)	5' TGGTCTCCTGCCCTGACA 3' 5' GCGGTGGCTGCTATTTATACTGAG 3'	221	64	-
Exon 1	5' CAGGCTCTCGTCGGAATA 3' 5' AGTTTATCAGGTCCAGGGTG 3'	238	64	-
Exon 2	5' TGTTCTCCTGATGTCCCTTC 3' 5' CAGCCTCTAACTTCTCCACA 3'	280	62	+
Exon 3	5' TAATCTCCTTCTTGCCTGCC 3' 5' CCCATTGTGTACCCTCTCC 3'	254	64	+

3.1.4 High resolution amplicon melting and automatic calling

HRM was carried out in the LightCycler 480. The samples were first heated at 95°C for 1 min to denature the double-stranded DNA. Upon denaturation, the samples were incubated at 40°C for 1 min to facilitate the formation of heteroduplex, and then progressively heated

from 65°C to 95°C at a ramp rate of 1°C/s with the fluorescent signal levels simultaneously acquired 25 times for every degree change.

The fluorescent melting curves generated were analysed using LC480 Gene Scanning software v1.5 (Roche Diagnostics, Indianapolis, Indiana, United States). All curves were normalized and temperature shifted before they were automatically assigned into different subgroup by the calling tool module of the software.

The automatic calling tool provided by the Gene Scanning software was used to differentiate the wild type from the genetic variants based on the melting curves. The sensitivity of the calling tool is the only parameter to be set and it ranges from 0 to 1. A lower sensitivity will produce a smaller number of subgroups while a higher sensitivity will yield a greater number of groups albeit with more false positives. A sensitivity setting of 0.15 was deemed to be optimal for our study. The identity of the variants in the various subgroups as assigned by the Gene Scanning software was determined by DNA sequencing.

3.1.5 DNA sequencing

Amplicons were treated with 5 units of exonuclease I (Fermentas, Burlington, Ontario, Canada) and 1 unit of shrimp alkaline phosphatase (Fermentas) at 37°C for 15 min to degrade any of the remaining primers. Subsequently, 2.0 μl of the PCR product was mixed with 0.16 μM of either the forward or reverse primer, and 1.0 μl of the BigDye terminators (Life Technologies, Carlsbad, California, United States) in a 5 μl reaction mix. The sequencing mix was incubated in the thermocycler with 35 cycles of 10s of denaturation at 96°C, 5s of primer annealing at 50°C and 4 min of primer extension at 60°C.

DNA incorporated with BigDye was precipitated using the ethanol/sodium acetate method. The precipitation mix consisting of 5.0 μl of BigDye incorporated DNA, 0.1M sodium

acetate and 62.5% ethanol was incubated at -20°C for 20 min. Upon incubation, the mixture was centrifuged at 13,000 rpm for 15 min to obtain the DNA pellet. The DNA pellet was washed with cold 70% ethanol and centrifuged at 13,000 rpm for 5 min. The supernatant was then aspirated and the pellet was left to dry in the dark. 12 µl Hi-Di formamide was added to the dried pellet and the mixture was loaded onto the sequencing plate. Sequencing was carried out in the 16-capillary ABI 3100 (Life Technologies) and analysed using sequencing analysis software v3.7 (Applied Biosystems).

3.1.6 *In silico* SNP discovery and *in silico* prediction of biological significance of polymorphisms

In silico SNP discovery using sequences deposited in public databases was performed to supplement our experimental approach. The SNPFINDER program (Buetow et al., 1999; http://lpgws.nci.nih.gov/perl/snpbr) which predicts SNPs based on deposited expressed sequence tags (EST). SNPs were discovered using Unigene *SAA1* cluster, Hs. 632144 and the analysis was carried out with an expect value of 0.01 to minimize false positive.

For non-synonymous SNP that is found in the coding region, the change is analysed for its likely impact on the protein function using ClusterW and BLOSUM62. ClusterW was used to determine whether the amino acid at the indicated position is well-conserved among various species. The amino acid sequence of human SAA1 (CAG47037.1) was aligned with that of rabbit (*Oryctolagus cuniculus;* AAB20616.1), Syrian hamster (*Mesocricetus auratus*; AAB27187.1), Mouse (*Mus musculus*; NP_0.331431.1), Rhesus monkey (*Macaca mulatto*; XP_001086137.1), Cheetah (*Acinonyx jubatus*; BAG06986.1). The impact of amino acid change on the primary structure of the protein was analysed by using the substitution matrix, BLOSUM62 (Henikoff and Henikoff, 1992). Positive values on the BLOSUM62 are

indicative of frequent and conservative substitution while negative values indicate rare and non-conservative substitution. The naming of the genetic variant is based on NM199161.2 for the exons and NG_021330.1 for the promoter.

3.2 Genetic association study

3.2.1 Study subjects

Samples from CAD patients who underwent coronary bypass graft surgery were used. All cases have at least 50% stenosis in at least one of the major coronary arteries as revealed by angiography. In addition, cases that have valve diseases, hypertension and diabetes were excluded from the study.

The controls were healthy subjects attending a routine health screening or rhinologic examination in the Ear, Nose and Throat clinic of the National University Hospital. Control subjects were screened for potential confounders through a combination of questionnaire, physical examinations and laboratory tests. The confounders that were screened include both personal medical history of CAD and diabetes. Tests that were conducted include glucose level quantification and electrocardiogram. Control subjects with these potential confounders were excluded from the study. Ethical approval from the university IRB was obtained for the usage of the DNA for the purpose of the study.

3.2.2 Genotyping by allele-specifc PCR

The SNP, c.-913G>A, was genotyped using allele-specific PCR. Two PCR reactions were set up for each sample; in one of the reactions the reverse primer has a 3' end that is complementary to the wild type while in the other reaction a primer with 3' end complementary to the variant was added. The universal forward primer used was 5'

GCAGATGCACACTACCA 3' while the reverse primers used were 5' GCGGAGTTGAGAGTTGGAGCACC 3' (wild type complementary) and 5' GCGGAGTTGAGAGTTGGAGCACT 3' (variant type complementary).

The reaction mix consists of 1 µl genomic DNA, 5X reaction buffer, 2.0mM Mg^{2+}, 0.2 mM dNTPs, 1 µM forward and reverse primers and 1 unit Taq Polymerase (Promega). Amplification conditions consist of an initial denaturation at 95°C for 2 min and 35 cycles of 30s of denaturation at 95°C, 30s of annealing at 61°C, and 30s of extension at 72°C. A final extension step at 72°C for 5 min was included and the samples were then loaded onto 2% agarose gel.

3.2.3 Genotyping by RFLP

208C>T, 224 C>T, and 269G>A were genotyped using RFLP. PCR was carried out in a 20 µl reaction mix containing 1 µl genomic DNA, 0.2 mM dNTPs (Fermentas), 0.5 µM forward and reverse primers and 0.4 units DNA polymerase (Finnzymes, Espoo, Finland). The primers used were 5' GCCAATTACATCGGCTCAG 3' (forward) and 5' TGGCCAAAGAATCTCTGGAT 3' (reverse) for 208C>T and 224C>T. 5' GCT CAC TCG CCT GAT TAT TA 3' (forward) and 5' ATACCCATTGTGTACCCTCT 3' (reverse) were used for the genotyping of 269 G>A. Amplification conditions consist of an initial denaturation at 95°C for 3 min and 30 cycles of 15s of denaturation at 96°C, 30s of annealing at 58°C, and 25s of extension at 72°C. A final extension step at 72°C for 2 min was included.

208C>T, 224C>T and 269G>A were genotyped using BanI, BclI, and NcoI restriction enzyme respectively. The digested products were resolved using 2.5% agarose gels. Gel was visualized under ultraviolet illumination and images were captured using ChemiDoc XRS (Bio-Rad Laboratories, Hercules, California, United States).

3.2.4 Data analysis

Allele frequencies were determined by the gene-counting method. Significant departure of genotype frequencies from Hardy–Weinberg expectation and the test of allele frequencies difference between cases and controls were determined by the chi-square test. The effects from confounding variables such as age, gender and BMI were adjusted using a binary logistic regression model. The odds ratios for the various SNPs were determined using co-dominant, dominant and recessive genetic models when possible. Odds ratios are presented with 95% confidence intervals (CI). Statistical significance is set at $p < 0.05$.

3.3 Functional study of p.Gly90Asp

3.3.1 Preparation of recombinant human SAA1

3.3.1.1 Plasmid construction

Both wild-type and variant SAA1 cDNAs were synthesized using custom gene synthesis service (Genscript, Piscataway, New Jersey, United States). The synthesized sequence contains the sequence 5' CATGGATCC**GATGATGATGATAAG** 3' at the 5' end which incorporates the BamHI restriction enzyme recognition site. The 3' end contains the sequence 5' CTGAGAAATACTGAGCTTCCTCGAATTCTGTCGACG 3' with the EcoRI recognition site. The cDNAs were subcloned into pET21-a (+) vector (Novagen, Madison, Wisconsin, United States). Subcloning service was provided by Genscript.

The pET21-a vector was transformed into E. coli strain BL21(DE3)pLysS competent cells (Novagen). 1 μl of plasmid vector was added to 20 μl of competent cells and the mixture was incubated on ice for 5 min, followed by incubation at 42°C for 30s. The tube was then placed on ice and 80 μl of SOC medium was added. Transformants were plated on agar plate

containing 300 μg/ml carbenicillin (Novagen) and 34 μg/ml chloramphenicol (Sigma Aldrich, St. Louis, Missouri, United States). Successful transformants were verified by sequencing the plasmid DNA extracted from the colony using a pair of primers, 5' TAATACGACTCACTATAGGG 3' (F) and 5' GCTAGTTATTGCTCAGCGG 3' (R).

3.3.1.2 Production of wild-type and variant human SAA1 protein

Successful clones of wild-type and variant SAA1 were grown overnight by innoculating a single colony in 10 ml LB medium supplemented with 500 μg/ml carbenicillin and 34 μg/ml chloramphenicol at 37°C. Overnight culture was then transferred into a flask containing 300 ml of LB medium supplemented with the same concentration of carbenicillin and chloramphenicol. The culture was incubated at 37°C until the OD_{600} of the medium reached 0.6 upon which expression of the recombinant protein was induced by the addition of 1mM iso-propylthio-ß-D-galactoside (IPTG). After 30 min, 200 μg/ml rifampicin (Novagen) was added to inhibit *E.coli* RNA polymerase. The culture was incubated for 3 hr before the cells were collected by centrifugation at 1000 rpm. Cell pellet obtained was lysed using a lysis buffer containing 4.29 mM Na_2HPO4, 1.47 mM KH_2PO_4, 2.7mM KCl, 0.137M NaCl, 0.1% Tween-20, 0.002% sodium azide supplemented with protease inhibitor cocktail (Roche). The lysate was homogenated and 150U of benzonase nuclease (Novagen) was added and the resulting mixture was incubated for 30 min at 4°C.

3.3.1.3 Purification of recombinant SAA

Recombinant SAA was purified from the cell lysate using immunoaffinity purification. T7-tag antibody agarose resin (Novagen) was loaded onto a centrifuge column (Thermo Scientific, Rockford, Illinois, United States). Resin was equilibrated by washing with 4 ml of

lysis buffer; the process was repeated 4 times. Cell lysate was then loaded and the lysate was washed 5 times with 4 ml of lysis buffer. Binded protein was eluted using 0.1M citric acid and the eluent was immediately neutralized with 2M Tris base. The purity of the eluted protein was ascertained by SDS-PAGE followed by Coomasie Blue staining. In addition, the functionality of the protein was verified by determining its ability to induce cytokine release from THP-1 dervied macrophages as outlined in 3.3.3.

3.3.1.4 Endotoxin removal and detection

Endotoxin was removed from the recombinant SAA protein using an endotoxin removal kit (Norgen Biotek, Thorold, Ontario, Canada). Purified protein was loaded onto a spin column prior to the addition of a proprietary Endotoxin Removal solution. The mixture was incubated for 5 min and centrifuged to remove the endotoxin. Endotoxin-free protein was eluted using the elution buffer supplied and the eluted protein was neutralized immediately upon elution. Endotoxin level in the protein was detected using a single test gel clot limulus amebocyte lysate kit (Lonza, Allendale, New Jersey, United States); both wild-type and variant protein were verified to have an endotoxin level of less than 0.0625EU/μg of protein.

3.3.1.5 Concentration and quantification of protein

The protein was dialysed and concentrated using a concentrator unit (Millipore, Billerica, Massachusetts, United States) with a molecular weight cutoff of 10kDa. Concentrated protein was quantified using Nanodrop (Thermo Scientific) and diluted to 1mg/ml using endotoxin-free water (Lonza). The recombinant protein was stored at -80^{o}C until use.

3.3.2 Cell culture of macrophages and neutrophils

Human monocytic leukaemia cell line, THP-1, was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, United States). Cells were grown in RPMI medium supplemented with 10% FBS (Life Technologies), 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified atmosphere. Cells were seeded at 2.5 X 10^6 cells/ml in a 6-well plate in the presence of 0.1 μg/ml phorbol myristate acetate (PMA) for 7 days to induce differentiation into macrophages. Differentiation of monocytes into macrophages was confirmed through an observable morphological change.

Human promyleocytic leukaemia cell line, HL-60, was purchased from ATCC. Cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified atmosphere. Cells were seeded at 1.0 X10^6 cells/ml and incubated with 1.3% DMSO for 5 days to induce differentiation into neutrophils. Cellular viability was performed on the fifth day using Trypan blue and differentiation of HL-60 cells into neutrophils was confirmed by Wright-Giemsa staining. Differentiated cells have multi-lobular nucleus which are stained purple.

3.3.3 Measurement of cytokines release from macrophages and neutrophils

THP-1 induced macrophages were incubated for 1 hr at 37°C in 1ml serum-free RPMI medium supplemented with 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. Wild-type and variant SAA were added into separate wells at a concentration of either 3 μg or 15 μg per ml of medium. Cells were incubated for 24 hours in a 5% CO_2 incubator. The supernatants were assayed for IL-8, TNF-α and MCP-1 using

enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Norcross, Georgia, United States). All assays were carried out in triplicates and according the instruction of the vendor. The colour intensity was measured at 450 nm using an ELISA plate reader (BioRad, Hercules, California, United States).

HL-60 induced neutrophils were incubated for 1 hr at 37°C in 1 ml serum-free IMDM medium supplemented with 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. Wild-type and variant SAA were added into separate wells at a concentration of either 4 μg or 20 μg per ml of medium. Cells were incubated for 24 hours in a 5% CO_2 incubator. The supernatants were assayed for IL-8 and MCP-1 using ELISA kits (RayBiotech).

3.3.4 Neutral cholesteryl ester hydrolase (nCEH) activity assay

Cholesteryl oleate (Sigma Aldrich) was dissolved in a solution containing 1.7%(v/v) polyoxyethylene 9 lauryl ether (Sigma Aldrich) and 0.8% (w/v) NaCl. Sodium taurocholate, sodium cholate hydrate, 4-aminoantipyrine, cholesterol oxidase, horseradish peroxidase Type II and porcine nCEH were obtained from Sigma Aldrich. A 1 ml reaction mix containing 700 μl potassium phosphate buffer (400 mM, pH 7.0), 16.7 μl sodium taurocholate (15% w/v), 16.7 μl sodium cholate hydrate (15% w/v), 33.3 μl horse radish peroxidase type II (50 units/ml), 166.7 μl cholesteryl oleate (8.6 mM), 16.7 μl phenol (5% w/v) and 20 μg of recombinant SAA1 were added into a curvette. The solution was mixed and allowed to equilibrate to 37°C. 16.7 μl each of 4-aminoantipyrine (1.76% w/v), cholesterol oxidase (30 units/ml) and porcine nCEH (0.40 units/ml) were added to the curvette. The mixture was mixed and incubated at 37°C for either 15 or 45 mins and the colour intensity was measured at 500nm.

3.3.5 Data analysis

Measurements were expressed as mean of 3 independent experiments. The difference between wild-type SAA1 treatment and variant SAA1 was compared using Student's *t*-test and was deemed significant for *P*-value <0.05.

3.4 Microarray study

3.4.1 Cell culture

THP-1 was grown in RPMI medium supplemented with FBS in a 6-well plate and induced to differentiate into macrophages as described in section 3.3.2. After 7 days, the medium was replaced with serum-free RPMI medium and 1 μg/ml of recombinant wild-type or variant SAA1 was added into the appropriate well. The cells were incubated for either 8 h or 24 h. Upon incubation, the cells were removed by scraping and the cell pellet was collected upon centrifugation.

3.4.2 RNA isolation and cRNA synthesis

RNA was extracted from cell pellet using a RNA extraction kit (Qiagen). Prior to RNA amplification, the integrity of RNA was verified using formaldehyde gel electrophoresis. RNA amplification was carried out with Illumina TotalPrep RNA amplification kit (Life Technologies). The amplification procedure consists of first strand and second strand cDNA synthesis, following which the double-stranded cDNA was purified and used for cRNA synthesis using biotinylated nucleotides. The concentration and integrity of the cRNA was determined using Nanodrop (Thermo Scientific) and Bioanalyser (Agilent, Santa Clara, California, United States) respectively. cRNA was stored at -20^{o}C prior to hybridization onto microarray chip.

3.4.3 Array hybridization and scanning

The hybridation mixture consists of 750 μg of cRNA and hybridization buffer that is supplied with the kit. Hybridisation mixture was heated at 65°C for 5 min and loaded onto Illumina HT-12 microarray chip (San Diego, California, United States). Microarray chip was incubated for 16 hr in a hybridization oven. Upon hybridization, the chip was washed using the proprietary buffer supplied and stained with Streptavidin Cy3. The stained chip was dried by centrifugation and the chip was read using a BeadArray reader.

3.4.4 Quantitative real-time PCR validation of microarray results

Real-time PCR was conducted to validate differential RNA concentration between wild-type SAA1, variant SAA1 and untreated control as identified through microarray analysis. The primers for real-time PCR were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). The cDNA sequences used for primer design are available from genome browser (http://genome.ucsc.edu/). The melting temperature for all primers was set as 60°C for easy optimization and running of the assay. The sequences of the primers are as shown in Table 3-2.

cDNA was synthesised from total RNA using a first strand cDNA synthesis kit (Fermentas) and quantified using Nanodrop. Real-time PCR was carried out using Roche light cycler 480. The reaction mixture consists of reaction master mix, 0.2 μM of forward and reverse primer and 30ng of cDNA in a 10 μl reaction mix. Amplification conditions consist of an initial denaturation at 95°C for 10 min, 45 cycles of 10s of denaturation at 95°C, 30s of annealing at 60 °C and 8s of extension at 72°C. The threshold cycle (C_T) value was determined using the accompanied software. The relative changes in gene expression between wild type SAA1,

variant SAA1 and untreated were determined using a software for gene expression analysis, REST 2009 (Qiagen).

Table 3-2. Primer sequences for real-time PCR.

Comparison in expression	Gene	Forward Primer / Reverse Primer
Wild-type vs untreated (8 h)	Baculoviral IAP repeat-containing protein 3 (*BIRC3*)	5' ATGCTTTGCTGTGATGGTG 3' 5' CGGATGAACTCCTGTCCTTT 3'
	Chemokine (C-C motif) ligand 3 (*CCL3*)	5' GGCTCTCTACAACCAGTTCT 3' 5' CTGGCTGCTCGTCTCAAAG 3'
	Chemokine (C-C motif) ligand 4-like 1 (*CCL4L1*)	5' GCTGCCTTCTGCTCTCTAGC 3' 5' ACCACAAAGTTGCGAGGAAG 3'
	Interleukin 23A (*IL23A*)	5' ACAGAAGCTCTGCACACTGG 3' 5' CCACACTGGATATGGGGAAC 3'
	Integrin, alpha 1 (*ITGA1*)	5' GTCCAGTTGGGAGAGGTGAA 3' 5' CTCCATTTGGGTTGGTGACT 3'
	Myristoylated alanine-rich C-kinase substrate (*MARCKS*)	5' ATGAGTGCCCAGTTCTCC 3' 5' AGTGTCGCCGTTTACCTT 3'
Wild-type vs untreated (8 h)	Oxidised low density lipoprotein receptor 1 (*OLR1*)	5' AAGGACCAGCCTGATGAGAA 3' 5' AGGCAAAGGACCCCTAGAGT 3'

Comparison in expression	Gene	Forward Primer / Reverse Primer
Wild-type vs untreated (8 h)	Plasminogen activator inhibitor 2 precursor (*SERPINB2*)	5' CATTCATCCTTCCGCTCTCT 3' 5' CTACTGCCTGGGGTTCTGAG 3'
Wild-type vs variant (8 h)	Bone morphogenetic protein receptor type II (*BMPR2*)	5' TCATTGCTTTGGCATCAGTC 3' 5' ATCAAGAGAGGGTTCGGATG 3'
	Prolyl 4-hydroxylase (*P4HA1*)	5' AAGACAGAAGAGGACAAGTTAGAA 3' 5' GCATTTACTGGATGCCCAAC 3'
Wild-type vs variant (24 h)	Acyl-coenzyme A thioesterase 8 (*ACOT8*)	5' TGTGAGACCCTCATTGACCA 3' 5' GGATGGGTTTACTGGCTTGA 3'
	CD63	5' TTGCTCTACGTCCTCCTGCT 3' 5' GTAGCCCCCTGGATTATGGT 3'
	Carboxylesterase 1 (*CES1*)	5' CACTCCTGCTGACTTGACCA 3' 5' CAGATGCCCAGGCGATATT 3'
	Integrin beta-1 binding protein 1 (*ITGB1BP1*)	5' CAGATGCAAGCAATGAGGAA 3' 5' CAGAGTCAAAAGCGGTGGAT 3'
Wild-type vs variant (24 h)	Lysyl oxidase homolog 3 (*LOXL3*)	5' AGCGAAAAGAGGGTCAACG 3' 5' AGGGAACAGAGGGAGAGGTG 3'
	Myosin 1f (*MYO1F*)	5' GTGCTTCTTCCCCAGATCAC 3' 5' GTAGGGCATCTGCTTGAAGG 3'

Comparison in expression	Gene	Forward Primer / Reverse Primer
Wild-type vs variant (24 h)	5'-AMP-activated protein kinase subunit gamma-1 (*PRKAG1*)	5' CACTACCACCCCCGTCTATG 3' 5' GGTCTTTTCTGCTGCCAGAT 3'
	Sterol regulatory element-binding protein 1 (*SREBF1*)	5' AGCTCAAGGATCTGGTGGTG 3' 5' GTTTCTGGTTGCTGTGTTGC 3'
Internal Control	Beta-2 microglobulin (*B2M*)	5' GTGCTCACGCTACTCTCTCT 3' 5' TCAATGTCGGATGGATGAAA 3'
	LIM and SH3 domain protein 1 (*LASP1*)	5' CCACGGAGAAGGTGAACTGT 3' 5' GTGAAGGACTGCTTGGGGTA 3'
	FXYD domain-containing io*n transport regulator 5 (FXYD5*)	5' CCTGTGTCTTCTCACCATCG 3' 5' GGAACCTGAATGTCCATGAT 3'

3.4.5 Data analysis

The quality of the microarray data was determined using Genome Studio (Illumina) while normalization and analysis were performed using Beadstudio and R/Bioconductor. For each treatment group, the average of the two readings was determined and fold change was determined between SAA1 and untreated at both 8 h and 24 h. For each treatment group, the average of the two readings was determined and fold change was determined at 8 h and 24 h. Genes with a differential expression of $\geq$1.5 fold (wild-type vs variant) or $\geq$ 2.0 fold

(wild-type vs untreated) were analysed using gene annotation software, GOEAST (http://omicslab.genetics.ac.cn/GOEAST/).

To find enriched pathways at 8 h, the upregulated genes were mapped onto PathwayAPI (Soh et al. 2010) which comprises of information extracted from KEGG (Kanehisa 2009), WikiPathways (Kelder et al. 2009; Pico et al. 2008) and IPA(Jimenez-Marin et al. 2009). The online repository is available at http://pathwayapi.com/. There are 4268 nodes and 35307 nodes, corresponding to 544 pathways. Statistical significance for pathway enriched was determined using hypergeometric method ($P < 0.05$).

For real-time PCR, statistical difference between two different treatment groups was determined using randomization and bootstrapping technique that is incorporated into the REST 2009 software (Qiagen). Readings from 3 independent experiments were used for the statistical analysis. Statistical difference between treatment and control in the quantification of the levels of chemokines was compared using Student's *t*-test ($P < 0.05$).

3.5 Elucidation of surface receptors of SAA1

3.5.1 Cell culture

RAW264 mouse macrophages (ATCC) were grown in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. 1.0 X 10^5 cells were seeded in a 6-well plate one day prior to the assay and on the day of assay the medium was replaced with serum free DMEM medium. 1 µg of recombinant human SAA1 and either 10 µg of anti-mouse TLR2 (eBiosience, San Diego, California, United States) antibody or 5 µl of anti-mouse SR-BI antibody (Novus Biologicals, Littleton, Colorado, United States) were added into the appropriate wells. The culture was incubated in a 5% CO_2 incubator at 37°C for 8 h

and the supernatant was collected. The cytokine levels in the supernatants were determined using ELISA as outlined in section 3.3.3.

3.5.2 Data Analysis

Results were obtained from the mean of 3 independent experiments. The difference between antibody treatment and untreated was compared using Student's *t*-test and was deemed significant for *P*-value <0.05. The *P*-value cut-off for significance when multiple comparisons were involved was adjusted by the Bonferroni correction.

4 *SAA1* SNPs SURVEY

4.1 Introduction

Chronic inflammation plays an important role in the pathogenesis of atherosclerosis. Given its immune regulatory role in protecting the body from infection and the inflammatory nature of atherosclerosis, A-SAA has increasingly been associated with atherosclerosis. A-SAA consists of SAA-1 and SAA2 of which SAA1 was reported to be the predominant form of A-SAA present in plasma and the precursor of fibrillar deposits in reactive amyloidosis (Yamada et al. 1999). The potential role A-SAA might play in atherosclerosis suggests that certain polymorphisms of *SAA1* might be associated with coronary artery disease (CAD). However, to date, no genetic variant screening has been conducted on *SAA1*.

Prior to the SNPs survey conducted in 2008, only 2 non-synonymous SNPs of *SAA1*, p.Ala70Val (rs1136743) and p.Ala75Val (rs1136747), were reported in publication. SNP survey was carried out in the exon and promoter region of *SAA1* as polymorphisms in these regions should have a more direct impact on the function and plasma concentration of SAA1. Variant screening was performed using cord blod DNA from 96 annoymous, unrelated Singaporean Chinese neonates delivered in the National University Hospital, Singapore. The method of HRM was used for the variant screening. The result is supplemented with data obtained from dbSNP and a SNP discovery program SNPFinder.

The SNPfinder program, (http://lpgws.nci.nih.gov/perl/snpbr), (Beutow et al, 1999) was used for SNP discovery using information deposited in UniGene. Unlike other programs, which mine SNP by alignment of deposited text-based sequences (Cox et al, 2001), SNPfinder analyses the DNA electrophoretogram of the accompanied sequence to ensure that allelic variant identified does not arise due to a sequencing error.

4.2 Results

4.2.1 SNPfinder analysis of deposited Unigene Expressed Sequence Tags (ESTs)

In silico discovery was performed in 2007 using SNPfinder. The program uses deposited genetic information from Unigene HS.632144 which consists of 11 mRNA sequences and 332 non-redundant EST sequences. ESTs were obtained from various human tissues including kidney, mammary gland, prostate, lung, liver and adipose. The list of SNPs as predicted by SNPfinder after analyzing deposited DNA electrophoretograms, together with the corresponding Blosum62 score is as shown in Table 4-1. A positive Blosum62 score is indicative of conservative amino acid substitution. In total, 18 non-synonymous SNPs were predicted by SNPFinder. The multiple sequence alignment of SAA1 from various species is as shown in Figure 4-1. The alignment of the sequences from ESTs and the subsequent prediction of the SNPs, p.Met42Thr, are shown in Figure 4-2. Due to its algorithms and its source data, SNPFinder does not predict SNP present in the promoter region.

Table 4-1. List of predicted *SAA1* SNPs by SNPFINDER.

SNP Ref	CDS SNP	Blosum 62 Score	Conserved in multiple organisms*	Region
rs11545475	p.Leu12Ser	-2	Yes	Exon 1
-	p.Leu14Pro	-3	Yes	Exon 1
-	p.Met42Thr	-1	Yes	Exon 2
-	p.Glu44Gly	-2	Yes	Exon 2
-	p.Tyr47His	2	No	Exon 2
-	p.Tyr53His	2	Yes	Exon 2
-	p.Tyr53Cys	-2	Yes	Exon 2
rs1136743	p.Ala70Val	0	No	Exon 2

SNP Ref	CDS SNP	Blosum 62 Score	Conserved in multiple organisms*	Region
rs11545468	p.Trp71Arg	-3	Yes	Exon 2
rs1136747	p.Ala75Val	0	No	Exon 2
-	p.Asp78Asn	1	Yes	Exon 3
-	p.Ile83Thr	-1	No	Exon 3
-	p.Gln84Pro	-1	Yes	Exon 3
rs1059559	p.Phe86Leu	0	No	Exon 3
-	p.Asp93Gly	-1	No	Exon 3
-	p.Ala96Val	0	Yes	Exon 3
rs17850140	p.Asn101Asp	1	Yes	Exon 3
-	p.Pro119Ser	-1	Yes	Exon 3

*Organisms include cheetah, hamster, monkey, mouse and rabbit.

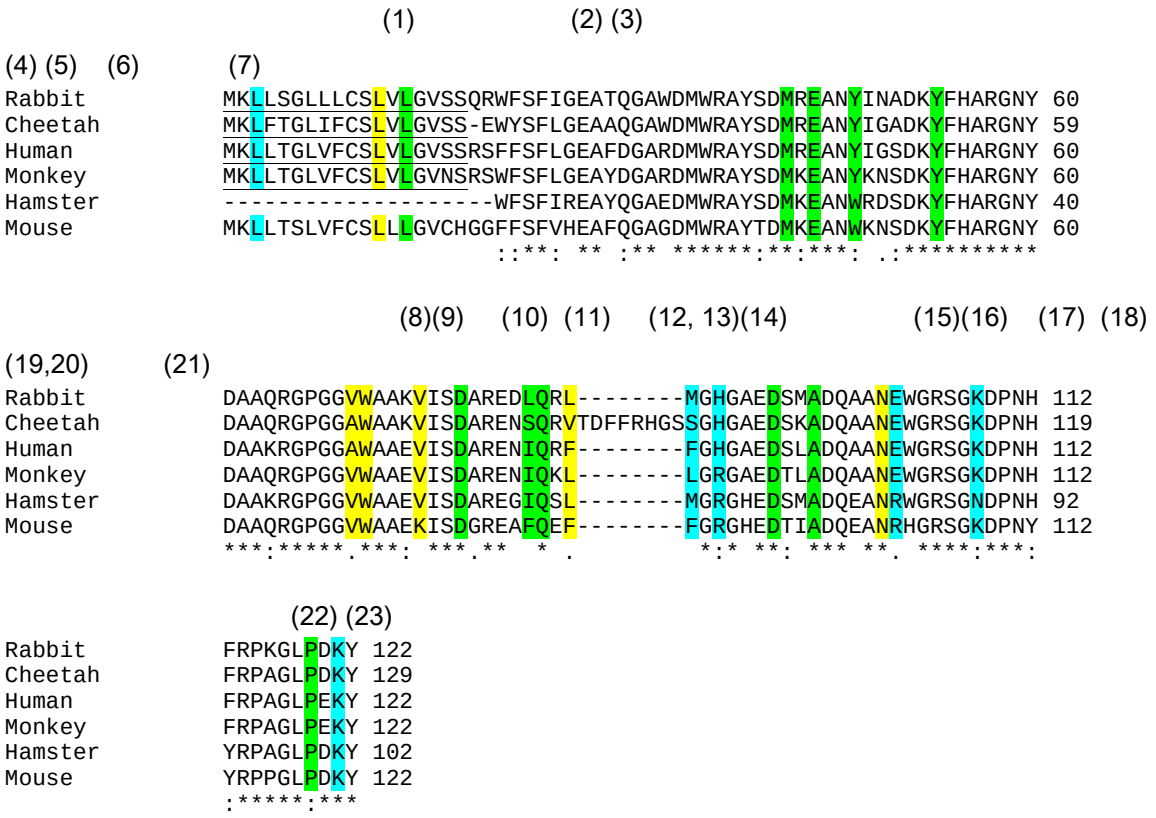


Figure 4-1. Multiple sequence alignment of SAA1 in Cheetah (BAG06986), Hamster (ABB27187), Human (CAG47037), Monkey (XP_001086137), Mouse (NP_033143) and Rabbit (AAB20616). The underlined sequence represents the signal peptide which will be cleaved to produce the mature protein. Highlighted in blue is the non-synonymous SNP identified by dbSNP which includes p.Leu3Pro (1), p.Phe87Ser(15), p.His89Arg (16), p.Glu102Lys (20), p.Lys108Arg (21) and p.Lys121Arg (23). The position highlighted in green are SNPs identified by SNPfinder which includes p.Leu14Pro (3), p.Met42Thr (4), p.Glu44Gly (5), p.Tyr47Cys (6), p.Tyr53His, p.Tyr53Cys (7), p.Asp78Asn (11), p.Ile83Thr (12), p.Gln84Pro (13), p.Asp93Gly (17), p.Ala96Val (18) and p.Pro119Ser (22). SNPs that are predicted by SNPfinder and listed in dbSNP are in yellow: p.Leu12Ser (2), p.Ala70Val (8), p.Trp71Arg (9), p.Ala75Val (10), p.Phe86Leu (14) and p.Asn101Asp (19).

Figure 4-2. Pictorial representation of a SNPfinder result. SNPfinder result showing the p.Met42Thr SNP using EST data deposited in Unigene. The highlighted blue line plus the adjacent bases encodes the codon for methionine (ATG) at the 42th position. A substitution of cytosine for thymine results in an amino acid change to threonine. The base substitution exists as a singleton.

4.2.2 SNPs survey using deposited data in dbSNP

A manual search of dbSNP in 2007 for polymorphisms in *SAA1* returns a total of 15 SNPs of which 12 are non-synonymous SNPs in the exon region and 3 are found in the promoter region (Table 4-2). SNPs in the intronic region and synonymous SNPs are not taken into consideration for the SNPs survey. Of the 3 SNPs in the promoter region, only 1 of them, c.-685C>G (rs1829575), is validated by frequency data as well as data published in journal (Moriguchi et al. 2005). Of the 12 non-synonymous SNPs identified, only 6 of the SNPs are validated by available frequency data, these include p.Ala70Val, p.Ala75Val, p.Phe86Leu, p.His89Arg, p.Glu102Lys, p.Lys108Arg.

To obtain a more meaningful compilation of the results from the *in silico* studies, the non-synonymous and non-conservative SNPs (with negative Blosum62 score) are consolidated together (Table 4-3). A total of 12 non-synonymous and non-conservative SNPs are obtained through the combined results of dbSNP and SNPFinder. None of the 12 is validated by frequency data in the dbSNP database, and hence a comparison with the actual variant screening of *SAA1* as documented in 4.2.3 will reflect the strength of the prediction.

Table 4-2. List of *SAA1* SNPs obtained from a manual search of dbSNP. For the SNPs in the coding region, only p.Ala70Val, p.Ala75Val, p.Phe86Leu, p.His89Arg, p.Glu102Lys and p.Lys108Arg have accompanied population data.

SNP Ref	CDS SNP/Promoter SNP	Blosum 62 Score	Conserved in multiple organisms	Region	Found in SNPfinder
rs7103956	c.-955C>T	-	-	Promoter	-
rs1829575	c.-685C>G	-	-	Promoter	-
rs7103441	c.-667G>C	-	-	Promoter	-
rs11545470	p.Leu3Pro	-3	Yes	Exon 1	No
rs11545475	p.Leu12Ser	-2	Yes	Exon 1	Yes
rs1136743	p.Ala70Val	0	No	Exon 2	Yes
rs11545468	p.Trp71Arg	-3	Yes	Exon 2	Yes
rs1136747	p.Ala75Val	0	No	Exon 2	Yes
rs1059559	p.Phe86Leu	0	No	Exon 3	Yes
rs1059560	p.Phe87Ser	-2	No	Exon 3	No
rs2229338	p.His89Arg	0	No	Exon 3	No
rs17850140	p.Asn101Asp	1	Yes	Exon 3	Yes
rs1059567	p.Glu102Lys	1	No	Exon 3	No
rs1059571	p.Lys108Arg	2	Yes	Exon 3	No
rs61745680	p.Lys121Arg	2	Yes	Exon 3	No

Table 4-3. Non-synonymous and non-conservative amino acid changes predicted by SNPfinder and dbSNP.

SNP Ref	CDS SNP	Blosum 62	Predictor/Database	Region
rs11545470	p.Leu3Pro	-3	dbSNP	Exon 1
rs11545475	p.Leu12Ser	-2	dbSNP/SNPFinder	Exon 1
-	p.Leu14Pro	-3	SNPFinder	Exon 1
-	p.Met42Thr	-1	SNPfinder	Exon 2
-	p.Glu44Gly	-2	SNPfinder	Exon 2
-	p.Tyr53Cys	-2	SNPfinder	Exon 2
rs11545468	p.Trp71Arg	-3	SNPfinder/dbSNP	Exon 2
-	p.Ile83Thr	-1	SNPFinder	Exon 3
-	p.Gln84Pro	-1	SNPFinder	Exon 3
rs1059560	p.Phe87Ser	-2	dbSNP	Exon 3
-	p.Asp93Gly	-1	SNPFinder	Exon 3
-	p.Pro119Ser	-1	SNPFinder	Exon 3

4.2.3 Variant screening of promoter and exons of *SAA1*

The promoter region and the 3 exons of *SAA1* were screened for novel variants using HRM. As the amplicon containing exon 2 has multiple melting domains, DNA sequencing was used to screen exon 2. A novel variant, c.92-5T>G, was found in the intron-exon boundary of exon 2. Two variants, p.Ala70Val and p.Ala75Val, were identified in exon 2 and a synonymous SNP, c.279C>T was identified in exon 3. c.-685C>G and c.-913G>A, were identified in the promoter region of *SAA1* of which the latter is novel (Table 4-4).

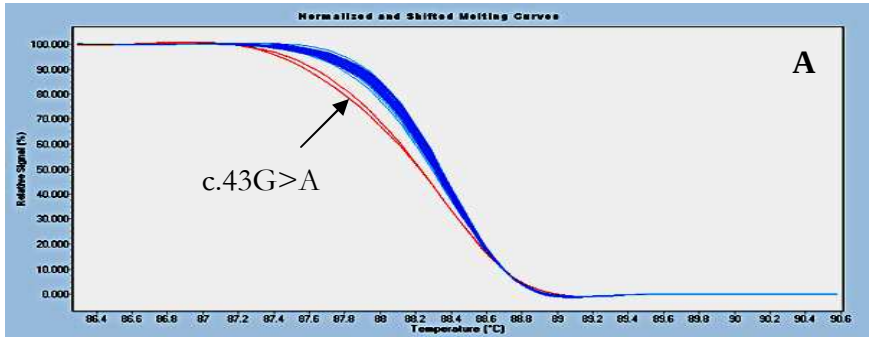
The temperature shifted curves and the difference plots for each of the 6 regions together with the corresponding DNA electrophoretograms are as shown in Figure 4-3 and Figure 4-

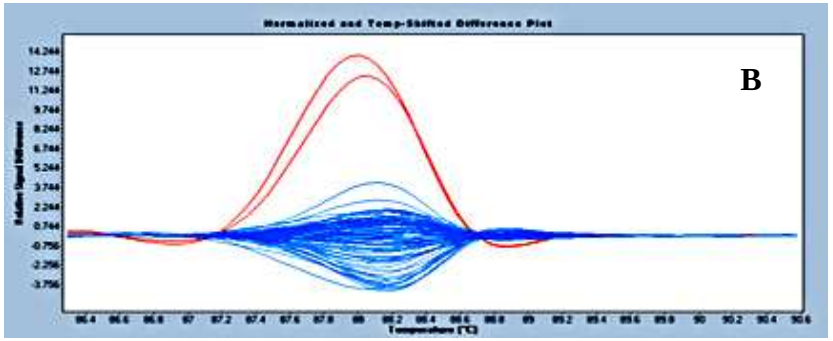
4. Although, c.43G>A was initially identified as a genetic variant, it was found to be a false positive due to the heterogenous amplification of *SAA1* and *SAA2*. Genetic variants were determined by the accompanied software which analyses changes in fluorescence levels from the melting curve. The various settings for the analysis of the melting curve are deemed to be appropriate if the temperature shifted melting curve and the difference plot correspond to each other.

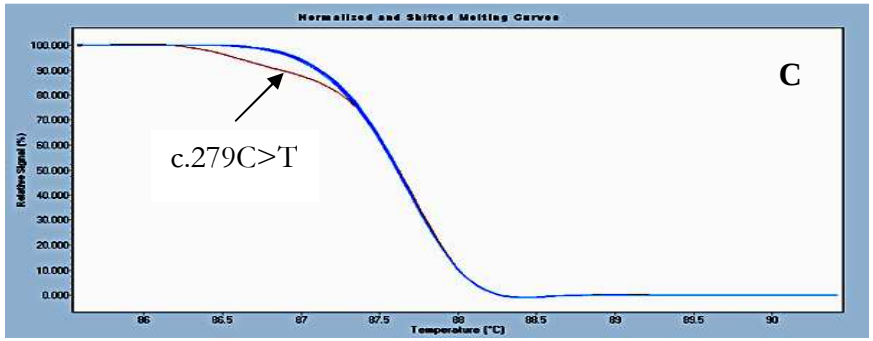
In summary, a novel SNP is discovered in the promoter region and there is no novel non-synonymous SNP identified.

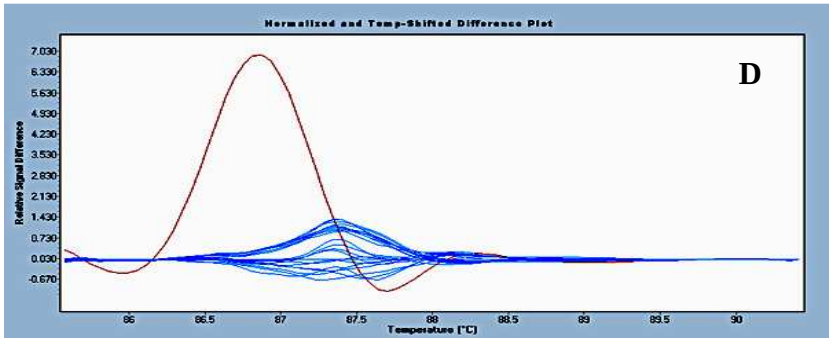
Table 4-4. Polymorphisms identified in the exons and promoter of *SAA1*. *c.92-5T>G, c.209C>T and c.224C>T were identified from the sequencing of exon 2; the rest of the genetic variants were identified using HRM.

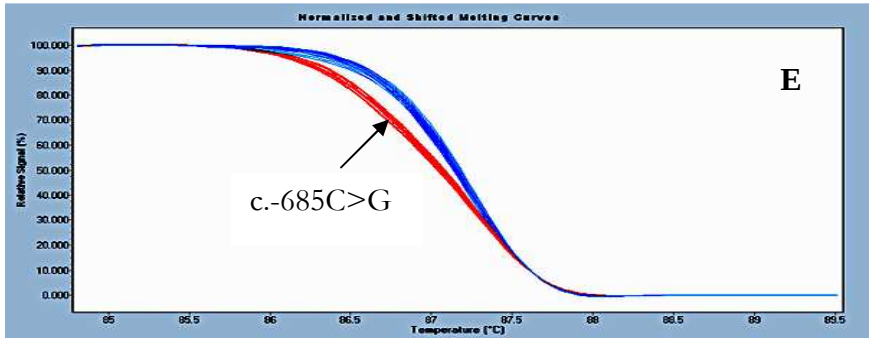
SNP	Position	SNP Ref	Novel variant
c.-913G>A	Promoter	-	Novel
c.-685C>G	Promoter	rs1829575	-
c.92-5T>G*	Intron	-	Novel
p.Ala70Val* (c.209C>T)	Exon	rs1136743	-
p.Ala75Val* (c.224C>T)	Exon	rs1136747	-
c.279C>T	Exon	rs15790	-

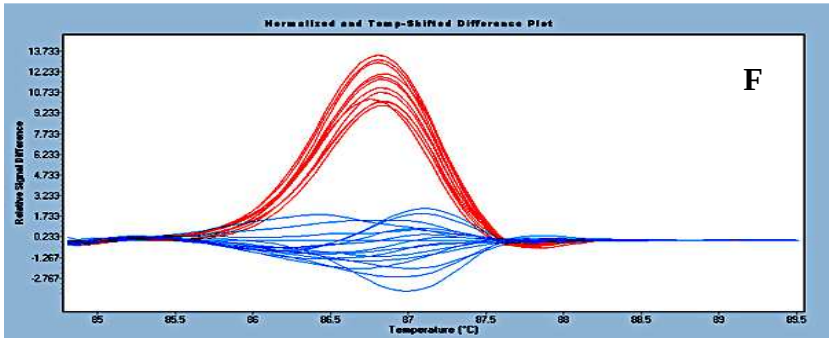

Normalized and Shifted Melting Curves
A
c.43G>A
Relative Signal (%)
Temperature (°C)


Normalized and Temp-Shifted Difference Plot
B
Temperature (°C)


Normalized and Shifted Melting Curves
C
c.279C>T
Relative Signal (%)
Temperature (°C)


Normalized and Temp-Shifted Difference Plot
D
Relative Signal Difference
Temperature (°C)


Normalized and Shifted Melting Curves
E
c.-685C>G
Relative Signal (%)
Temperature (°C)


Normalized and Temp-Shifted Difference Plot
F
Relative Signal Difference
Temperature (°C)

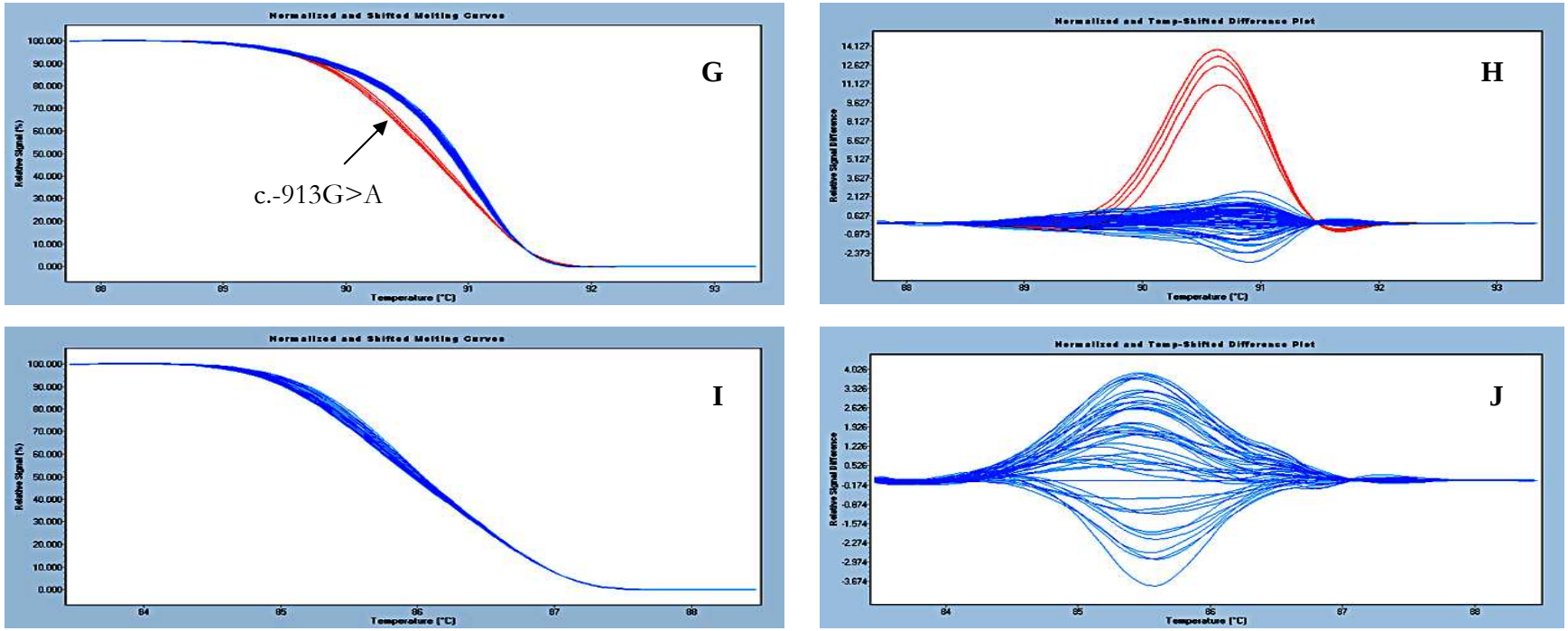
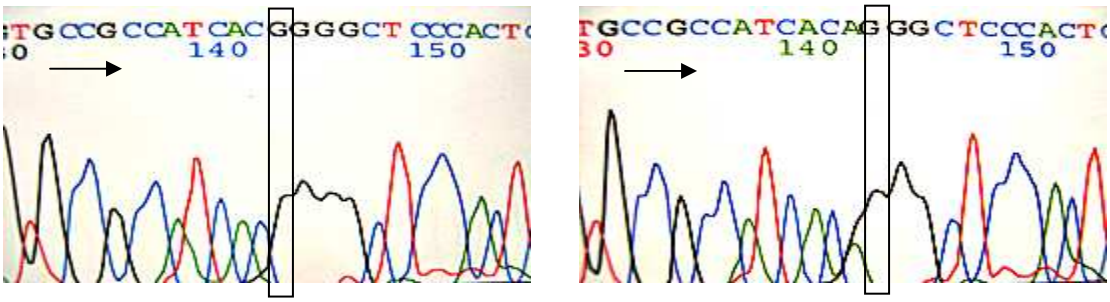
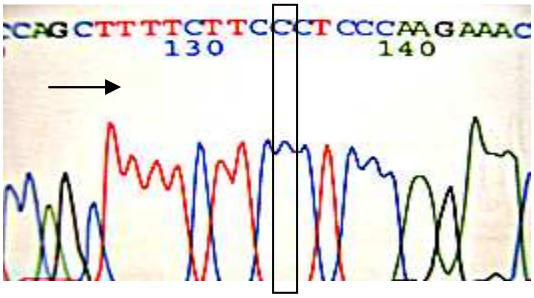


Figure 4-3. Normalised high resolution melting curves and the corresponding difference plots. The variants are differentiated from the wild-type by the relative difference in their normalized fluorescence. The wild-types are all coloured blue while variants are in various colours. (A-B) Melting curves and difference plots of Exon 1. (C-D) Melting curves and difference plots of Exon 3. (E-F) Melting curves and difference plots of promoter segment 1 (c.-857 to c.-637). (G-H) Melting curves and difference plots of promoter segment 2 (c.-1088 to c.-809). (I-J) Melting curves and difference plots of promoter segment 3 (c.-1305 to c.-1029).

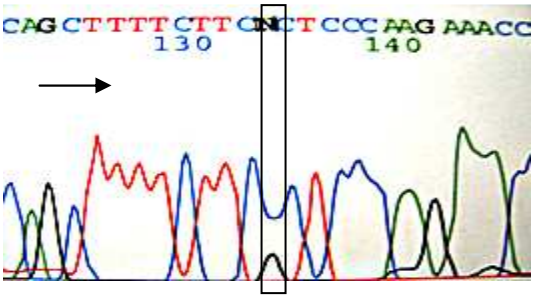


(A)

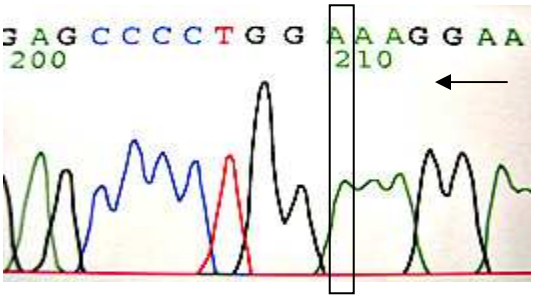
(B)



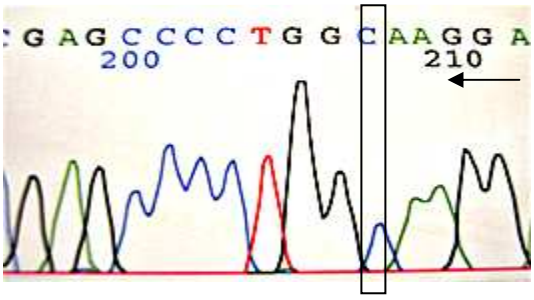
(C)



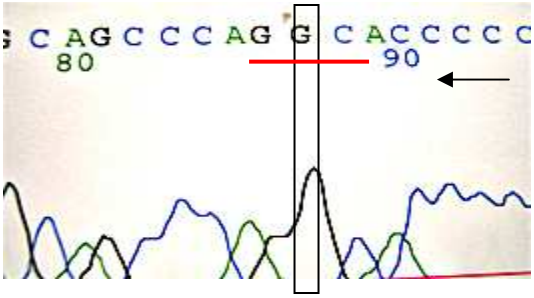
(D)



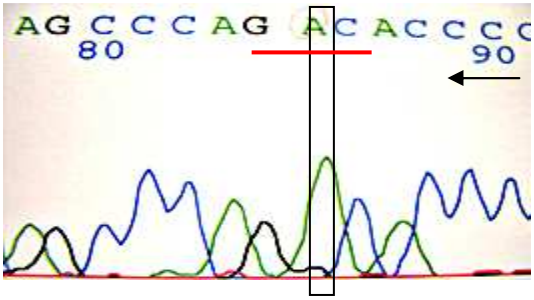
(E)



(F)



(G)



(H)

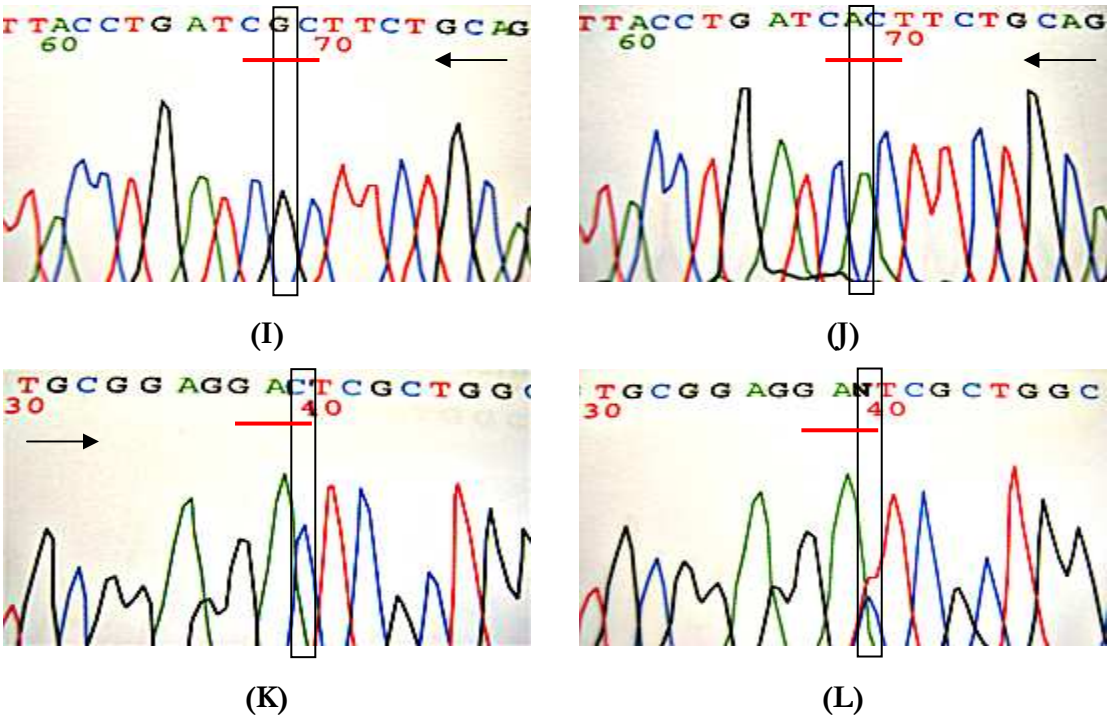


Figure 4-4. Electrophoretograms of the various identified SNPs. The arrow indicates the orientation of the DNA sequence in the 5' to 3' direction (→: coding strand; ←: reverse complementary strand). All SNPs are named according to the location of the nucleotide substitution in the coding strand. (A-B): Wild-type G allele and variant A allele of c.-913G>A.. (C-D): Wild type C allele and variant G allele (present in heterozygote) of c.-685C>G. (E-F): Wild-type T and variant G allele of c.92-5T>G. (G-H): Wild-type C allele and variant T allele of p.Ala70Val. GCC encodes for alanine while GTC encodes for valine in the coding strand. (I-J): Wild type T allele and variant C allele of p.Ala75Val. GTG encodes for valine while GCG encodes for alanine in the coding strand. (K-L): Wild type C allele and variant T allele (present in heterozygote) of D93D, both GAC and GAT encodes for aspartic acid.

4.3 Discussion

4.3.1 SNPs survey using *in silico* SNPFINDER and dbSNP

SNPFINDER and dbSNP offer a convenient way to retrieve and predict SNPs on the gene of interest. However, the information provided by these data-mining tools need to be carefully analysed as false-positives can arise as some of the underlying data are not validated. For the current study, there are differences between the results from SNPFINDER and dbSNP as compared to the variant screening performed using HRM. In total, there are 23 non-synonymous SNPs in the coding region predicted by SNPFINDER and dbSNP, however, only 2 non-synonymous SNPs were found through the variant screening by HRM. In comparing the results from the various programs and the actual variant screening, only non-synonymous SNPs were taken into consideration.

The results obtained from dbSNP were analysed and only 6 of the non-synonymous SNPs reported by dbSNP were found to be validated by population data. Genotyping was thus performed on one of the SNP, pHis89Arg, using 200 cord blood DNA samples to validate the data. Based on dbSNP, p.His89Arg (rs2229338) has a genotype frequency of 0.138 in a mixed population of sample size of 58. There is, however, no variant allele of rs2229338 found in our cord blood DNA samples. Instead, a rare allele adjacent to rs2229338, p.Gly90Asp, was identified. There is a 90% sequence similarity between SAA1 and SAA2 and out of the 6 validated variants identified by dbSNP, 4 of them, p.Phe86Leu, p.His89Arg, p.Glu102Lys and p.Lys108Arg, are the corresponding wild-type alleles for *SAA2* (Figure 4-5). As such, it is speculated that the variant alleles identified on dbSNP could be due to a heterogenous sequencing of both *SAA1* and *SAA2*. To validate our speculation, 8 DNA samples were amplified to yield amplicons containing either *SAA1* or *SAA2*. The amplicons were genotyped for p.Phe86Leu; the allele encoding for phenylalanine was found in the

amplicon containing *SAA1* and the allele encoding for leucine was found in the amplicon containing *SAA2*. Thus, upon excluding the four non-synonymous SNPs, the number of validated non-synonymous SNPs is agreeable with that obtained from the variant screening.

```
SAA2_AAA64800.1 MKLLTGLVFCSLVLSVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNY 60
SAA2_AAA64801.1 MKLLTGLVFCSLVLSVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNY 60
SAA1_CAG47037.1 MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNY 60
SAA1_AAA64799.1 MKLLTGLVFCSLVLGVSSRSFFSFLGEAFDGARDMWRAYSDMREANYIGSDKYFHARGNY 60
                **************.******************************************

SAA2_AAA64800.1 DAAKRGPGGAWAAEVISNARENIQRLTGHGAEDSLADQAANKWGRSGRDPNHFRPAGLPE 120
SAA2_AAA64801.1 DAAKRGPGGAWAAEVISNARENIQRLTGRGAEDSLADQAANKWGRSGRDPNHFRPAGLPE 120
SAA1_CAG47037.1 DAAKRGPGGAWAAEVISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPNHFRPAGLPE 120
SAA1_AAA64799.1 DAAKRGPGGVWAAEAISDARENIQRFFGHGAEDSLADQAANEWGRSGKDPNHFRPAGLPE 120
                *********.****.**:*******: *:************:*****:************

SAA2_AAA64800.1 KY 122
SAA2_AAA64801.1 KY 122
SAA1_CAG47037.1 KY 122
SAA1_AAA64799.1 KY 122
                **
```

Figure 4-5. Multiple sequence alignment of the primary sequence of SAA1 and SAA2. Amino acids that differ between SAA1 and SAA2 are highlighted in green.

Majority of the SNPs predicted by SNPFINDER are novel SNPs that have not been deposited in dbSNP. Over half of the non-synonymous SNPs are non-conservative SNPs with a negative Blosum62 score. In total, 8 non-synonymous SNPs were predicted to be found in exon 2, however, when sequencing were performed on 96 DNA samples, only 2 non-synonymous SNPs were identified. Thus, the results derived from SNPFINDER either consists of a large number of false positives or that most of the polymorphisms predicted are rare mutants. As the results from SNPFINDER are based on EST data, there are inherent errors associated with it. ESTs obtained from tumour tissue can contribute to an increase in false discovery rate. The large number of non-synonymous SNPs predicted by SNPFINDER and the close proximity of some of the SNPs (p.Met42Thr, p.Glu44Gly,

p.Tyr47His, p.Ile83Thr, p.Gln84Pro, p.Phe86Leu) suggest the the likely occurence of false positives. Deficiency of EST mining program and its associated false discovery rate in predicting SNP were also reported in a few studies (Buetow et al. 1999; Cox et al. 2001).

SNP data from large scale genome projects that were subsequently archieved in dbSNP are more reliable than SNP predicted from mining program. The high level of identity between *SAA1* and *SAA2* results in heterogenous sequencing of both genes of A-SAA*;* heterogenous sequencing could likely possibly account for the differential number of SNPs observed between dbSNP and the variant screening conducted.

4.3.2 SNPs survey by the method of HRM

HRM has been increasingly used for genetic variant screening (Dagar et al. 2009). For the screening of a single gene, the direct method of gene sequencing is too expensive and time consuming. In addition, much time is needed to analyse the electropherograms. One significant advantage of HRM over both DNA sequencing and DHPLC is its ease of setup. While in DHPLC, the sample is analysed one at a time, in HRM the entire plate of amplified products is analysed simultaneously within a short interval of time. However, certain modifications were made to the experimental protocol to ensure that the results are accurate and reproducible. As the Roche's HRM master mix that is optimized for use with the Roche 480 appears to interfere with DNA amplification, an alternative double stranded binding dye, LC green, was used. Amplification was also carried out in a separate thermocycler as amplification is inefficient in the Roche 480. Lastly, LCgreen was added prior to PCR as the results were inaccurate when the dye was added post-amplification. Variant screening by HRM was successful after the above modifications were made.

Although, HRM might not be able to differentiate between wild-type and variant homozygote for certain genetic variants, the method is able to consistently differentiate heterozygote from the homozygote. As the frequency of heterozygote variant is generally higher than homozygote variant, HRM can be used for variant screening without the use of additional probes. Pilot study was conducted on known variants and a sensitivity setting of 0.15 for the analysis of the melting curves was found to be sufficient for the detection of genetic variants with minimal false positives. HRM was used for the variant screening of all the promoters and exons of *SAA1* except for exon 2. As exon 2 has two melting domains, the melting curves are no longer smooth and as such it is not optimal for analysis by HRM (Figure 4-6). The presence of multiple melting domains might interfere with the analysis of the melting curves and result in inaccurate analysis. The inability to screen amplicons that contain multiple melting domains is the main drawback of HRM. However, its reproducibility and ability to differentiate genetic variants efficiently should render it a suitable method for high throughput genetic variant screening.

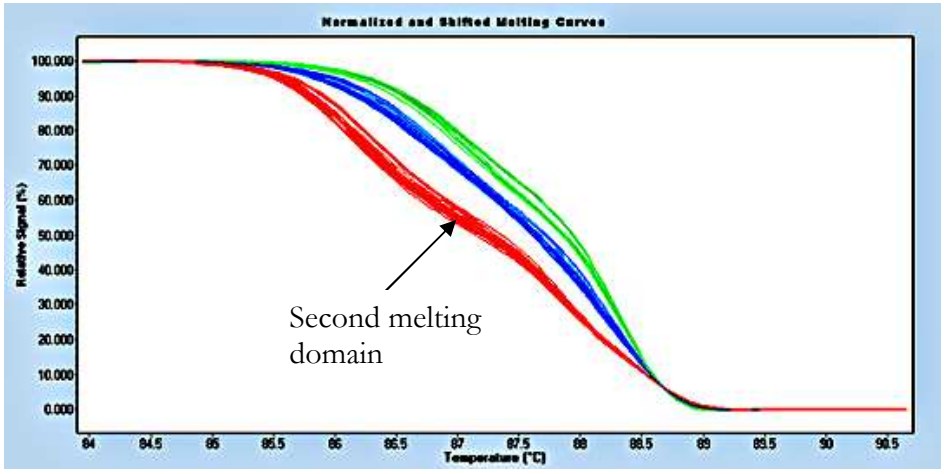


Figure 4-6. Multiple melting domains disrupt an otherwise smooth melting curve. As a result, the analysis is inaccurate. Melting curve analysis is thus unsuitable under such condition.

4.3.3 Significance of variant screening of *SAA1*

SAA1 is one of the major APPs produced during an APR and as such its functional roles are probably critical for survival. The variant screening of *SAA1* reflects its evolutionary conserved status as few genetic variants were discovered. The variant, c.-913G>A, does not lie in known transcription factor binding sites and as such it might not exert significant effects on SAA1 production. p.Ala70Val and p.Ala75Val are the only two non-synonymous SNPs present in *SAA1*; however, alanine and valine are structurally similar and thus the Blosum62 score is 0. The high prevalence of both genetic variants and the nature of the substitution suggest that the functional effects exerted by both variants might be similar to the wild-type. However, it will be interesting to investigate the differential functional effects of a rare mutant, p.Gly90Asp, against wild-type SAA1. p.Gly90Asp is the only genetic variant identified in which the amino acid substitution is non-conservative. The genetic association study conducted on these variants as documented in the following section will help determine whether these variants have altered structure that can alter the susceptibility to CAD.

4.3.4 Caveats of SNP survey

Genetic variant screening was conducted using cord blood DNA from Chinese neonates. Other races were excluded from the study as limited DNA samples are available for the Malays and the Indians. The sample size for the variant screening is only 96 which allows for the detection of SNP with allele frequency of $\geq$ 1%. The variant screening shows that there are only a small number of non-synonymous SNPs; as SAA1 plays a central role in the immune system, it is possible that non-synonymous SNPs might be deleterious and hence its frequency of occurrence is low. As it is difficult to set a limit on the sample size to detect

rare mutant, the design of the variant screening did not allow for the discovery of rare mutant. Nonetheless, through the validation of dbSNP, a rare mutant, p.Gly90Asp was discovered.

5 Association study of *SAA1* SNPs in Singapore Chinese population

5.1 Introduction

As the study of the role SAA1 plays in the pathogenesis of atherosclerosis only gathers pace in the past few years, there has been no prior genetic association studies of genetic variants of *SAA1* with CAD conducted. Genetic epidemiology study is also limited by the number of known genetic variants of *SAA1*. There are, however, two recent genetic association studies conducted on genetic variants of *SAA1*; a non-synonymous SNP of *SAA1* (rs12218) was found to be associated with increased carotid intima-media thickness (cIMT) (Xie et al. 2010). In another study, rs1520887, located thousands of bases upstream of *SAA1* 5'UTR, was reported to be associated with slightly lower HDL level. However, there was no association of the SNP with cIMT in an African-American population (Carty et al. 2009).

A number of genetic association studies of *SAA1* with other inflammatory diseases such as RA, Turkish familial Mediterranean fever (FMF) and Behcet's disease were conducted previously (Table 5-1). Majority of the studies involve association studies of 2 haplotypes (p.[Val70Ala; Ala75Val]; p.[Ala70Val; Val75Ala]) of *SAA*1 with susceptibility to amyloidosis in patients with the abovenamed diseases. In the Turkish population, the p.[Val70Ala; Ala75Val] haplotype is associated with higher susceptibility to amyloidosis in patients with FMF and Behcet's disease (Utku et al. 2007; Yilmaz et al. 2003). However, in the Japanese population, both p.Ala70Val and the haplotype p.[Ala70Val; Val75Ala] are associated with increased susceptibility to amyloidosis in patients with RA (Ajiro et al. 2006; Nakamura et al. 2006) . Hence, the SNPs might be genetically linked with other polymorphisms that might account for the increased susceptibility to amyloidosis. However, there has not been any genetic association study conducted to study the association between these genetic variants

with susceptibility to CAD. As the pathogenesis of amyloidosis and CAD are wholly different, it is unkown whether these genetic variants will alter the susceptibility to CAD.

Case-control genetic association study was conducted in two phases. In the first phase, about 150-200 cases and controls were used for each SNP of interest to determine the approximate odds ratio as well the the frequency of the variant allele. The approximate sample size required for each SNP was then determined from the obtained result; when the odds ratio is around 1, the study for that SNP was not extended.

Table 5-1. Association of genetic variants/haplotypes of *SAA1* with susceptibility to certain medical conditions in patients with FMF, Hyper-IgD, Behcet's disease, amyloidosis and RA.

SAA1 SNP	Reference	Study Population	Area of Study	Outcome
p.[Val70Ala; Ala75Val]	(Yilmaz et al. 2003)	Turkish population, case-control study	FMF	Higher susceptibility to amyloidosis
p.[Val70Ala; Ala75Val]	(van der Hilst et al. 2005)	Dutch population, case-control study	Hyper-IgD	No susceptibility towards IgD
p.[Val70Ala; Ala75Val]	(Kelkitli et al. 2006)	Turkish population, case-control study	FMF/ Amyloidosis	Higher susceptibility to amyloidosis in patients with FMF. Haplotype also has a higher prevalence in patients with amyloidosis.
c.-637C>T	(Akar et al. 2006)	Turkish population, case-control study	FMF	Higher susceptibility to amyloidosis

SAA1 SNP	Reference	Study Population	Area of Study	Outcome
p.[Val70Ala; Ala75Val]	(Utku et al. 2007)	Turkish population,	Behcet's disease	Higher susceptibility to amyloidosis
p.Ala70Val	(Ajiro et al. 2006)	Japanese population	RA	Higher susceptibility to amyloidosis
c.-637C>T	(Ajiro et al. 2006)	Japanese population	RA	Higher susceptibility to amyloidosis
p.[Ala70Val; Val75Ala]	(Nakamura et al. 2006)	Japanese population	RA	Higher susceptibility to amyloidosis
p.[Ala70Val; Ala75Val]	(Yamada et al. 2001)	Japanese population	RA	Higher serum SAA1 levels

5.2 Results

5.2.1 Population demographics

The population demographics of both cases and controls are as shown in Table 5-2. CAD patients are significantly older with a mean of 59.7 as compared to 42.1 in controls. BMI is also significantly higher in cases. There is a higher portion of smokers in CAD patients making up 52.8% of the sample population as compared to 15.6% in healthy controls. HDL cholesterol is, however, significantly higher in healthy controls.

Table 5-2. Population demographics of Chinese cases and controls used in the study.

Variables	CAD- n = 1261	CAD+ n = 800	*P*-value
Age (years)	42.1±13.2	59.7±8.8	<0.0001
BMI (kg/m^2)	22.9±3.6	24.2±3.6	0.0009
Sex (Male) (%)	40.9	76.7	<0.0005
TC (mM)	5.5±1.1	4.6±1.2	<0.0001
HDL-C (mM)	1.5±0.4	1.0±0.3	<0.0001
LDL-C (mM)	3.5±1.0	3.3±6.6	0.298
TG (mM)	1.4±1.1	1.8±1.0	<0.0001
Smokers* (%)	15.6	52.8	<0.0005

* Both smokers and ex-smokers are classified as smokers in the table.

5.2.2 Single locus case control association study of c.-913G>A, c.-637C>T, c.209C>T (p.Ala70Val) and c.224C>T (p.Ala75Val)

Case control association study was initially conducted with a smaller sample size of cases and controls for 5 selected SNPs, c.-913G>A, c.-637C>T, c.209C>T, c.224C>T and c.269G>A (p.Gly90Asp). However, the study was not extended beyond the small sample size for 4 of the SNPs as the odds ratio between cases and controls were not sufficiently high. c.269G>A has an initial odds ratio of > 2.0 and as such the study was extended to include a bigger

sample population. The association study of c.269G>A (p.Gly90Asp) is reported in section 5.2.2.

5.2.2.1 Genotyping of c.-913G>A, c.-637C>T, c.209C>T and c.224C>T

c.-913G>A was genotyped using allele-specific PCR while c.-637C>T, c.209C>T and c.224C>T were genotyped using RFLP (Figure 5-1). The enzymes used were BsrBI, BanI and BclI for c.-637c>T, c.209C>T and c.224C>T respectively. For genotyping by allele-specific PCR, two separate amplification reactions are needed; one contains a primer that is complementary to the wild-type while the other contains a primer that is complementary to the variant. A heterozygote has two visible bands when the PCR products for both reactions are run side by side.

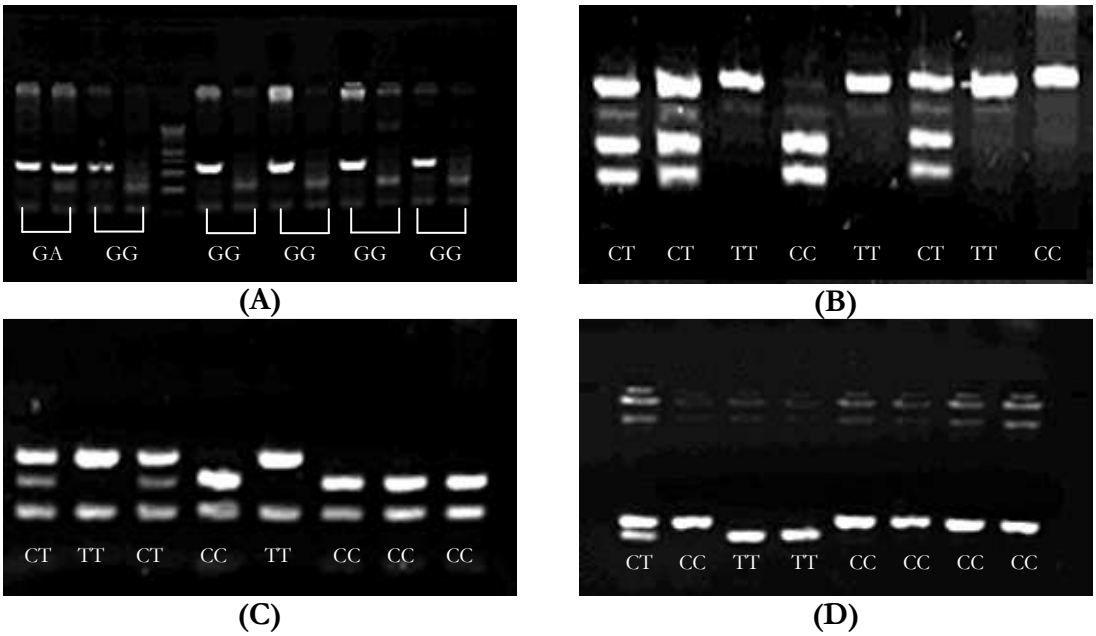


Figure 5-1. Genotyping results of (A) c.-913G>A (B) c.-637C>T (C) c.209C>T (D) c.224C>T. c.-913G>A was genotyped using allele-specific PCR while c.-637C>T, c.209C>T and c.224C>T were genotyped using RFLP.

5.2.2.2 Genotype and allele frequency

The genotype frequencies do not deviate significantly from Hardy-Weinberg expectation (HWE) except for the SNP c.-637C>T in CAD patients (Table 5-3). The *P*-values for HWE for CAD patients were shown for reference; the genotype frequencies might deviate from HWE as the cases are a selected group and thus its allele frequencies might not conform to HWE. There are no significant differences in the allele frequencies of c.-913G>A, c.-637C>T, c.209C>T and c.224C>T between cases and controls. Odds ratio of the variant allele c.-637C>T, c.209C>T and c.224C>T are 1.21, 1.10 and 0.95 respectively, all of which do not deviate largely from 1 (Table 5-4). The *P*-values were not corrected for the two-stage design and multiple testing as they were not statistically significant before correction. Although, the odds ratio is relatively lower for c.-913G>A, a large sample size is needed to determine its association with CAD due to its low allele frequency. Given that the level of association of the 4 SNPs are likely to be weak, the sample size was not increased further.

Table 5-3. Genotypes distribution and allele frequencies of healthy controls and CAD patients for c.-913G>A, c.-637C>T, c.209C>T and c.224C>T.

	Healthy Controls			CAD Patients		
	Frequency	HWE χ^2	*P*-value	Frequency	HWE χ^2	*P*-value
c.-913G>A						
GG	178	0.051	0.821	186	0.021	0.885
GA	6			4		
AA	0			0		
Allele						
A	0.984			0.990		
G	0.016			0.010		

	Healthy Control			CAD Patients		
	Frequency	HWE χ^2	*P*-value	Frequency	HWE χ^2	*P*-value
c.-637C>T						
CC	89	0.627	0.428	52	5.86	0.0155
CT	110			98		
TT	27			21		
Allele						
C	0.637			0.591		
T	0.363			0.409		
c.209C>T						
CC	77	3.36	0.067	79	3.60	0.058
CT	80			93		
TT	10			14		
Allele						
C	0.700			0.675		
T	0.300			0.325		
c.224C>T						
CC	114	0.102	0.749	132	0.124	0.724
CT	128			122		
TT	33			31		
Allele						
C	0.647			0.675		
T	0.353			0.325		

Table 5-4. Odd ratios of the variant allele of the various SNPs of SAA1 and their association with CAD.

SNP	Odds ratio	95% CI	*P*-value
c.-913G>A	0.64	0.18-2.29	0.492
c.-637C>T	1.22	0.91-1.62	0.182
c.209C>T	1.12	0.82-1.55	0.460
c.224C>T	0.87	0.68-1.12	0.290

5.2.2.3 Odds ratio of c.-637C>T, c.209C>T and c.224C>T as analysed using different genetic models

For c.-637C>T, c.209C>T and c.224C>T, genetic association were further determined using codominant, dominant and recessive genetic models. This analysis was not carried out for c.-913G>A as the homozygous variant was not present in either the controls or cases. For c.-637C>T, under the dominant model the odds ratio is 1.49. However, the odds ratio under the recessive model is 1.03, thus the choice of genetic model used might have important significance for c.-637C>T. For c.209C>T and c.224C>T, the odds ratio were close to 1 regardless of the genetic models that were used.

Table 5-5. Odds ratio of c.-637C>T, c.209C>T and c.224C>T as determined using codominant, dominant and recessive genetic models.

SNP	Model	Genotype	Odds ratio	95% CI	*P*-value
c.-637C>T	Codominant	CC	1.00		
		CT	1.52	0.98-2.36	0.16
		TT	1.33	0.68-2.59	
	Dominant	CC	1.00		
		CT + TT	1.49	0.98-2.27	0.063
	Recessive	CC + CT	1.00		
		TT	1.03	0.56-1.90	0.92
c.209C>T	Codominant	CC	1.00		
		CT	1.13	0.73-1.75	0.72
		TT	1.36	0.57-3.26	
	Dominant	CC	1.00		
		CT + TT	1.16	0.76-1.77	0.49
	Recessive	CC + CT	1.00	0.55-2.96	
		TT	1.28		0.57
c.224C>T	Codominant	CC	1.00		
		CT	0.82	0.58-1.17	0.51
		TT	0.81	0.47-1.41	
	Dominant	CC	1.00		
		CT + TT	0.82	0.59-1.15	0.25
	Recessive	CC + CT	1.00		
		TT	0.90	0.53-1.51	0.68

5.2.3 Single locus case control association study of c.269G>A

c.269G>A was genotyped by RFLP using NcoI (Figure 5-2). DNA from 1243 healthy controls and 800 CAD patients were used in the study. The genotype frequencies do not deviate significantly from Hardy-Weinberg expectation (Table 5-6). There is no significant difference in the allele frequencies between cases and controls. The odds ratio for the variant allele is 0.86 (CI: 0.50 -1.47, *P*-value = 0.57). There is no correction needed for multiple comparison as c.269G>A is the only SNP that was determined for genetic association using a larger sample size.

However, in an initial study of 590 healthy controls and 547 CAD patients, the odds ratio was 2.72 (CI: 1.05-7.03, *P*-value = 0.032) (Appendix 5-1 and 5-2). The A allele which encodes for aspartic acid instead of glycine for the C allele has a higher frequency in CAD patients.

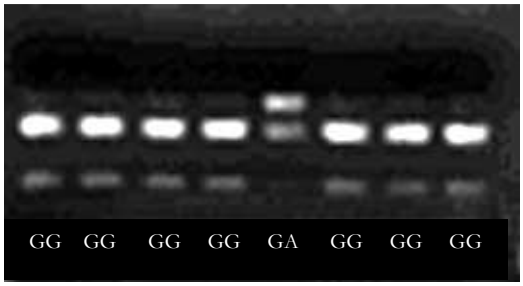


Figure 5-2. Genotyping results of c.269G>A. RFLP was used to genotype the samples; the restriction enzyme used was NcoI.

Table 5-6. Genotypes distribution and allele frequencies of healthy controls and CAD patients for c.269G>A.

	Healthy Control			CAD Patients		
	Frequency	HWE χ^2	*P*-value	Frequency	HWE χ^2	*P*-value
c.269G>A						
GG	1205	0.30	0.58	779	0.14	0.71
GA	38			21		
AA	0			0		
Allele						
G	0.985			0.987		
A	0.015			0.013		

5.2.4 Genotyping results of the 5 SNPs after adjustment for age, gender and BMI

For c.637C>T, c.209C>T and c.224C>T, there are slight differences in the odds ratio for the variant allele after adjustment for age, gender and BMI. The odds ratios are still insignificant as before the adjustment. Upon adjustment, there is a bigger change in the odds ratio for both c.-913G>A and c.269G>A. The odds ratio for c.-913G>A was 0.91 before adjustment and 0.64 after adjustment. For c.269G>A, the odds ratio was 0.85 before adjustment and 1.61 after adjustment.

Table 5-7. Odds ratio of c.-913G>A, c.-637C>T, c.209C>T, c.224C>T and c.269G>A after adjustment for age, gender and BMI.

SNP	Model	Genotype	Odds ratio	95% CI	*P*-value
c.-913G>A	NA	GG	1.00		
		GA	0.91	0.09-9.25	0.51
c.-637C>T	Codominant	CC	1.00		
					0.29
		CT	1.78	0.86-3.66	
		TT	1.47	0.47-4.66	
	Dominant	CC	1.00		
					0.13
		CT + TT	1.72	0.86-3.46	
	Recessive	CC + CT	1.00		
					0.94
		TT	1.04	0.36-3.01	
c.209C>T	Codominant	CC	1.00		
					0.89
		CT	0.91	0.45-1.84	
		TT	1.25	0.31-5.01	
	Dominant	CC	1.00		
					0.88
		CT + TT	0.95	0.48-1.87	
	Recessive	CC + CT	1.00		
					0.7
		TT	1.31	0.34-5.03	

SNP	Model	Genotype	Odds ratio	95% CI	*P*-value
c.224C>T	Codominant	CC	1.00		
					0.7
		CT	1.15	0.63-2.12	
		TT	0.80	0.33-1.91	
	Dominant	CC	1.00		
					0.86
		CT + TT	1.05	0.60-1.86	
	Recessive	CC + CT	1.00		
					0.48
		TT	0.74	0.33-1.69	
c.269G>A	NA	GG	1.00		
		GA	1.61	0.68-3.80	0.28

5.2.5 Sample size determination for the various SNPs

The sample size required for the various SNPs are as shown in Table 5-7. The sample size was determined based on the initial genotype frequencies obtained from the genotyping performed in the initial smaller sample size and that it will have a power of 80%.

Table 5-8. Sample size determination for all 5 SNPs

SNP	Sample size required in each group
c.-913G>A	6469
c.-637C>T	1728
c.209C>T	5310
c.224C>T	4546
c.269G>A	2215

5.3 Discussion

5.3.1 Choice of SNPs for genotyping and genotyping methods

Genetic association study was carried out on five selected variants of *SAA1*. As there are limited number of known genetic variants of *SAA1*, the non-synonymous variants, c.209C>T and c.224C>T were selected. Although, the two variants, c.-913G>A and c.-637C>T, do not lie in known transcription factor binding site, genetic association was also carried out to test its possible association with CAD. Lastly, genotyping was also carried out on the rare mutant, c.269G>A. c.269G>A was chosen as it is the only non-synonymous SNP that was discovered from the variant screening with a non-conservative amino acid substitution.

HRM was not used to genotype any of the variants as no positive control was available for the optimization of -913G>A and c.269G>A. As such, RFLP and allele-specific PCR were used for the genotyping as both are inexpensive and the accuracy of the genotyping of each allele can be validated.

5.3.2 Genotyping results of c.-913G>A, c.-637C>T, c.209C>T and c.224C>T

A smaller sample size was used for the genetic association study of c.-913G>A, c.-637C>T, c.209C>T and c.224C>T. In this smaller sample size, the allele frequencies do not appear to differ between healthy controls and CAD patients before and after adjustment for age, gender and BMI. As the odds ratio for the variant allele of all the 4 SNPs does not differ greatly from 1, and a much larger sample was not needed to attain a power of 80% the sample size was not increased. One caveat of this study is that the sample size was not extended based on the odds ratio obtained from an initial sample size of 200 to 300. However, it was reported that a smaller sample size has a tendency to overestimate the odds

ratio (Nemes et al. 2009) and as such a larger sample size is unlikely to yield an odds ratio that deviates significantly from 1 since the odds ratio that was obtained from this smaller sample was close to 1.

c.-913G>A is a novel SNP and no prior genetic association study has been performed on the SNP. The predicted transcription factor binding sites of *SAA1* include SP1 (c.-668 to c.-660), NF-kappaB (c.-720 to c.-712), NF-IL6/STAT (-814 to -798) and AP2 (c.-891 to c.-880) (Thorn and Whitehead 2002). c.-913G>A is not located at any of the predicted transcription factor binding site of *SAA1*, as such the substitution might not have any effect on the expression of SAA1 and thus has little association with CAD. The case is similar for c.-637C>T which is not located near any transcription factor binding site. However, the T allele was found to be associated with higher susceptibility to amyloidosis in patients with RA (Akar et al. 2006). The association between serum SAA1 level and amyloidosis is, however, unknown. Given that polymorphism in the promoter will have a greater impact on expression level than on the function of the protein, the increased susceptibility to amyloidosis is probably not directly due to the base substitution. Instead, c.-637C>T could probably be genetically linked with other allele that could explain the susceptibility to amyloidosis.

There have been a number of studies involving either C.209C>T or haplotypes of C.209C>T and C.224C>T. However, as mentioned to date no study has been performed on the genetic association of these SNPs with CAD. Our study shows that both the variant alleles in c.209C>T and c.224C>T are not genetically associated with CAD. As both alanine and valine have similar structural properties, the functional impact of these two substitutions on CAD susceptibility is probably very subtle. Further doubts on the non-synonymous substitution having a functional impact can be gathered from the studies of the TC and CT

haplotypes in Turkish and Japanese populations. In the Turkish population, the TC haplotype was associated with increased susceptibility to amyloidosis (Yilmaz et al. 2003), however, the contrasting haplotype, CT, was associated with increased susceptibility to amyloidosis in the Japanese population (Nakamura et al. 2006). Thus, there is a possibility that the non-synonymous substitutions do not account for the phenotypic change observed. The genetic association study carried out for these SNPs do not have sufficient power. To attain a power of 80%, the sample sizes required as shown in Table 5-7 are sufficiently large due to a relatively low odds ratio of most of the SNPs. Due to the likely benign effects of these SNPs and the large sample sizes required, the study was not extended. For -269G>A, as the amino change is non-conservative, a functional study was carried out to further characterized the SNPs as a larger population could not be recruited.

5.3.3 Genotyping result of -269G>A and significance of results of genetic association study

There is no genetic association of -269G>A with CAD in our study of 1243 healthy controls and 800 CAD patients. The odds ratio for the variant allele is 0.86 before adjustment and 1.61 after adjustment for age, gender and BMI. The odds ratio before adjustment is in contrast with the initial study of 547 CAD patients and 590 healthy controls. In the initial study, -269G>A was associated with CAD and the odds ratio for the variant allele was 2.72 (CI: 1.05-7.03, *P*-value = 0.032). This difference could be due to the low frequency of the variant allele and thus the result might be different with a bigger population size. Another area where discrepancy might arise is that SAA is also involved in a number of inflammatory diseases such as rheumatoid arthritis. These diseases were not identified as potential confounders when the sample subjects were first recruited. This could partly account for the

observation that the allele frequency of the variant in the cases is consistent between the initial sample group and the additional sample group that is subsequently included. In contrast, the allele frequency of the variant in the controls is not consistent between the original group and the additional group of samples that is subsequently added. Although, the genetic association of -269G>A could not be ascertained, this is the only SNP that was found to be significantly associated with CAD in the initial study, a more stringent study can be carried out in the future if a bigger population size can be recruited for the study.

As SAA1 is a relatively small protein of only 104 amino acids for the mature protein, a non-conservative substitution of amino acid might have a significant functional effect. In addition, a multiple sequence alignment of protein sequences from 6 organisms suggest that the 90th position is well conserved among the 6 organisms with no variant amino acid present. Due to the low allele frequency, there are in total only 6 and 15 heterozygotes present in the healthy controls and CAD patients group respectively. As the corresponding serum samples for the heterzygotes were depleted, it is not possible to study whether the allele alters the levels of inflammatory and atherogenic markers. Further characterization of -269G>A (p.Gly90Asp) will have to be performed by functional assays as outlined in Chapter 7.

5.3.4 Caveats of genetic association study

The genetic association study did not include the measurement of the levels of SAA1 and inflammatory mediators in both cases and controls. These levels were not determined as the plasma samples from our subjects were depleted. SAA1 level was also not monitored as there is no commercial kit that is specific for SAA1 due to its sequence similarity to SAA2.

There are significant differences in the age and gender distribution between cases and controls. The healthy controls have a mean age of 42.1 yr while the cases have a mean age of 59.7 yr. CAD cases also have a higher proportion of male comprising 76.7% of the sample size as compared to 40.9% for healthy controls. As the susceptibility to CAD increases with age, there is a concern that the healthy controls might contain subjects that might develop CAD at an older age. The odds ratio might be altered under such circumstances. Ideally, an alternative way to ameliorate the selection bias is to use younger cases compared with older controls, however, this is difficult to accomplish in this study due to two concerns. Firstly, the selection of cases was based on subjects with more than 50% stenosis in major arteries and subjects that meet these criteria are usually older. It is also harder to recruit significant number of healthy controls that are older than cases. Secondly, as CAD is a multi-factorial disease, genetic association study should be conducted with a large population size and there is substantial difficulty to recruit significant number of subjects that fit these criteria. We took the next best alternative by statistically adjusting for the confounding effect of age in our analysis. Age is included in the logistic regression model when determining the strength of association.

Although there are a number of other SNPs of *SAA1* listed on dbSNP, those SNPs were not included in the study. The focus of the study is on SNPs that have a direct impact on either the structure of SAA1 or on its expression level as these SNPs are more likely to have an impact on disease susceptibility. Thus, only genetic association study of non-synonymous SNPs and SNPs in the promoter region of SAA1 were investigated. Furthermore, these SNPs can be functionally characterized and its specific role in disease susceptibility investigated. This could not be performed for SNPs that are located in the introns. Haplotypes analysis was also not carried out as no SNP was found to be independently

associated with CAD. Ideally, a large scale SNP chip assay will allow for a more comprehensive association study of *SAA1*; however a much larger sample size would be needed for the study to attain significant power. Furthermore, the number of SNPs deposited in the SNP database at the time of the study was not large enough for the need to undertake such a high throughput assay. Nonetheless, the study manages to achieve the basic objective of studying the genetic association of common known variants and a rare mutant with CAD.

5.3.5 Future works

In a well-conserved protein such as SAA1, the presence of a non-conservative substitution such as in p.Gly90Asp might have a significant effect. However, this mutant has a low allele frequency and thus a larger population size is needed for a genetic association study of sufficient power. The sample size should be increased if sufficient number of cases and controls can be recruited in the future. In addition, healthy controls should also be screened for whether they suffer from inflammatory diseases such as rheumatoid arthritis as SAA was reported to play a role in the pathogenesis of these diseases. The impact of p.Gly90Asp on the plasma levels of inflammatory cytokines such as IL-8, TNF-α and SAA1 should also be determined if a larger population sample can be recruited.

As CAD is a mutli-factorial disease, the contribution of other SNPs will determine the susceptibility of an individual to the disease. A multiple SNPs analysis will allow for the combined effects of these SNPs on CAD susceptibility to be studied. Through this study, the synergy of certain subsets of genes might be discovered and this will allow for a better understanding of the pathogenesis of CAD at a gene level. The SNPs that might be used for this study include inflammatory genes such as *IL-16* (Wu et al. 2011), *CX3CR1* (Sirois-

Gagnon et al. 2011), *IL-6*, *IL1-B* (Rios et al. 2010), *CD14* (Rizzello et al. 2010) and *TNF* (Ghazouani et al. 2009), all of which were found to be associated with CAD. Similarly, the levels of inflammatory cytokines and lipids could be measured to determine whether the combined effects of multiple SNPs would have an impact on the levels of these proteins.

6 Functional study of p.Gly90Asp

6.1 Introduction

The rarity of non-conservative SNP of *SAA1* suggests that SAA1 might play a critical role in the regulation of important life processes in humans. p.Gly90Asp is the only non-conservative SNP that was identified from our variant screening. In addition, p.Gly90Asp has a high odds ratio in the performed genetic association study which is rare for a multifactorial disease such as CAD. The characterization of p.Gly90Asp will help identify possible processes that are affected due to the amino acid substitution and explain the association of the SNP with CAD.

As there is limited information on the functional domains of SAA1, the choice of functional assays to be conducted was based on the reported effects that SAA1 has on macrophages. This is the first time that functional characterization of genetic variants of *SAA1* was conducted using *in vitro* assays involving cell culture. Previously, the only characterization of p.Gly90Asp involves the electrofocusing of both the wild-type and variant proteins. Variant SAA1 was reported to be more acidic with a pI value of 6.1 as compared to 6.5 in wild-type SAA1 (Kluve-Beckerman et al. 1991). A-SAA induces the secretion of pro-inflammatory cytokines (Song et al. 2009; Yang et al. 2006), facilitates cholesterol efflux in macrophages (Tam et al. 2008) and increases nCEH activity. Induction of pro-inflammatory cytokines is deemed to be atherogenic while the latter two are most probably atheroprotective. Differences in the induced level of cytokines or the rate of cholesterol efflux and nCEH activity between wild-type or variant SAA1 treatment will help account for the association of p.Gly90Asp with CAD. In addition, the induced changes in global gene expression levels in macrophages upon treatment with either wild-type or variant SAA1 treatment were also determined. The combination of both approaches will help characterize p.Gly90Asp given

the limited information that is known about the structure of SAA1 as well as its functional effects.

Functional assays were carried out using THP-1 derived macrophages. Upon treatment with PMA, THP-1 monocytes differentiate into human macrophages. As THP-1 monocyte is one of the few cell lines that can be used to derive macrophages, it was used for the assay. The macrophages were used as they are crucial for both atherogenesis and the development of atherosclerotic lesion. Both recombinant wild-type and variant SAA1 were produced using *E.coli*. Glycosylation is not required to produce mature SAA1 hence *E.coli* is suitable for the production of the recombinant protein (Marsche et al. 2007). Recombinant SAA1 was used instead of transfecting SAA1 DNA into macrophages as endogenous production of SAA1 is limited in the macrophages. The major sources of SAA1 production are the liver and adipose tissues. Hence, the likely impact of variant SAA1 on macrophages could probably be due to its altered binding with cell surface receptors as SAA1 has limited ability to translocate across cell membrane (Kinkley et al. 2006). In addition to macrophages, neutrophils were used in the initial functional assays to determine the level of inflammatory cytokines secreted when incubated with SAA1. Neutrophils were used to determine whether the differential production of cytokines observed in THP-1-dervied macrophages could be observed in other immune cells. The importance of neutrophils to CAD has been highlighted in a number of studies that were reported after this work was completed; these studies will be mentioned in the discussion of this chapter.

6.2 Results

6.2.1 Production of IL-8, TNF-α and MCP-1 from THP-1 macrophages

When macrophages were incubated with variant SAA1, the amount of IL-8, MCP-1 and TNF-α produced were significantly lower as compared to wild-type SAA1 treatment (Figure 6-1 to Figure 6-3). At 3 μg/ml of SAA1, the decrease in IL-8, MCP-1 and TNF-α production were 57%, 50% and 39% respectively. Although, the amount of cytokines secreted were less for variant SAA1, there were still a great induction of secretion of IL-8 and TNF-α from macrophages; IL-8 and TNF-α levels increased by 5 and 60 fold respectively as compared to untreated control when macrophages were incubated with 3 μg/ml of variant SAA1.

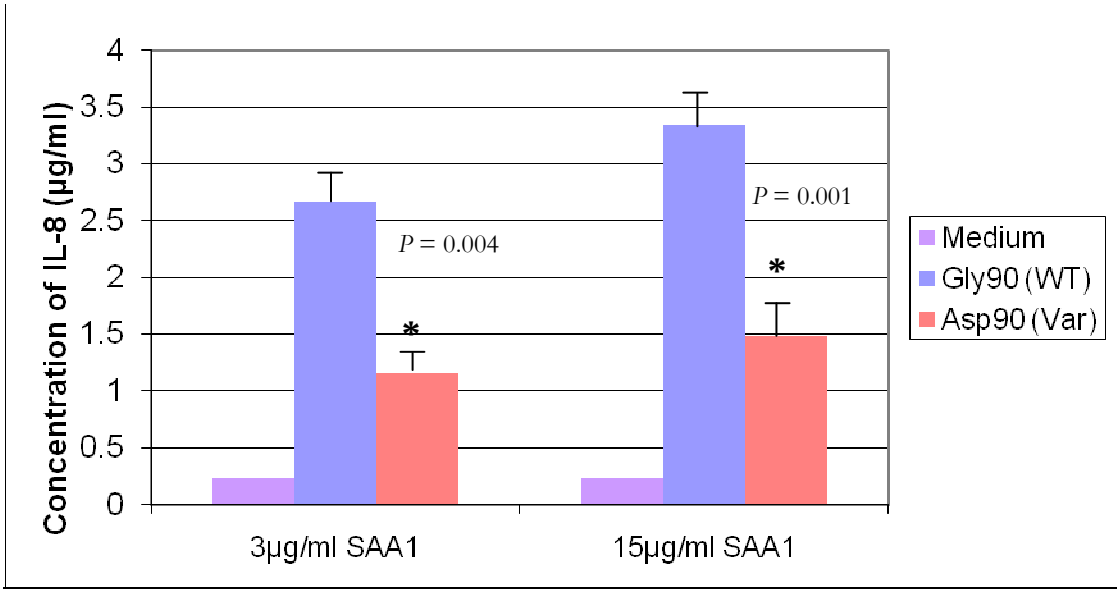


Figure 6-1. Differential effects of wild-type and variant SAA1 treatment on IL-8 secretion by THP-1 derived macrophages. Cells were incubated with SAA1 for 24 h and the supernatants were assayed for IL-8 using ELISA. Error bars represent standard deviations (n =3). *P <0.005 for variant SAA1 vs wild-type SAA1.

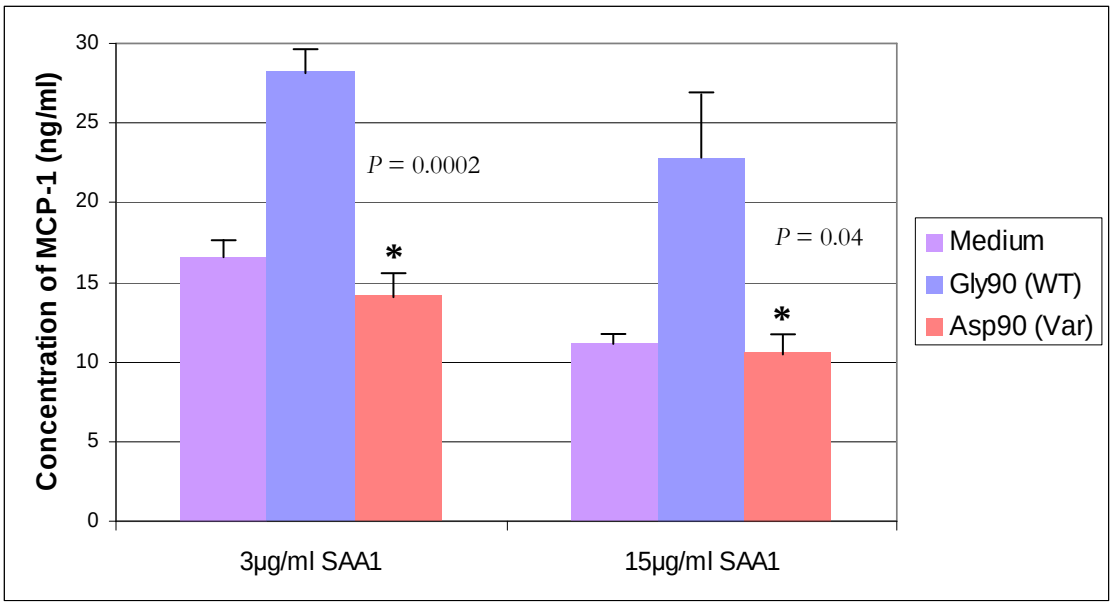


Figure 6-2. Differential effects of wild-type and variant SAA1 treatment on MCP-1 secretion by THP-1 derived macrophages. Cells were incubated with SAA1 for 24 h and the supernatants were assayed for MCP-1 using ELISA. Error bars represent standard deviations (n =3). *P<0.05 for variant SAA1 vs wild-type SAA1.

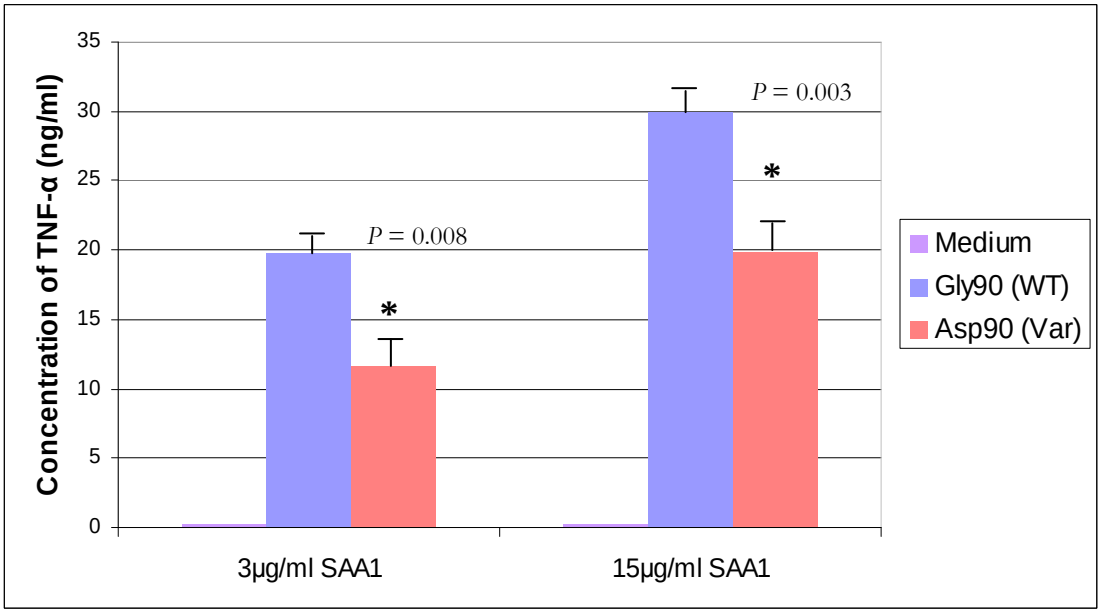


Figure 6-3. Differential effects of wild-type and variant SAA1 treatment on TNF-• secretion by THP-1 derived macrophages. Cells were incubated with SAA1 for 24 h and the supernatants were assayed for TNF-• using ELISA. Error bars represent standard deviations (n =3). *P<0.01 for variant SAA1 vs wild-type SAA1.

6.2.2 Production of IL-8 and MCP-1 from neutrophils like differentiated HL-60 cells

Variant SAA1 has a lower cytokine induction capability as compared to wild-type SAA1 (Figure 6-4 to Figure 6-5). The differences were significant for the production of MCP-1 at both concentrations of SAA1. At 3 μg/ml of SAA1, there was a decrease of 23.5% as compared to wild-type SAA1 (P = 0.003) and a decrease of 40% when the concentration of SAA1 was increased to 15 μg/ml (P =0.001). Although, there was a general decrease in IL-8 production of 10% (3 μg/ml of SAA1) and 21.2% (15 μg/ml of SAA1) when variant SAA1 was incubated with the neutrophils as compared to wild-type SAA1, the differences were not significant. Variant SAA1 retains its cytokine induction capability with a 7.8 fold increase of IL-8 and a 14 fold increase of MCP-1 as compared to the untreated control when neutrophils were treated with 3 μg/ml of variant SAA1.

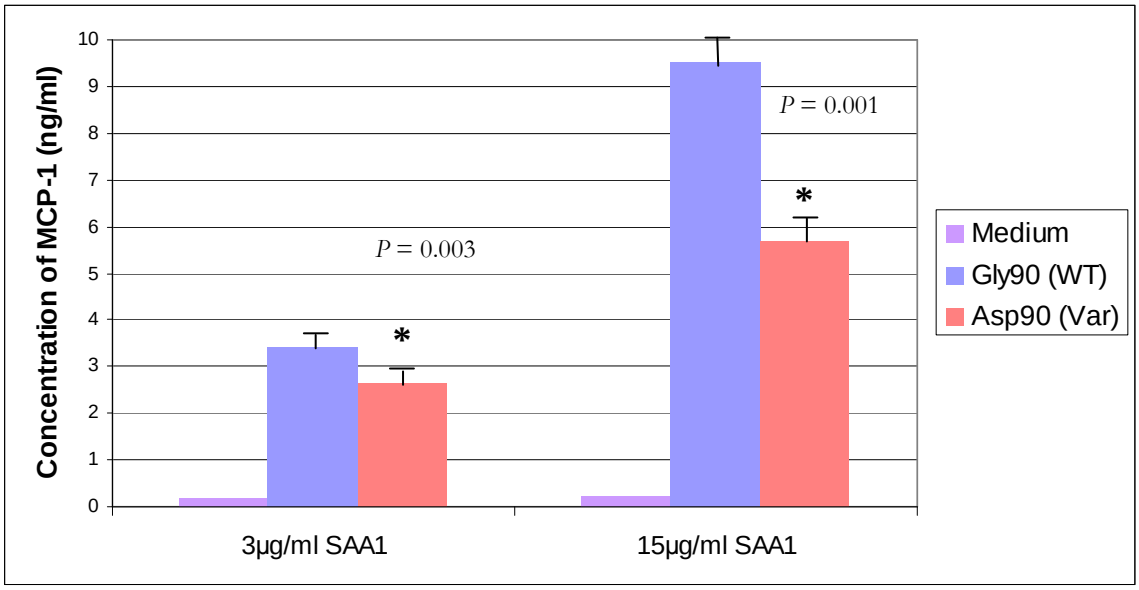


Figure 6-4. Differential effects of wild-type and variant SAA1 treatment on MCP-1 secretion by HL-60 derived neutrophils. Cells were incubated with SAA1 for 24 h and the supernatants were assayed for MCP-1 using ELISA. Error bars represent standard deviations (n =3). *P<0.005 for variant SAA1 vs wild-type SAA1

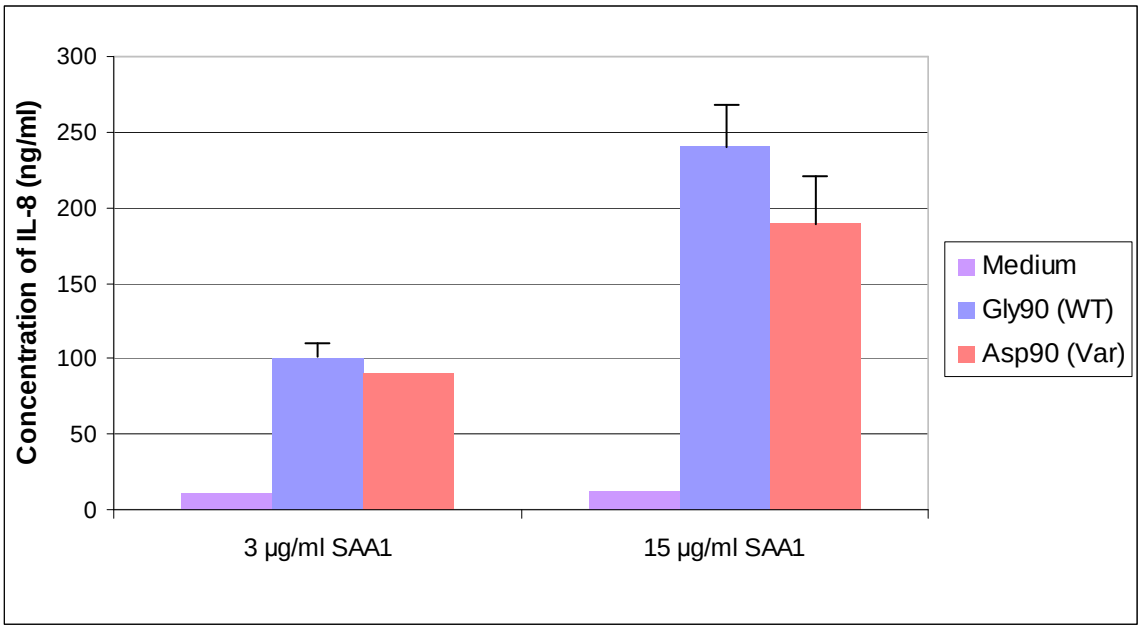


Figure 6-5. Differential effects of wild-type and variant SAA1 treatment on IL-8 production by HL-60 derived neutrophils. Cells were incubated with SAA1 for 24 h and the supernatants were assayed for IL-8 using ELISA. Error bars represent standard deviations (n =3).

6.2.3 Effects of SAA on nCEH activity

Both wild-type and variant SAA1 have no effect on cholesterol esterase activity (Figure 6-6). A-SAA from mouse was found to stimulate an increase in cholesterol esterase activity by 3-7 fold (Lindhorst et al. 1997). In another study, mouse SAA2 was found to have an impact on cholesterol esterase activity whereas mouse SAA1 has negligible impact on cholesterol esterase activity (Tam et al. 2005). Since A-SAA consists of both SAA1 and SAA2, the observed effects might be from either one of SAA1 or SAA2. The experiment was repeated using apoSAA (Peprotech, Rocky Hill, New Jersey, United States) which has a 99% sequence similarity to SAA1 and similarly there was no apparent effect on cholesterol esterase activity (data not shown). Hence, it is safe to conclude that SAA1 has no effect on cholesterol esterase activity and that the association of the genetic variant with CAD is not due to an altered activity in cholesterol esterase.

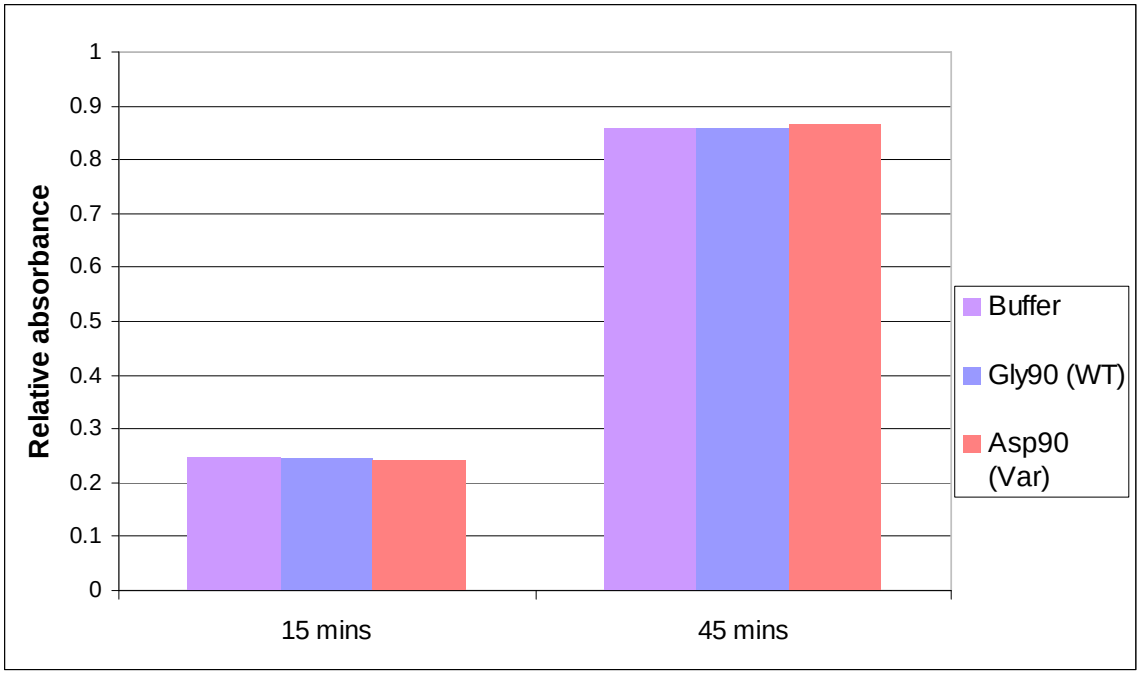


Figure 6-6. Effects of SAA1 on nCEH activity. Both wild-type and variant SAA1 have negligible effects on nCEH activity. An *in vitro* mixture containing cholesteryl oleate, horse radish peroxidase, 4 amino-antipyrine, cholesterol oxidase and porcine cholesterol esterase was incubated in the presence of recombinant SAA1 at 37°C. The colour intensity was measured at 500nm . Data are expressed as the mean of 3 independent experiments.

6.2.4 Microarray studies of wild-type SAA1 (Gly90) and variant SAA1 (Asp90) in THP-1 derived macrophages

6.2.4.1 Differential gene expression between wild-type SAA1 and variant SAA1 at 8 h

In total, there were 67 genes that were differentially downregulated between wild-type and variant SAA1 with a fold difference of more than 1.5. However, the functions of most of the genes in the list were relatively unkown; genes that were differentially upregulated and with function that might play a role in atherosclerosis are shown in Table 6-1. Proline 4-hydroxylase (P4HA1) is required for the synthesis of collagen (Kivirikko et al. 1990) and inhibition of collagen synthesis was shown to increase the rate of degradation of extracellular matrix (Greenwel et al. 2000; Verrecchia et al. 2002). Mutations in the bone morphogenetic

protein receptor, type II (*BMPR2*) gene are associated with primary pulmonary hypertension (PPH) and account for 50% of familial cases of PPH (Deng et al. 2000). PPH results from the development of obstructive lesions in small pulmonary arteries leading to heart failure (Tobin and Celeste 2006).

There are no genes that were differentially upregulated between wild-type and variant SAA1 with a fold difference of less than 0.67. As the fold difference between wild-type and variant SAA1 is very small, the chance of picking up a false positive is relatively high. One possible way to reduce false positive is to observe the ratio of wild-type SAA1/untreated. A low ratio of less than 2 suggests that the gene is not activated upon SAA1 treatment and hence the possibility of differential expression between wild-type and variant SAA1 treatment is lower. Nonetheless, the results were validated using real-time PCR so that a conclusion on the genes that were differentially expressed between the two variants of SAA1 can be drawn.

Table 6-1. Decreased relative genetic expression upon variant SAA1 treatment as compared to wild-type SAA1 treatment after 8 h of treatment. Fluorescence signals were normalized and the mean from two independent experiments were calculated. Fold difference was obtained by taking the ratio of fluorescence signal from wild-type treatment to that of variant SAA1 treatment.

Gene (Gene Symbol)	Fold difference (Wild-type /Variant SAA1)	Fold difference (Wild-type SAA1/Untreated)
APAF1 interacting protein (*APIP*)	1.62	1.44
Fibrinogen-like 1 (*FGL1*)	1.61	1.35
Granulocyte chemotactic protein 2 (*CXCL6*)	1.60	3.18
Plasminogen activator inhibitor-2 (*SERPINB2*)	1.59	4.45
Bone morphogenetic protein receptor, type II (*BMPR2*)	1.54	1.20

Gene (Gene Symbol)	Fold difference (Wild-type SAA1/Variant SAA1)	Fold difference (Wild-type SAA1/Untreated)
Prostaglandin G/H synthase and cyclooxygenase (*PTGS2*)	1.53	2.71
Proline 4-hydroxylase (*P4HA1*)	1.50	1.16

6.2.4.2 Differential gene expression between wild-type SAA1 and variant SAA1 at 24 h

3 genes were differentially downregulated between wild-type and variant SAA1 with a fold difference of more than 1.5 (Table 6-2). However, these genes are not well characterized with unknown functional roles. There were 1110 genes that were differentially upregulated between wild-type and variant SAA1 with a fold difference of less than 0.67. As there were too many genes that were differentially downregulated only a selected few with either atherogenic or atheroprotective roles are shown in Table 6-3. These genes include lysyl oxidase-like 3 (*LOXL3*), which was reported to be associated with diet induced atherosclerosis in rabbit (Kagan et al. 1981) and carboxylesterase 1 (*CES1*), which regulates intracellular triacylglycerol accumulation and is associated with obesity (Ko et al. 2009; Marrades et al. 2010) . The list of the top 50 genes is listed in appendix 6-1.

Table 6-2. Decreased relative genetic expression upon variant SAA1 treatment as compared to wild-type SAA1 treatment after 24 h; data is obtained from the analysis of microarray result.

Gene (Gene Symbol)	Fold difference (Wild-type SAA1/Variant SAA1)	Fold difference (Wild-type SAA1/Untreated)
Additional sex combs like 2 (*ASXL2*)	1.62	1.91
Transmembrane protease, serine 7 (*TMPRSS7*)	1.58	1.03
Zinc finger protein 749 (*ZNF749*)	1.51	1.36

Table 6-3. Increased relative genetic expression upon variant SAA1 treatment as compared to wild-type SAA1 treatment after 24 h of treatment.

Gene (Gene Symbol)	Fold difference (Wild-type SAA1/Variant SAA1)	Fold difference (Wild-type SAA1/Untreated)
CD63	0.43	0.58
Myosin (*MYO1F*)	0.53	0.73
Sterol regulatory element binding transcript factor 1 (*SREBF1*)	0.55	0.90
Acyl-CoA thioesterase 8 (*ACOT8*)	0.56	0.81
Protein kinase, AMP-activated, gamma 1 non-catalytic subunit (*PRKAG1*)	0.57	0.88
Carboxylesterase 1 (*CES1*)	0.58	0.76
Lysyl oxidase-like 3 (*LOXL3*)	0.58	0.79

6.2.4.3 Real-time PCR validation of microarray result

For the validation of the microarray result at 8 h, five genes were chosen for validation by real-time PCR. Genes were chosen based on their functional effects and its possible contribution to atherosclerosis. *SERPINB2* and *PTGS2* had decreased expression upon variant SAA1 treatment as compared to wild-type SAA1. There does not appear to be any differential expression in *BMPR2*, *P4HA1* and *CXCL6* between SAA1 and wild-type SAA1 treatment. *PTGS2* and *SERPINB2* were upregulated upon SAA1 treatment (wild-type SAA1/untreated $\geq$ 2) while *BMPR2* and *P4HA1* have wild-type SAA1/untreated ratio of < 2. Thus, the differentially expressed genes between wild-type and variant SAA1 treatments are probably those genes with a wild-type SAA1/untreated ratio of > 2 and a high ratio for wild-type SAA1/variant SAA1. Using this criteria, it is safe to conclude that only 2 genes were differentially regulated between wild-type and variant SAA1 at 8 h (Table 6-4).

For the 24 h incubation, all the genes listed in Table 6-3 were not differentially expressed between wild-type and variant SAA1 treatment (Table 6-5). This is within expectation, as genetic induction of expression by SAA1 appears to cease at 24 hours with wild-type SAA1/untreated having values of 0.58 to 1.15.

Table 6-4. Real time PCR verification of microarray results at 8 h. Real-time PCR results was obtained from 3 independent assays and analysed using a software for real time PCR analysis, REST 2009. Differential expression was observed in SERPINB2 and PTGS2.

Gene	**Expression**	**Standard Error**	***P*-value**
CXCL6	1.29	0.77-2.22	0.325
SERPINB2	1.76	1.42-2.18	<0.0001
BMPR2	1.04	0.95-1.13	0.426
PTGS2	1.39	1.13-1.66	0.029
P4HA1	1.07	0.99-1.13	0.249

Table 6-5. Real time PCR verification of microarray results at 24 h. Real-time PCR results were obtained from 3 independent assays. Differential expression was not observed in any of the genes.

Gene	**Expression**	**Standard Error**	***P*-value**
CD63	0.96	0.79 - 1.15	0.695
MYO1F	1.00	0.92 - 1.10	0.965
SREBF1	1.11	0.89 - 1.30	0.434
ACOT8	0.95	0.87 - 1.08	0.532
PRKAG1	1.00	0.90 - 1.13	0.835
CES1	1.08	0.93 - 1.22	0.418
LOXL3	1.14	0.97 - 1.38	0.36
ITGB1BP1	0.91	0.76 - 1.06	0.447

6.3 Discussion

6.3.1 Effects of SAA1 treatment on cytokine production in macrophages and neutrophils

As variant SAA1 is associated with susceptibility to CAD, it was originally speculated that variant SAA1 will induce a higher level of pro-inflammatory cytokines as compared to the wild-type. However, results show that variant SAA1 induces lower level of pro-inflammatory cytokines. Although, this run contary to our expectation, the result is plausible as the substitution could probably alter the structure of mature SAA1 protein. In our study, the surface receptors, TLR2 and CLA-1, account for part of the cytokine induction property of SAA1 (Chapter 8.3). As such, the amino acid substitution might alter the structure of SAA1 and affects its affinity with the surface receptors. As a reduction of the level of pro-inflammatory cytokines is most probably athero-protective, the association of the genetic variant with CAD could possibly be due to a change in expression level of other genes that are simulataneously activated by the same surface receptors.

Differential induction of inflammatory cytokines by p.Gly90Asp was observed in both macrophages and neutrophils. However, a greater difference was observed in macrophages for both IL-8 and MCP-1. This could probably be due to the differential regulation of cytokine induction in the 2 different immune cells; the macrophages could probably have more surface receptors and less negative regulation on its production of cytokines. In summary, the variant has a functional impact on cytokine production in immune cells and this could probably be attributed to its altered affinity to cell surface receptors.

6.3.2 Effects of SAA1 treatment on cholesterol storage and metabolism

One of the proposed anti-atherogenic potential of SAA1 is its role in preventing accumulation of cholesteryl ester in cells. This can take place through a dual stimulation of

increased cholesterol efflux from macrophages (Tam et al. 2008) and increasing the activity of nCEH (Lindhorst et al. 1997). In a preliminary study, there was a small difference in cholesterol efflux from THP-1 derived macrophages between the wild-type and the variant, however, this difference was observed only at a concentration of 40μg/ml SAA1. As the concentration of SAA1 in the wall of the coronary artery is still unknown, it is doubtful whether 40μg/ml SAA1 is physiologically relevant. However, given that the serum concentration of SAA1 in obese subjects is 20μg/ml (Lappalainen et al. 2008), a concentration in excess of 40μg/ml might not be physiologically relevant, and as such cholesterol efflux assay was not carried out after the preliminary study.

The nCEH assay shows that SAA1 does not have any effect on nCEH activity. In an earlier study, acute-phase HDL was shown to stimulate an increase in nCEH activity (Lindhorst et al. 1997). However, in another study, short peptides corresponding to SAA2 amino acid sequence but not SAA1 was shown to induce a positive effect on nCEH activity (Tam et al. 2005). This result clarifies the two earlier published studies and shows that SAA1 does not have any effect on nCEH activity and that earlier observed effects could be due to SAA2. As technical difficulty was encountered during the process of obtaining the nCEH from THP-1 macrophages, porcine nCEH was used instead. However, the usage of porcine nCEH should have little interferance on the result, as previous study reported also involved the use of porcine nCEH. Thus, from the result, the association of variant of SAA1 with CAD is not due to its effects on nCEH activity and cholesterol efflux.

6.3.3 Differential effects of wild-type and variant SAA1 on global expression level in macrophages

As results from the earlier functional assays did not appear to explain the association of the variant with CAD, a microarray study was initiated. Two time points of 8 h and 24 h were

used for the study. Due to the inherent cost of the chips, only two samples for each treatment and timepoint were used. The results were compared and analysed with real-time PCR to ensure its validity.

The initial results show that only a few genes were differentially expressed between wild-type and variant SAA1 at 8 h while there were many more genes that were differentially expressed at 24 h. However, real-time PCR shows that most of the genes that were deemed to be differentially regulated at 24 h through microarray analysis were false-positives. Various steps were taken to eliminate false positives. To select for genes for validation by real time PCR, two steps were taken; genes that were deemed to be differentially upregulated or downregulated by microarray were first functionally annotated. For the 8 h timepoint, only 7 genes were identified with functional roles that might contribute to athersoclerosis. Upon functional annotation of genes, genes that have a high wild-type/variant SAA1 ratio and genes that were both differentially expressed between wild-type and variant as well as between wild-type and untreated were selected for validation by real-time PCR. A wild-type/untreated ratio of > 2 together with a high value of wild-type/variant was found to be more reliable in determining whether the genes were differentially regulated. At 24 h, none of the genes that were selected for validation by real-time PCR were deemed to have significant differential expression. This was not surprising given that induction of global expression by SAA1 in macrophages ceased at 24 h and that most of the genes shown in Table 7-3 have wild-type SAA1/untreated ratio of less than 2.0. As the analysis of microarray results was carried out in a comprehensive manner, it can be concluded that only two genes (*PTGS2* and *SERPINB2*) were differentially expressed at 8 h and no genes was differentially regulated at 24 h.

Of the 2 differentially expressed genes at 8 h, PTGS2 is a mediator of inflammation while SERPINB2 can exert atherogenic or athero-protective effects depending on where it is expressed. Endothelial expression of SERPINB2 is potentially atherogenic as it inhibits fibrinolysis through its inhibition of tissue plasminogen activator and urokinase plasminogen activator (uPA). Production of SERPINB2 by the macrophages in atherosclerotic lesion is athero-protective as it has a negative impact on THP-1 cell proliferation and differentiation (Yu et al. 2002a). SERPINB2 has anti-apoptotic property and its inhibiton of uPA prevents uPA-mediated extracellular matrix degradation which helps to reduce the invasion of migratory cells (Kumar and Baglioni 1991). Lastly, SERPINB2 is a negative modulator of pro-inflammatory Th1 responses and thus it helps to regulate the amount of inflammatory cytokines in atherosclerotic lesion (Schroder et al. 2010). Taken together, SERPINB2 might have an influence on CAD through its regulatory role in key processes of atherosclerosis such as matrix degradation and pro-inflammatory activity in the lesion. The decreased expression of *SERPINB2* upon variant SAA1 treatment as compared to wild-type SAA1 might account for the association of variant SAA1 with CAD. In our study, there was no quantification of the level of SERPINB2 both in the cellular lysate and the supernatant as THP-1 cells are not able to produce functional SERPINB2 and thus its level cannot be quantified with available ELISA kit (Gross and Sitrin 1990).

NF-kappaB is a reported downstream target of A-SAA (Zhao et al. 2007). All of the genes that were found to be differentially regulated in our functional study are reported downstream targets of NF-kappaB (Schreiber et al. 2006; Wang et al. 2008a; Wang et al. 2008b). NF-kappaB is a downstream target of several cell surface receptors of acute-phase SAA such as TLR2 (Baranova et al. 2005; Cheng et al. 2008) and CLA-1 (Baranova et al. 2005). Thus, it is likely that the impaired affinity of variant SAA1 for its surface receptors

affects efficiency of downstream signaling resulting in lower activation of NF-kappaB targets. Although, NF-kappaB mediates pro-inflammatory actvity which is essentially atherogenic, a few studies have highlighted its athero-protective role. In one study, mice with diminished NF-kappaB activity developed atherosclerotic lesion that was twice the size in normal mice (Idel et al. 2003) . In another study, mice with induced macrophage specific deficiency of I-kappaB kinase beta (IKK2) have a 62% increase in lesion area (Kanters et al. 2003). Thus, NF-kappaB has athero-protective functions through its induction of athero-protective genes, many of which are not currently known. Based on previous published studies, it is possible that SERPINB2 is one of the athero-protective genes that are induced by NF-kappaB.

6.3.4 Intrepretation of results of the functional assays

Previously, there has been uncertainty over the role SAA1 plays in atherosclerosis, in particular whether it is atherogenic or athero-protective. The identification of a non-synonymous SNP that has a significant association with CAD allows us to clarify the role SAA1 plays in CAD. The first section of the functional assay involves the quantification of cytokine levels secreted by macrophages upon induction by either wild-type or variant SAA1. Although, the results are unexpected, they are clarified through the microarray analysis. Microarray analysis identifies a few genes that were differentially expressed and all of which are downstream targets of NF-kappaB. Although, IL-8, MCP-1 and TNF-• are not deemed to be differentially expressed in the microarray analysis, this is not unexpected as the protein levels of IL-8, MCP-1 and TNF-• decreased by only 57%, 50% and 39% respectively when they were treated with 3 μg/ml SAA1. Furthermore, microarray analysis only quantifies the relative amount of transcripts at that time interval. Nonetheless, the results of the study

indicate that a major impact of the substitution is ithe possible alteration of the efficiency of NF-kappaB signaling. Both atherogenic and athero-protective genes such as SERPINB2 are regulated by Nf-kappaB and it is possible that the balance is disrupted in variant SAA1.

In addition, the studies also show that there are no differential expression in genes that are associated with lipid metabolism between wild-type and variant SAA1. Thus, the association of the variant with CAD is most probably not associated with altered regulation in lipid storage. However, the effects of wild-type and variant SAA1 has on cholesterol efflux in the presence of components of the extracellular matrix remain to be ascertained, however this area is technically harder to accomplish.

6.3.5 Caveats of functional characterization of p.Gly90Asp

Functional characterization of the variant protein was carried out at 3µg/ml and 15µg/ml for the quantification of cytokines secreted by macrophages. For the microarray analysis, the concentration used was lowered to 1µg/ml. Initial concentrations of 3 and 15µg/ml of SAA1 were used as this is close to the serum SAA1 concentration in healthy subjects and patients with metabolic syndrome respectively. There is, however, a concern that the concentration of SAA1 in the walls of the coronary artery might be different than that in the blood. As the microarray analysis includes the stimultaneous study of the differential effects between wild type and variant and between wild-type and untreated (Chapter 7), the concentration for the microarray analysis was lowered so that the global gene expression analysis between SAA and untreated could be conducted at a concentration that addresses this concern. Due to the inherent cost of the chips, only one concentration of SAA1 was used, nonetheless, the study was able to identify the NF-kappaB pathway as the likely pathway to be impacted by the amino acid substitution. There is a limitation in the number

of time points that were used for the microarray analysis. Originally, it was thought that a 8 and 24 h timepoint would be sufficient to monitor the differential genetic expression in the macrophages. However, given the result, it would be interesting to see whether there is a greater degree of differential expression at 4 hr.

THP-1 derived macrophages is one the few commercially available macrophages that resembles human macrophages. One major disadvantage of THP-1 derived macrophages is that PMA is needed to induce the differentiation of monocytes into macrophages. The morphology of the differentiated cells was however monitored carefully to ensure that the differentiated macrophages were morphologically similar for each independent experiment. A better alternative would be PBMCs; however, these primary cells were not available as human subjects need to be recruited.

The surface receptors are most likely responsible for the functional effects induced by SAA1 in the macrophages. However, it is not possible to directly inhibit the various receptors to determine whether the surface receptors are responsible for the differential production of cytokines. As the morphology of THP-1 derived macrophages changes when it is induced to differentatiate at low confluence, a large amount of antibodies are needed to inhibit a substantial amount of the surface receptors. Thus, the assay was not carried out, however, the cell surface receptors, TLR2 and CLA-1, were found to be partly responsible for the induction of inflammatory cytokines by SAA1 using mouse RAW264 macrophages (Chapter 8).

6.3.6 Future works

Functional studies were carried out using an immortalized cell line which might have a differential cellular response as compared to a primary cell line. It is expected that SAA1 will

be able to induce the secretion of a significant amount of inflammatory cytokines from primary cells as similar effects were observed with recombinant human A-SAA in a reported study (Song et al. 2009). In addition, surface receptors of SAA1 such as TLR2 and CLA-1 were found to be expressed in human primary macrophages (Erdman et al. 2009; Gallego et al. 2011). Nonetheless, the study should be verified in primary cell line to ascertain the results from THP-1 macrophages and to determine whether the global gene expression is different in the primary cell line.

The binding affinity of the genetic variant to each of the surface receptors of SAA1 could be investigated and determined using ELISA. Recombinant fusion protein of each of the five surface receptors and the Fc region can be prepared using a mammalian system and the binding efficiency is measured by determining the fluorescence levels after SAA1 is allowed to bind to the recombinant cell surface receptors.

There are currently five reported surface receptors of A-SAA and all these five receptors including FPRL-1 and RAGE are expressed in monocytes or macrophages (Dragomir et al. 2011; Ernst et al. 2004). As such, the downstream signaling pathways of these receptors which include ERK1/2, p38, Jak-STAT and NF-kappaB might be altered between wild-type and variant. The pathway that accounts for the differential induction could be investigated through the selective inhibition of surface receptors of SAA1.

Prior to this study, the importance of neutrophils to CAD was not exactly known and the pathogenesis of atherosclerosis has been mainly attributed to the interplay of macrophages, lymphocytes and fibroblasts. However, recent studies have indicated the importance of neutrophils to the pathogenesis of CAD: neutrophils were found to infiltrate arteries during the intial stages of atherosclerosis (Drechsler et al. 2010) and that human neutrophil peptides were reported to play a role in the formation of foam cells (Quinn et al. 2011). The

functional study of p.Gly90Asp could thus be extended to neutrophils so that the functional effects of the variants and its impacts on CAD can be better understood.

7 Gene expression profiling of THP-1 derived macrophages upon treatment with SAA1

7.1 Introduction

With the completion of the genetic variant screening, genetic association and functional studies of SAA1 and its variant, there are sufficient evidence to support the association of SAA1 with CAD. The discovery of the genetic association of p.Gly90Asp with CAD suggests that SAA1 is not merely a marker of chronic inflammatory disease and that it has a functional role in the pathogenesis of atherosclerosis.. As an evolutionary conserved protein, the role it plays in survival as well as its role in atherosclerosis remains to be answered. To solve this conundrum, the global genetic expression that is induced in THP-1 derived macrophages upon treatment with SAA1 was studied. The microarray analysis will help identify genes and pathways that are activated upon SAA1 treatment; this will be helpful in determining the possible roles SAA1 plays in atherosclerosis and the scope of its functional effects.

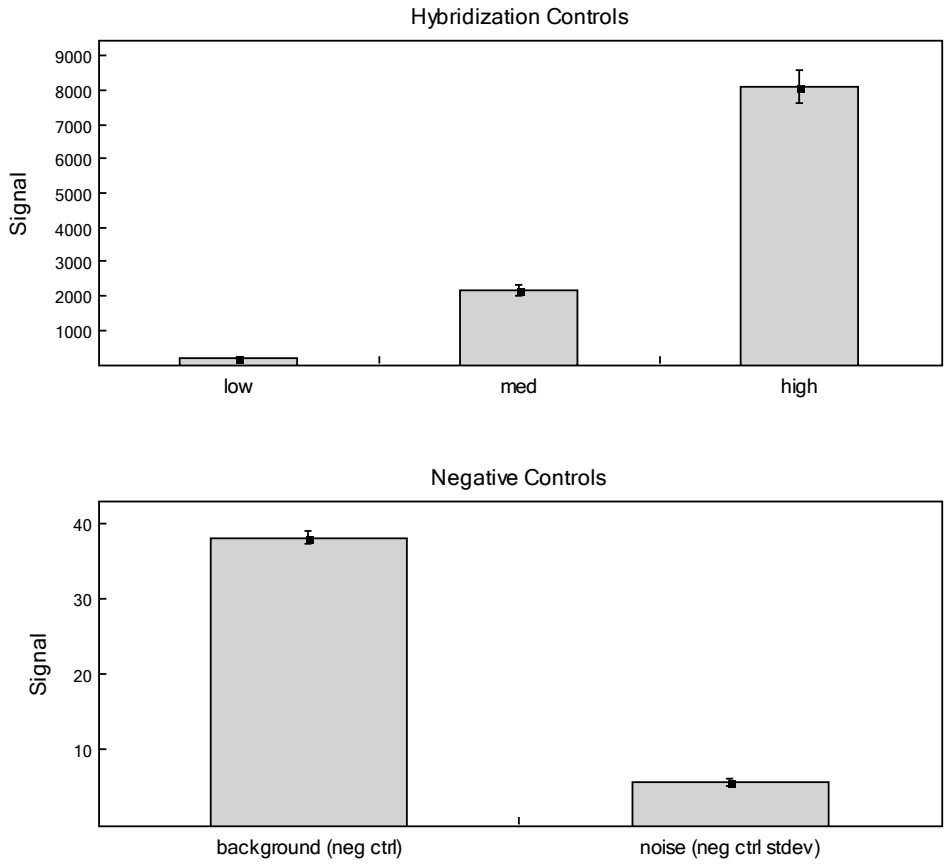
Previous published studies have focused on characterizing the likely role A-SAA plays in atherosclerosis using a recombinant SAA with an amino acid sequence that is a hybrid of SAA1 and SAA2. To round up the study of SAA1, two of the reported surface receptors of A-SAA, TLR2 and CLA-1, were ascertained for their involvement as surface receptors of SAA1. Due to technical difficulties in using THP-1 derived macrophages for this particular study, RAW264 macrophages were used instead. This study will support the hypothesis in Chapter 7 where the altered affinity with the surface receptors is believed to explain for the lower genetic expression of cytokines and other genes.

7.2 Results

7.2.1 Microarray analysis

7.2.1.1 Quality of microarray data

The quality of the microarray data was first verified using Genome Studio which compares signals from the internal controls used in the assay (Figure 7-1). The signals from the hybridization controls were in the correct order with the 'high-signal' hybridization control having the greatest intensity. Both the negative controls have low signal intensities. For the low stringency test, the signal levels were according to expectation with the perfect match (pm) probe having a higher signal than the mismatch (mm2) probe. Housekeeping genes have a total signal intensity that was much greater than the sum of the signals from all other genes.



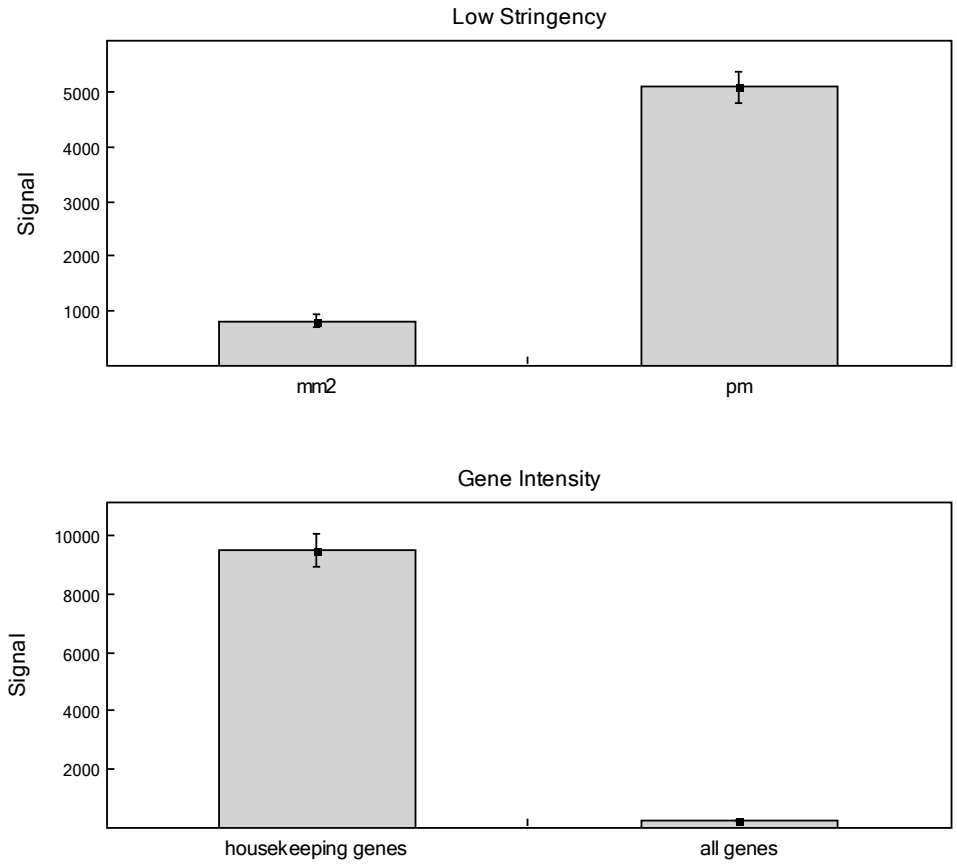


Figure 7-1. Quality of microarray data. Data quality was verified through set a of internal controls (hybridisation, negative, low stringency, gene intensity). All the signals from the four parameters were according to expectation and indicative of good quality data. Signals from hybridisation controls were in the appropriate order with the 'high signal' hybridisation control having the highest signal; negative controls have low signal intensity; signal for perfect probe (pm) > mismatched probe (mm2) and signal for housekeeping genes > all genes.

7.2.1.2 Effects of SAA1 on gene expression in THP-1 derived macrophages at 8 h

In total, 94 genes were upregulated with a fold difference of $\geq$ 2. The top ten most upregulated genes when human macrophages were treated with SAA1 are as shown in Table 7-1. More than half of the top ten genes are involved in immune regulatory processes, these includes chemokine (C-C motif) ligand 4 (*CCL4*), tumor necrosis factor, alpha-induced protein 6 (*TNFAIP6*), (C-C motif) ligand 1 (*CCL1*), chemokine (C-C motif) ligand 3 (*CCL3*), *IL23A*, and *IL-8*. The chemokines, *CCL1*, *CCL3*, *CCL4* and *IL-8* were highly

upregulated and made up 4 of the top 10 upregulated genes. *CCL4* has the highest fold increase of 21.6 while *IL-8* has a 6.6 increase in expression. *TNFAIP6* and lysosomal-associated membrane protein 3 (*LAMP3*) both have possible roles in matrix reorganization due to the presence of hyaluronic acid binding domain present in the protein.

There was only one gene that was downregulated with a fold difference of $\leq$ -2. Lysyl oxidase homolog 4 precursor (*LOXL4*) has a fold difference of -2.1. Differentially expressed genes are classified according to their functional roles which are based on information provided by gene annotation database, GoEAST. To minimize false positive, only genes with high *P*-value for the function indicated were included. The lists of genes classified according to their functions are shown in the following section.

Table 7-1. Top 10 upregulated genes when THP-1 derived macrophages were incubated with SAA1 for 8 h.

Gene symbol	Gene	Fold difference
CCL4	Chemokine (C-C motif) ligand 4	21.6
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	17.6
CCL1	Chemokine (C-C motif) ligand 1	16.4
CCL3	Chemokine (C-C motif) ligand 3	10.3
SOD2	Superoxide dismutase 2, mitochondrial	8.4
LAMP3	Lysosomal-associated membrane protein 3	8.3
IL23A	Interleukin 23, alpha subunit p19	7.8
IL-8	Interleukin 8	6.6
SLC2A6	Solute carrier family 2 (facilitated glucose transporter), member 6	5.9
MCOLN2	Mucolipin 2	5.3

7.2.1.2.1 Differentially expressed genes involved in angiogenesis

Angiogenesis is associated with the pathogenesis of atherosclerosis as it provides oxygen and nutrients supply to the atherosclerotic plaque and has possible role in tissue remodeling. There are 7 SAA1 upregulated genes with functional role in angiogenesis: *IL-8*, tumour necrosis factor, alpha-induced protein 2 (*TNFAIP2*), chemokine (C-C motif) ligand 2 (*CCL2*), interleukin 1, beta (*IL1B*), zinc finger CCCH-type containing 12A (*ZC3H12A*), *IL1A* and syndecan (*SDC4*) (Table 7-2). The fold differences are 6.6, 3.7, 2.9, 2.9, 2.6, 2.4 and 2.1 respectively. ZC3H12A is a reported angiogenic factor and promotes angiogenesis partly through the upregulation of cadherin 12 and cadherin 19 (Niu et al. 2008). IL1A was shown to promote angiogenesis *in vivo* through its stimulation of VEGF synthesis and release (Salven et al. 2002). Mice lacking SDC4 was reported to have impaired angiogenesis in the granulation tissue (Echtermeyer et al. 2001). *SERPINB2* and *CD82*, which are involved in anti-angiogenesis, were upregulated with a fold difference of 4.5 and 2.9 respectively. As there are more pro-angiogenic genes upregulated, it is possible that SAA1 stimulates angiogenesis to facilitate healing of damaged tissue and that the angiogenesis response is regulated by the expression of anti-angiogeneic proteins.

Table 7-2. Changes in gene expression of genes involved in angiogenesis.

Gene symbol	Gene	Fold difference
IL-8	Interleukin 8	6.6
TNFAIP2	Tumour necrosis factor, alpha-induced protein 2	3.7
CCL2	Chemokine (C-C motif) ligand 2	2.9
IL1B	Interleukin 1, beta	2.9
ZC3H12A	Zinc finger CCCH-type containing 12A	2.6
IL1A	Interleukin 1, alpha	2.4

Gene symbol	Gene	Fold difference
SDC4	Syndecan 4	2.1
*SERPINB2**	Plasminogen activator inhibitor-2	4.5
*CXCL10**	Chemokine (C-X-C motif) ligand 10	2.9
*CD82**	CD82 molecule	2.9

*Indicated genes have functional role in anti-angiogenesis

7.2.1.2.2 Differentially expressed genes involved in apoptotic process

Seven anti-apoptotic genes were upregulated upon SAA1 treatment. The most upregulated anti-apoptotic gene is *SERPINB2* with a fold difference of 4.5 (Table 7-3). SERPINB2 was reported to inhibit TNF-induced apoptosis in HeLa cells and HT-1080 fibrosarcoma cells (Dickinson et al. 1995; Kumar and Baglioni 1991). Baculoviral IAP repeat-containing 3 (BIRC3) inhibits both caspase 7 and 9 while TNF receptor-associated factor 1 (TRAF1) is known to be an anti-apoptotic agent. The effect of the anti-apoptotic genes is probably regulated with a corresponding upregulation of apoptotic genes, tumor necrosis factor (*TNF*), B-cell CLL/lymphoma 3 (BCL3) and tumor necrosis factor receptor superfamily, member 9 (*TNFRSF9*). The induction of anti-apoptotic genes could possibly be a protective mechanism as the phagocytosis of pathogens and the subsequent degradation of such pathogens can induce oxidative stress in macrophages. This hypothesis is in part supported by the upregulation of superoxide dismutase 2, mitochondrial (*SOD2*) upon SAA1 treatment.

Table 7-3. Changes in gene expression of genes involved in apoptosis or anti-apoptotic activity

Gene symbol	Gene	Fold difference
SERPINB2	Plasminogen activator inhibitor-2	4.5
ADORA2A	Adenosine A2a receptor	3.1
IER3	Immediate early response 3	2.7
BIRC3	Baculoviral IAP repeat-containing 3	2.4
TRAF1	TNF receptor-associated factor 1	2.1
HBEGF	Heparin-binding EGF-like growth factor	2.1
PIM2	Pim-2 oncogene	2.0
*TNF**	Tumor necrosis factor	2.7
*BCL3**	B-cell CLL/lymphoma 3	2.1
*TNFRSF9**	Tumor necrosis factor receptor superfamily, member 9	2.1

*Indicated genes have functional role in apoptosis.

7.2.1.2.3 Differentially expressed genes involved in inflammatory processes

Genes involved in the regulation of immune processes form the bulk of the genes that were upregulation upon SAA1 treatment. In total, 16 pro-inflammatory genes were upregulated. SAA1 is a massive inducer of chemokines: *CCL4*, *CCL1*, *CCL3*, *IL-8*, Granulocyte chemotactic protein 2 (*CXCL6*), Chemokine (C-X-C motif) ligand 10 (*CXCL10*) and chemokine (C-C motif) ligand 2 (*CCL2*) were all upregulated (Table 7-4). 4 anti-inflammatory genes, *TNFAIP6*, *SERPINB2*, *ZC3H12A* and *CD83*, were also upregulated. The large number of pro-inflammatory genes that were upregulated suggests that one of the major functions of SAA1 is to stimulate the migration of immune cells to site of injury.

Table 7-4. Changes in gene expression of genes involved in inflammatory or anti-inflammatory activity.

Gene symbol	Gene	Fold difference
CCL4	Chemokine (C-C motif) ligand 4	21.6
CCL1	Chemokine (C-C motif) ligand 1	16.4
CCL3	Chemokine (C-C motif) ligand 3	10.3
IL23A	Interleukin 23, alpha subunit p19	7.8
IL-8	Interleukin 8	6.6
CD40	CD40 molecule	4.9
CXCL6	Granulocyte chemotactic protein 2	3.2
CXCL10	Chemokine (C-X-C motif) ligand 10	2.9
IL1B	Interleukin 1, beta	2.9
CCL2	Chemokine (C-C motif) ligand 2	2.9
IL18R1	Interleukin 18 receptor 1	2.8
TNF	Tumor necrosis factor	2.7
PTGS2	Prostaglandin-endoperoxide synthase 2	2.7
IL1A	Interleukin 1, alpha	2.4
CCL20	Chemokine (C-C motif) ligand 20	2.2
CSF2	Colony stimulating factor 2	2.0
*TNFAIP6**	Tumor necrosis factor, alpha-induced protein 6	17.6
*SERPINB2**	Plasminogen activator inhibitor-2	4.5
*ZC3H12A**	Zinc finger CCCH-type containing 12A	2.6
*CD83**	CD83 molecule	2.1

*Indicated genes have functional role in anti-inflammation.

7.2.1.2.4 Differentially expressed genes involved in phagocytosis

Five genes with functional role in phagocytosis were upregulated with fold difference ranging from 4.6 for myristoylated alanine-rich protein kinase C substrate (*MARCKS*) to 2.7 for V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (*SRC*) (Table 7-5). MARCKS, hemopoietic cell kinase (*HCK*) and neutrophil cytosolic factor 1 (*NCF1*) are notable genes of the Fc gamma receptor-mediated phagocytosis pathway (KEGG: ko04666). MARCKS and HCK are involved in the regulation of the actin cytoskeletion which is important for phagocytosis while NCF1 is important for the digestion of bacteria by initiating respiratory burst.

Table 7-5. Changes in gene expression of genes involved in phagocytosis.

Gene symbol	Gene	Fold difference
MARCKS	Myristoylated alanine-rich protein kinase C substrate	4.6
ADORA2A	Adenosine A2a receptor	3.1
HCK	Hemopoietic cell kinase	2.8
NCF1	Neutrophil cytosolic factor 1	2.8
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	2.7

7.2.1.2.5 Differentially expressed genes with possible role in tissue remodeling/wound healing

In total, there are 11 genes with possible role in tissue remodeling that were upregulated (Table 7-6). The potential for a role in tissue remodeling is based on the ability of the gene to interact with the components of the extraceullar matrix. Based on gene annotation, CD44 has functional role in collagen binding while both CD44 and TNFAIP6 are able to bind to

hyaluronic acid. As tissue remodeling is an essential process of wound healing, it is possible that these genes also have a role in wound healing. SDC4 is upregulated in the dermis of skin wounds (Gallo et al. 1996) and SDC4 knockout mice have delayed wound repair (Echtermeyer et al. 2001).

Table 7-6. Changes in gene expression of genes involved in tissue remodeling/wound healing.

Gene symbol	Gene	Fold difference
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	17.6
LAMP3	Lysosomal-associated membrane protein 3	8.3
IL23A	Interleukin 23, alpha subunit p19	7.8
LEPREL1	Leprecan-like 1	3.6
PTGS2	Prostaglandin-endoperoxide synthase 2	2.7
BIRC3	Baculoviral IAP repeat-containing 3	2.4
CD44	CD44 molecule	2.4
ITGA1	Integrin, alpha 1	2.4
SDC4	Syndecan 4	2.1
HBEGF	Heparin-binding EGF-like growth factor	2.1
PIM2	Pim-2 oncogene	2.0
LOXL4	Lysyl oxidase homolog 4 precursor	-2.1

7.2.1.3 Effects of SAA1 on gene expression in THP-1 derived macrophages at 24 h

There are 12 SAA1 upregulated genes with fold difference of more than 2 (Table 7-7). Stimulation of genetic expression in THP-1 derived macrophages appears to cease at 24 h with fewer genes upregulated. In addition, the fold differences of upregulated genes at 24 h are lower as compared to 8 h. In general, the genes that were upregulated at 24 h are a subset

of the upregulated genes at 8 h albeit at a lower expression level. Only three genes, Bobby sox homolog (*BBX*), histone cluster 1, H4c (*HIST1H4C*) and PRP40 pre-mRNA processing factor 40 homolog A (*PRPF40A*), were not differentially upregulated at 8 h. However, the functional roles of these 3 genes are currently not established.

Three genes were downregulated with a fold difference of less than -2. However, other than *LOXL4*, there is currently limited information on the function of both Fc fragment of IgG binding protein (*FCGBP*) and *CD300A*. Hence, the pattern of genetic expression in 24 h is not wholly different from that at 8 h in that the genes that were upregulated at 24 h were similarly upregulated at 8 h. Thus, the functional impact of SAA1 can be studied by just analyzing the genetic expression pattern at 8 h.

Table 7-7. Genes that were differentially expressed upon treatment with SAA1 at 24 h.

Gene symbol	Gene	Fold difference
CCL1	Chemokine (C-C motif) ligand 1	7.3
SERPINA9	Serpin peptidase inhibitor, clade A, member 9	3.5
EBI3	Epstein-Barr virus induced 3	3.4
SOD2	Speroxide dismutase 2, mitochondrial	3.1
CCL4	Chemokine (C-C motif) ligand 4	2.6
CCL3	Chemokine (C-C motif) ligand 3	2.3
LEPREL1	Leprecan-like 1	2.3
BBX	Bobby sox homolog (Drosophila)	2.2
IL-8	Interleukin 8	2.0
HIST1H4C	Histone cluster 1, H4c	2.0
PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)	2.0

Gene symbol	Gene	Fold difference
LAMP3	Lysosomal-associated membrane protein 3	2.0
FCGBP	Fc fragment of IgG binding protein	-2.5
LOXL4	Lysyl oxidase-like 4	-2.2
CD300A	CD300a molecule	-2.0

7.2.1.4 Enriched pathways upon treatment with SAA1 at 8 h

The upregulated genes at 8 h were analysed for pathway enrichment using PathwayAPI. The top 20 pathways are as shown in Table 7-8 and the results are agreeable with the earlier classification of genes according to their functions. A number of pathways involved in immune regulation were enriched including cytokine-cytokine receptor intereaction (*P*-value = 2.41 X 10^{-22}), hematopoietic cell lineage (*P*-value = 5.19 X 10^{-11}), toll-like receptor signaling (*P*-value = 5.31 X 10^{-8}), inflammatory response (*P*-value = 1.21 X 10^{-6}), IL10 signaling (*P*-value = 4.08 X 10^{-5}). TNF-α/NF-kB signaling (*P*-value = 1.11 X 10^{-4}), IL6 signaling (*P*-value = 1.71 X 10^{-4}) and IL-1 signaling pathway (*P*-value = 1.05 X 10^{-3}). SAA1 also induces pathways that are important for lipid homeostasis with 2 pathways indicated: mitochondrial LC-Fatty acid beta oxidation (*P*-value = 4.62 X 10^{-5}) and adipogenesis (*P*-value= 2.07 X 10^{-4}). SAA1 treatment also appears to simulate the process of angiogenesis as various pro-angiogenic pathways were enriched: epidermal growth factor receptor 1 signaling (*P*-value = 1.58 X 10^{-3}), TGF-ß signaling (*P*-value = 3.75 X 10^{-3}), VEGF signaling (*P*-value = 1.29 X 10^{-2}).

Table 7-8. Enriched pathways upon treatment with SAA1 for 8 h.

Pathway	*P*-value
Cytokine-cytokine receptor interaction	2.41×10^{-22}
Hematopoietic cell lineage	5.19×10^{-11}
Toll-like receptor signaling	5.31×10^{-8}
Inflammatory response	1.21×10^{-6}
Myometrial relaxation and contraction	2.29×10^{-5}
GnRH signaling pathway	3.51×10^{-5}
IL-10 signaling	4.08×10^{-5}
Mitochondrial LC-Fatty acid beta oxidation	4.62×10^{-5}
TNF-α/NF-kB signaling pathway	1.11×10^{-4}
IL-6 signaling	1.71×10^{-4}
Gap junction	1.82×10^{-4}
Adipogenesis	2.07×10^{-4}
Apoptosis signaling	2.51×10^{-4}
p38 MAPK signaling	4.72×10^{-4}
Focal adhesion	4.87×10^{-4}
IL-1 signaling pathway	1.05×10^{-3}
Cell adhesion molecules	1.29×10^{-3}
Adipocytokine signaling pathway	1.38×10^{-3}
EGFR1 signaling pathway	1.58×10^{-3}
JAK/Stat signaling	2.04×10^{-3}

7.2.2 Validation of microarray results using real-time PCR

Microarray results were validated using real-time PCR. Nine genes from four of the functional role categories (apoptosis, inflammation, phagocytosis and tissue remodeling) were chosen. All of the 9 genes were found to be significantly upregulated (Table 7-9). The fold changes were quite similar for 7 of the 9 genes; however, the fold changes for *SERPINB2*, and *CCL1* were noticeably higher when compared to the microarray results (Table 7-10). As all the 9 genes were upregulated, the pattern of induction of genetic expression in THP-1 derived macrophages as determined by microarray is generally accurate. In addition, the correlation coefficient between the results from microarray and real-time PCR is 0.81 (Figure 7-2).

Table 7-9. Validation of microarray results using real-time PCR. Fold changes between SAA1 treatment and untreated were determined using B2M as an internal control for all genes except SERPINB2 and CCL1. FXYD5 was used as an internal control for SERPINB2 and CCL1.

Gene	Fold changes	95% confidence interval	*P*–value
BIRC3	3.2	2.9 – 3.5	<0.0001
SERPINB2	14.7	8.9 – 20.8	0.023
CCL1	24.7	13.9-68.9	0.022
CCL3	9.8	8.6 – 10.8	<0.0001
CCL4	13.4	11.5 – 16.5	<0.0001
MARCKS	6.0	5.1 – 7.1	<0.0001
IL23A	24.4	20.6 – 29.5	<0.0001
ITGA1	2.3	1.8 – 3.3	<0.0001
OLR1	2.6	2.2 – 3.1	<0.0001

Table 7-10. Comparision of fold changes between that determined by real-time PCR and microarray.

Gene	Real time PCR	Microarray
BIRC3	3.2	2.4
CCL3	9.8	10.3
IL23A	24.4	7.8
ITGA1	2.3	2.4
MARCKS	6.0	4.6
OLR1	2.6	3.4
CCL4	13.4	17.0
SERPINB2	14.7	4.5
CCL1	24.7	16.4

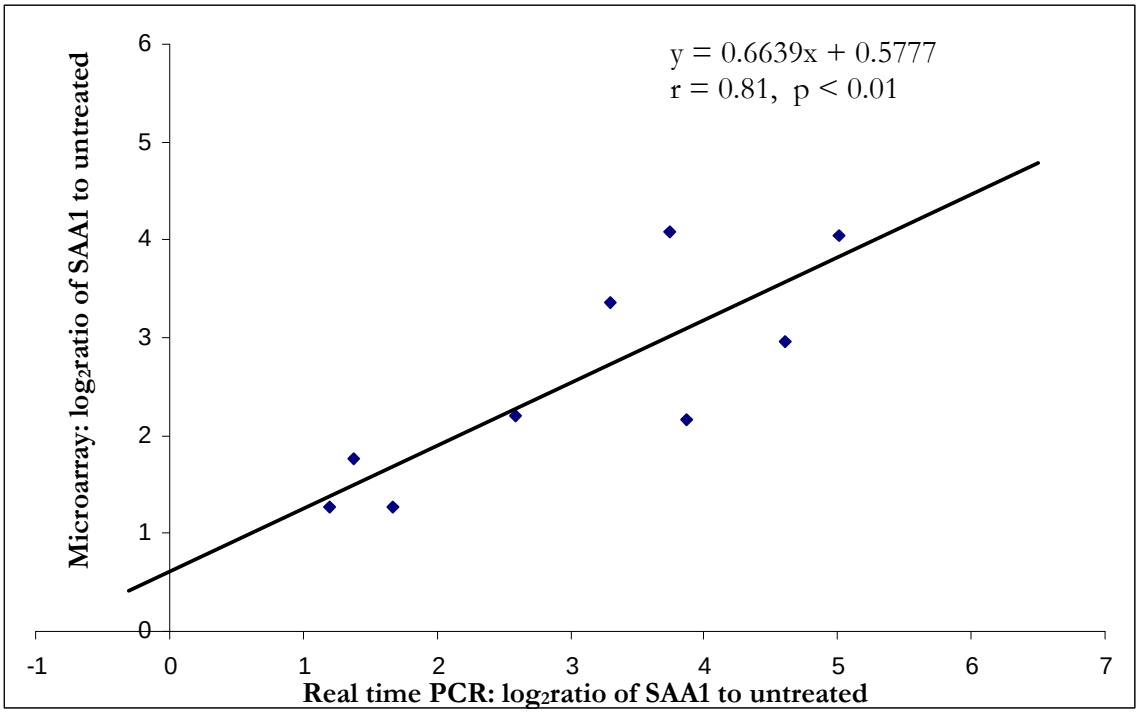


Figure 7-2. Correlation coefficient between microarray data and real time PCR. The genes used for the determination of r were as displayed in Table 7-10.

7.2.3 Effects of SAA1 on chemokines production

Secretion of CCL1, CCL3 and CCL4 were significantly increased upon treatment with 1 µg/ml or 3 µg/ml SAA1 (Figure 7-3 to Figure 7-5). Upon treatment with 1 µg/ml SAA1, the secretion of CCL1, CCL3 and CCL4 were increased by 14, 20 and 27 fold respectively. The result is consistent with the high expression level obtained from real-time PCR whereby the genetic expression of CCL1, CCL3 and CCL4 increased by 24.7, 9.8 and 13.4 fold respectively.

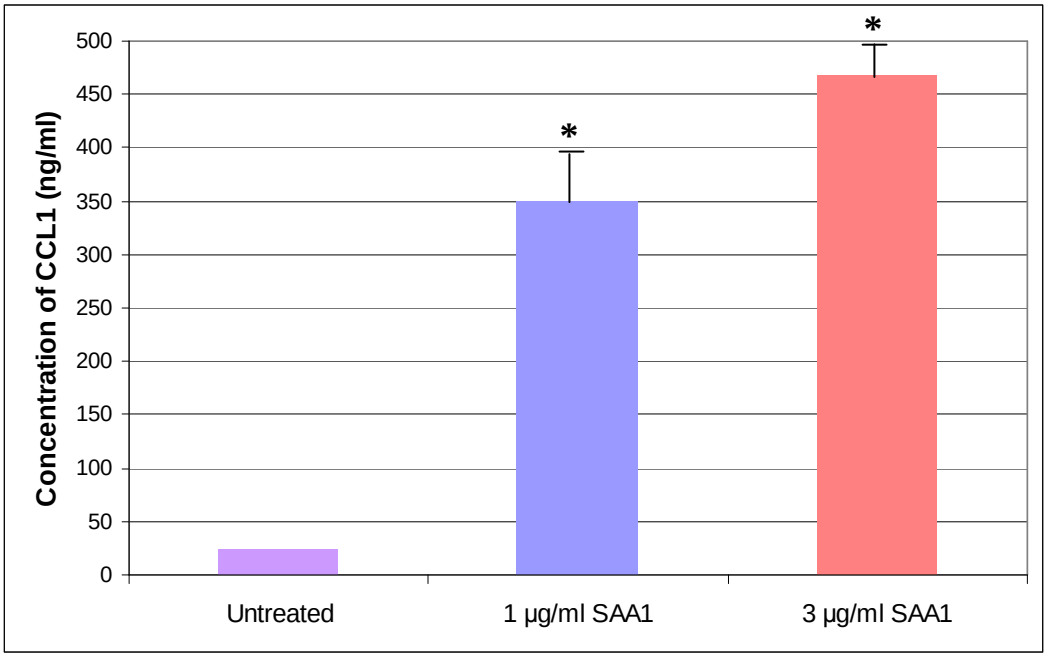


Figure 7-3. Effects of varying concentrations of recombinant human SAA1 on the secretion of CCL1 from THP-1 monocytes derived macrophages. 2.5 X 10^6 cells were incubated with recombinant SAA1 for 24 hours and the supernatants were assayed for CCL1 using ELISA. Error bars represent standard deviations. (n = 3). *P<0.01 for recombinant human SAA1 vs untreated.

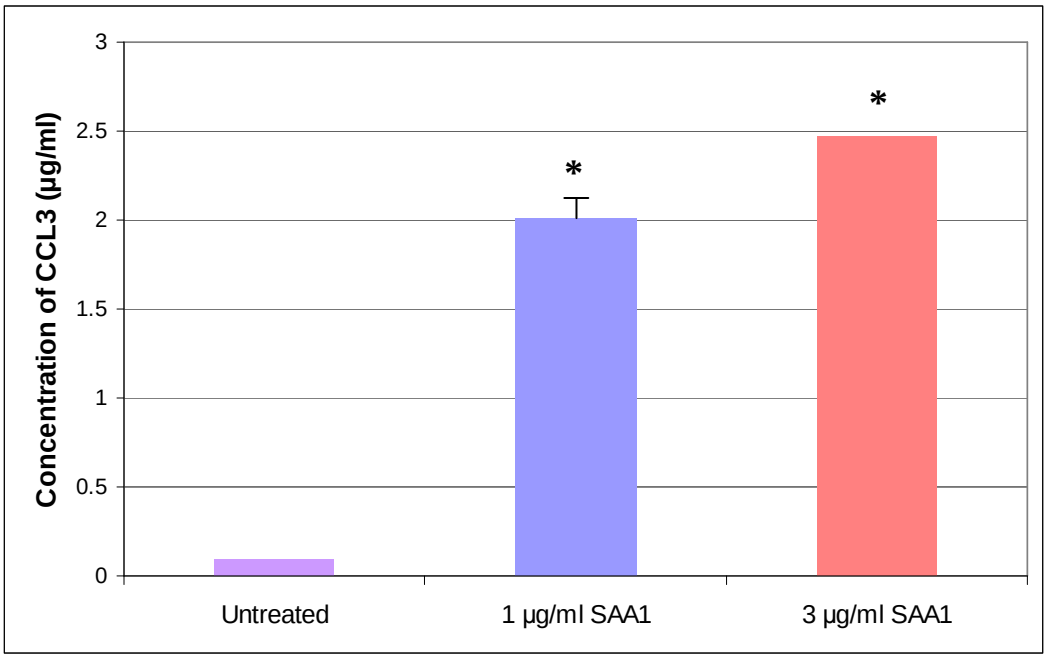


Figure 7-4. Effects of varying concentrations of recombinant human SAA1 on the secretion of CCL3 from THP-1 monocytes derived macrophages. Error bars represent standard deviations. (n = 3). *P<0.01 for recombinant human SAA1 vs untreated.

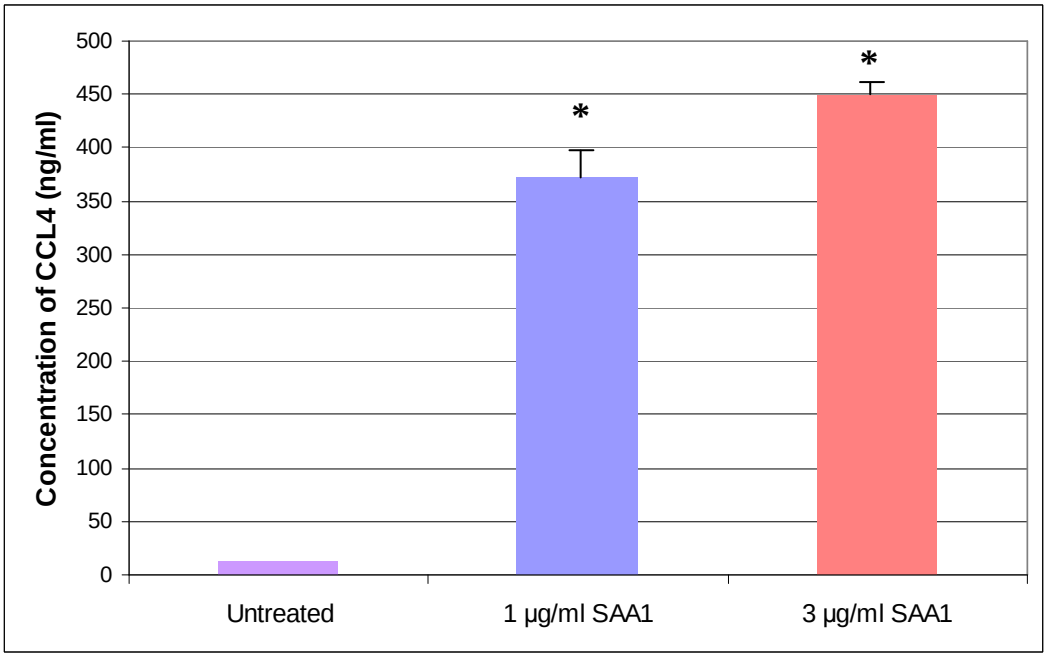


Figure 7-5. Effects of varying concentrations of recombinant human SAA1 on the secretion of CCL4 from THP-1 monocytes derived macrophages. Error bars represent standard deviations. (n = 3). *P<0.01 for recombinant human SAA1 vs untreated.

7.2.4 Surface receptors of SAA1

Induction of secretion of TNF-α by SAA1 in THP-1 derived macrophages is partly dependent on the presence of both TLR2 and CLA-1 (Figure 7-6). The concentration of TNF-α was significantly reduced in the presence of either TLR2 antibody or CLA-1 antibody; TNF-α decreased by 42.8% and 34.9% in the presence of TLR2 and CLA-1 antibody respectively.

Secretion of MCP-1 upon induction by SAA1 in THP-1 derived macrophages is partly dependent on TLR2 (Figure 7-7); the concentration of MCP-1 decreased by 47.1% upon treatment with TLR2 antibody and SAA1. However, unlike the secretion of TNF-α, CLA-1, appears to play a role in the negative regulation of MCP-1 production in THP1-derived macrophages; the secretion of MCP-1 increased by 96.3% in the presence of CLA-1 antibody.

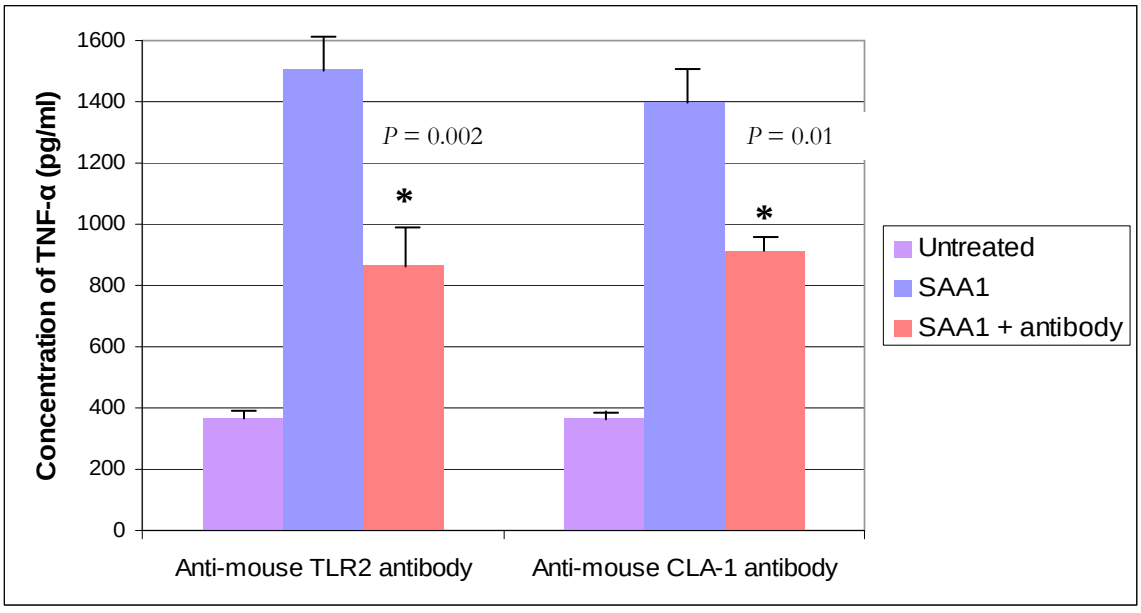


Figure 7-6. Effects of blocking TLR2 and CLA-1 surface receptors on TNF-α secretion. Both TLR2 and CLA-1 surface receptors are required for the production of TNF-α. The concentration of TNF-α was significantly lower in the presence of either TLR2 or CLA-1 antibody. Error bars represent standard deviations (n =3). *$P < 0.01$ for recombinant human SAA1 vs untreated.

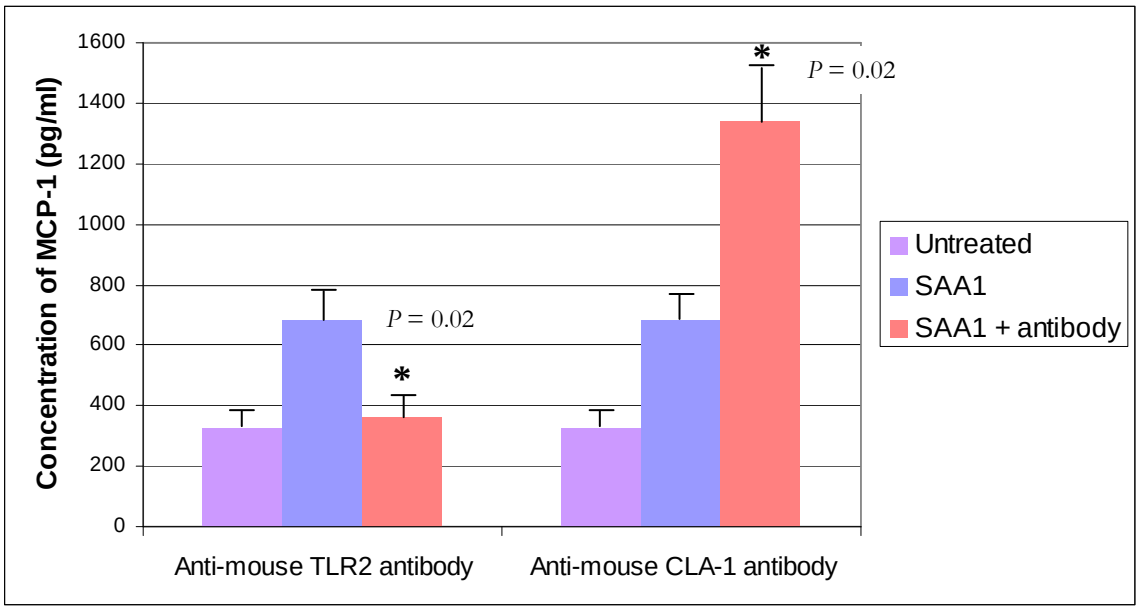


Figure 7-7. Effects of blocking TLR2 and CLA-1 surface receptors on MCP-1 secretion. The concentration of MCP-1 produced was significantly reduced upon treatment with TLR2 antibody, however, production of MCP-1 was significantly increased in the presence of CLA-1 antibody. Error bars represent standard deviations (n =3). *$P < 0.01$ for recombinant human SAA1 vs untreated.

7.3 Discussion

7.3.1 Effects of SAA1 on gene expression profile in THP-1 derived macrophages

SAA1 is a significant component of A-SAA. The exact role of SAA1 in innate immunity and in CAD, however, still remains unknown. As such, a microarray profiling assay was initated to determine the possible pathways that SAA1 induces in macrophages. The knowledge of the genes and pathways that are induced in the presence of SAA1 will help clarify the roles of SAA1 both in innate immunity and CAD.

Microarray analysis shows that a significant number of genes involved in inflammation, angiogenesis, phagocytosis and tissue remodeling were upregulated. *In silico* pathway enrichment analysis also shows that pathways regulating inflammation, angiogenesis and tissue remodeling were enriched. The chemokines, *CCL1*, *CCL3*, *CCL4* and *IL-8* were

highly upregulated upon SAA1 treatment. Upregulation of the chemokines as well as genes that are involved in toll-like receptor response, inflammatory response, cell adhesion molecules and TNF-α signaling pathway indicate that SAA1 has major roles to play in acute inflammation.

In addition to the mobilization of immune cells, SAA1 increases the phagocytic activity of macrophages through the induction of genes such as *MARCKS* which alters the morphology of macrophages to facilitate phagocytosis. SAA1 also facilitates the healing of the damaged tissue by inducing the expression of pro-angiogenic genes and genes with function in tissue remodeling. Angiogenesis helps to promote the healing of damaged tissues as newly formed blood capillaries are able to supply nutrients that are needed for healing. Although, the regulatory pathways and exact functional roles of the various genes listed with tissue remodeling functions are not known, emerging studies have highlighted their importance in tissue remodeling. CD44 is a reported cell surface receptor of hyaluronan and recruits MMP7 and HBEGF to the surface of various tissues (Yu et al. 2002b). Thus, as an APP, SAA1 plays multiple roles in acute inflammation which include stimulating the migration of immune cells, facilitating phagocytosis by macrophages and aiding in the healing of tissues that are damaged due to infection or trauma.

To analyse whether SAA1 plays an atherogenic or atheroprotective role in CAD, it is important to consider the regulation and source of production of SAA1. SAA1 is secreted in large amount by the liver during an APR. However, the production of SAA1 during an acute-phase response is tightly regulated and its concentration returns to base level after a few days (Marhaug et al. 1986). As atherosclerosis is a gradual process, it is unlikely the A-SAA that is produced by the liver plays an important role in the disease pathogenesis as APR is generally short-lived. Furthermore, as SAA1 has limited ability to move across the cell

membrane, it is likely that serum SAA1 concentration has little impact on atherosclerosis. However, the perivascular adipose tissue might be an important source of SAA1. As there is no physical barrier between the perivascular adipose tissue and the macrophages residing in the wall of the arteries, there is a likely exposure of macrophages to SAA1 produced by the adipose tissue. Furthermore, the adipose tissue is a major source of SAA under non acute-phase condition (Yang et al. 2006) and the size of epicardial adipose tissue was also reported to correlate with risk factors for CAD (Iacobellis et al. 2003; Wang et al. 2009).

SAA1 stimulates the production of inflammatory cytokines that is beneficial when the body is under stress, however, a continuous stimulation of macrophages by SAA1 might be potentially atherogenic. The large induction of chemokines production can lead to the migration and accumulation of macrophages in the walls of the coronary artery; this could probably account for the finding in a study in which mice with large lesions have a high upregulation of SAA1 in macrophages (Smith et al. 2006). In addition, chronic upregulation of genes involve in angiogenesis and tissue remodeling is potentially atherogenic. However, it is important to note that SAA1 does stimulate the expression of atheroprotective genes such as SERPINB2 which might help regulate the population of immune cells in the lesion. The balance between the atherogenic and athero-protective roles of SAA1 might however be altered under condition of chronic production of SAA1.

7.3.2 Cell-surface receptors of SAA1

Previous studies have indicated that TLR2 and CLA-1 are surface receptors for A-SAA. In this study, TLR2 and CLA-1 are shown to be cell surface receptors of SAA1. However, the two surface receptors have differential roles in the secretion of cytokines in macrophages. TLR2 contributes positively to the secretion of both TNF-α and MCP-1 while CLA-1

increases the secretion of TNF-α but decreases the secretion of MCP-1. Thus, the production of cytokines as well as the change in genetic expression induced by SAA1 in the macrophages is likely to be regulated by a complex interplay of signaling pathways involving various cell surface receptors.

Due to technical difficultly, this assay involves the use of mouse RAW264 macrophages instead of human THP-1 derived macrophages. As the morphology of RAW264 macrophages is more consistent even under low confluency, it is more suited for the assay. As both TLR2 and CLA-1 are generally well-conserved between mouse and human, the usage of mouse macrophages instead of human macrophages should have little interference on the result. Furthermore, both antibodies that were used for the assay are able to recognize both the human and mouse form of the receptors.

For the statistical analysis, Bonferroni correction was used to correct for multiple comparisons. There are other methods to adjust for multiple testing; these include Sidak, bootstrapping and Hochberg. However, these methods are either not significantly less conservative than Bonferroni as in the case of Sidak or require more complex calculations as in the case of Hochberg.

The expression of both TLR2 and CLA-1 receptors in RAW264 macrophages were not verified. RAW264 macrophages were reported to express CLA-1 (Baranova et al. 2002; Schafer et al. 2009) in its basal state and that RAW264 macrophages have been used in a number of studies involving TLR2 agonists (Curry et al. 2004; Xu et al. 2007). Thus, the observed results are unlikely to be a false-positive although further verification through a binding assay as described in the following section will confirm these results.

7.3.3 Future works

The identification of the cell surface receptors for SAA1 is a preliminary study in this thesis. For this preliminary study, only two possible surface receptors of SAA1, TLR2 and CLA-1, were investigated. Three other receptors, TLR-4, FPRL1 and RAGE, were not investigated as suitable inhibitors or antibodies which do not have any direct interfering effect on cytokine secretion could be found at the time of the study. Nonetheless, these three surface receptors should be investigated to ascertain their role in cytokine production mediated by SAA1. In addition, as it is not feasible to introduce four neutralizing antibodies simultaneously, inhibition should be carried out by blocking a single receptor initially and determining which blockage has the greatest effect on cytokine production. As numerous receptors are likely to play a role in cytokine production, the synergistic effects of these receptors should be studied. This could be investigated through the simultaneous inhibition of two receptors. In addition, the binding of SAA1 to these five receptors should be determined through an *in-vitro* binding assay; this will confirm the role of these surface receptors both as a binding partner and a mediator of cytokine production. The effects of A-SAA on global gene expression in primary endothelial cells have been studied in our lab and the data can be found in GEO (GSE6241). The results also support an atherogenic role for A-SAA with upregulation of genes involved in inflammation, cell adhesion and plaque stability. It will be useful to determine the effects of SAA1 on gene expression in adipocytes. Adipocytes are an important secretor of adipokines such as cytokines and metalloproteinases which have important roles in metabolic syndrome and cardiovascular diseases. Both microarray and protein array study can be performed. The protein array study will allow for the detection of 182 adipokines that are known to be secreted by adipocytes. As perivascular adipocytes are located in close proximity to sites of

lesion development, the adipokines that are induced by SAA1 might contribute to atherogenesis. As there are clear differences in the global gene expression between endothelial cells and macrophages, it is expected that adipocytes would have a different gene expression profile. The results from this study will allow for a more comprehensive understanding of the role of SAA1 in lesion development.

8 CONCLUSION AND FUTURE WORKS

Before the commencement of this study, little is known about the roles of SAA1 during an APR as well as in the pathogenesis of CAD. Earlier studies indicate that SAA1 might have a potential atherogenic role due to its ability to induce tissue factor from endothelial cells as well as inflammatory cytokines from macrophages. However, the role of SAA1 in atherogenesis is contradicted by its reported positive effects on cholesterol efflux and metabolism. As such, this study was initiated to clarify certain aspects of the functional role of SAA1.

The variant screening of SAA1 suggests that SAA1 is a well-conserved protein. Only a few SNPs are present in the exons and the promoter region of *SAA1*. There are only two non-synonymous SNPs present in *SAA1*, p.Ala70Val and p.Ala75Val, both of which were found to have no association with CAD. Previously, these two SNPs were associated with amyloidosis in patients with various diseases such as FMF and RA. However, our study shows that they have no association with CAD. Unlike p.Ala70Val and p.Ala75Val, p.Gly90Asp involves an amino acid substitution that is non-conservative in nature. This is the only non-synonymous SNP of SAA1 identified that involved a non-conservative amino acid change. The SNP, however, does not have any association with CAD. Our functional analysis, however, also shows that the mutant protein has functional difference in terms of the extent of its induction of inflammatory cytokines and SERPINB2. Two cell surface receptors, TLR2 and CLA-1, were found to be partly responsible for the production of inflammatory cytokines in macrophages in a separate study. Furthermore, as SAA1 is unlikely to move across the cell membrane, the amino acid substitution in the mutant probably affects the binding of SAA1 to cell surface receptors and alters downstream signaling.

In order to establish the role of SAA1 in CAD, a microarray analysis was performed. The induction of genes involved in inflammation, chemotaxis, wound healing, tissue remodeling and phagocytosis support the role of SAA1 in innate immunity as well as being a prominent component of acute phase protein. This induction of gene expression is probably tightly controlled as APR which produces a massive amount of SAA1 is generally short lived. However, a steady source of production by perivascular adipocytes could potentially be atherogenic as it stimulates the migration of monocytes and activates important pathways that are potentially atherogenic.

Functional study shows that the variant protein induces lower levels of inflammatory cytokines in macrophages and neutrophils. In addition, microarray analysis shows that the variant induces a lower level of atheroprotective *SERPINB2*. A consideration of our results from the mutant characterization study and the microarray analysis (wild-type treatment vs untreated) suggests that chronic production of SAA1 is possibly atherogenic, however certain genes induced by SAA1 such as *SERPINB2* might have atheroprotective effects and could be potential therapeutic targets.

Perivascular adipocyte is an emerging area of study for both metabolic syndrome and CAD. The adipocytes secrete various proteins that are crucial for the pathogenesis of chronic diseases. The importance of perivascular adipocytes in CAD can be drawn from a study in which segment of coronary arteries that are adjacent to adipose tissues were found to be more prone to atherosclerosis (Ishikawa et al. 2009). Future works should involve *in vivo* studies conducted in SAA1 partial knockout mice. Studies should involve the quantification of SAA1 in region adjacent to the adipose tissue and whether this level correlates with lesion development. In addition, studies of the functional roles of SERPINB2 as well as its effects

on the gene expression in macrophages will help further our understanding on the possible atheroprotective effects of SERPINB2.

BIBLIOGRAPHY

Ajiro J, Narita I, Sato F, Saga D, Hasegawa H, Kuroda T, Nakano M, Gejyo F (2006) SAA1 gene polymorphisms and the risk of AA amyloidosis in Japanese patients with rheumatoid arthritis. Mod Rheumatol 16: 294-9

Akar N, Hasipek M, Ozturk A, Akar E, Tekin M (2006) Serum amyloid A1 -13 T/C alleles in Turkish familial Mediterranean fever patients with and without amyloidosis. J Nephrol 19: 318-21

Aliprantis AO, Yang RB, Weiss DS, Godowski P, Zychlinsky A (2000) The apoptotic signaling pathway activated by Toll-like receptor-2. EMBO J 19: 3325-36

Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, Kelvin DJ, Oppenheim JJ (1994) Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. J Exp Med 180: 203-9

Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R (2005) Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 366: 1267-78

Balogh K, Patocs A, Majnik J, Racz K, Hunyady L (2004) Genetic screening methods for the detection of mutations responsible for multiple endocrine neoplasia type 1. Mol Genet Metab 83: 74-81

Baranova I, Vishnyakova T, Bocharov A, Chen Z, Remaley AT, Stonik J, Eggerman TL, Patterson AP (2002) Lipopolysaccharide down regulates both scavenger receptor B1 and ATP binding cassette transporter A1 in RAW cells. Infect Immun 70: 2995-3003

Baranova IN, Vishnyakova TG, Bocharov AV, Kurlander R, Chen Z, Kimelman ML, Remaley AT, Csako G, Thomas F, Eggerman TL, Patterson AP (2005) Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. J Biol Chem 280: 8031-40

Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B (2007) Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med 357: 2109-22

Betts JC, Cheshire JK, Akira S, Kishimoto T, Woo P (1993) The role of NF-kappa B and NF-IL6 transactivating factors in the synergistic activation of human serum amyloid A gene expression by interleukin-1 and interleukin-6. J Biol Chem 268: 25624-31

Bloomfield D, Carlson GL, Sapre A, Tribble D, McKenney JM, Littlejohn TW, 3rd, Sisk CM, Mitchel Y, Pasternak RC (2009) Efficacy and safety of the cholesteryl ester transfer protein inhibitor anacetrapib as monotherapy and coadministered with atorvastatin in dyslipidemic patients. Am Heart J 157: 352-360 e2

Braunwald E (1997) Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med 337: 1360-9

Breslow JL (1997) Cardiovascular disease burden increases, NIH funding decreases. Nat Med 3: 600-1

Brody AR, Soler P, Basset F, Haschek WM, Witschi H (1981) Epithelial-mesenchymal associations of cells in human pulmonary fibrosis and in BHT-oxygen-induced fibrosis in mice. Exp Lung Res 2: 207-20

Buetow KH, Edmonson MN, Cassidy AB (1999) Reliable identification of large numbers of candidate SNPs from public EST data. Nat Genet 21: 323-5

Cai H, Song C, Endoh I, Goyette J, Jessup W, Freedman SB, McNeil HP, Geczy CL (2007) Serum amyloid A induces monocyte tissue factor. J Immunol 178: 1852-60

Cannon CP, Dansky HM, Davidson M, Gotto AM, Jr., Brinton EA, Gould AL, Stepanavage M, Liu SX, Shah S, Rubino J, Gibbons P, Hermanowski-Vosatka A, Binkowitz B, Mitchel Y, Barter P (2009) Design of the DEFINE trial: determining the EFficacy and tolerability of CETP INhibition with AnacEtrapib. Am Heart J 158: 513-519 e3

Carty CL, Heagerty P, Heckbert SR, Enquobahrie DA, Jarvik GP, Davis S, Tracy RP, Reiner AP (2009) Association of genetic variation in serum amyloid-A with cardiovascular disease and interactions with IL6, IL1RN, IL1beta and TNF genes in the Cardiovascular Health Study. J Atheroscler Thromb 16: 419-30

Casas JP, Cooper J, Miller GJ, Hingorani AD, Humphries SE (2006) Investigating the genetic determinants of cardiovascular disease using candidate genes and meta-analysis of association studies. Ann Hum Genet 70: 145-69

Cheng N, He R, Tian J, Ye PP, Ye RD (2008) Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. J Immunol 181: 22-6

Cheng X, Lu SH, Cui Y (2010) ECRG2 regulates ECM degradation and uPAR/FPRL1 pathway contributing cell invasion/migration. Cancer Lett 290: 87-95

Chiu JJ, Lee PL, Chen CN, Lee CI, Chang SF, Chen LJ, Lien SC, Ko YC, Usami S, Chien S (2004) Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin expressions induced by tumor necrosis factor-[alpha] in endothelial cells. Arterioscler Thromb Vasc Biol 24: 73-9

Cho WC, Yip TT, Cheng WW, Au JS (2010) Serum amyloid A is elevated in the serum of lung cancer patients with poor prognosis. Br J Cancer 102: 1731-5

Cocco E, Bellone S, El-Sahwi K, Cargnelutti M, Buza N, Tavassoli FA, Schwartz PE, Rutherford TJ, Pecorelli S, Santin AD (2010) Serum amyloid A: a novel biomarker for endometrial cancer. Cancer 116: 843-51

Coffelt SB, Tomchuck SL, Zwezdaryk KJ, Danka ES, Scandurro AB (2009) Leucine leucine-37 uses formyl peptide receptor-like 1 to activate signal transduction pathways, stimulate oncogenic gene expression, and enhance the invasiveness of ovarian cancer cells. Mol Cancer Res 7: 907-15

Collins FS, Brooks LD, Chakravarti A (1998) A DNA polymorphism discovery resource for research on human genetic variation. Genome Res 8: 1229-31

Connolly M, Marrelli A, Blades M, McCormick J, Maderna P, Godson C, Mullan R, FitzGerald O, Bresnihan B, Pitzalis C, Veale DJ, Fearon U (2010) Acute serum amyloid A

induces migration, angiogenesis, and inflammation in synovial cells in vitro and in a human rheumatoid arthritis/SCID mouse chimera model. J Immunol 184: 6427-37

Couttet P, Fromont-Racine M, Steel D, Pictet R, Grange T (1997) Messenger RNA deadenylylation precedes decapping in mammalian cells. Proc Natl Acad Sci U S A 94: 5628-33

Cox D, Boillot C, Canzian F (2001) Data mining: Efficiency of using sequence databases for polymorphism discovery. Hum Mutat 17: 141-50

Cremona M, Calabro E, Randi G, De Bortoli M, Mondellini P, Verri C, Sozzi G, Pierotti MA, La Vecchia C, Pastorino U, Bongarzone I (2010) Elevated levels of the acute-phase serum amyloid are associated with heightened lung cancer risk. Cancer 116: 1326-35

Curry H, Alvarez GR, Zwilling BS, Lafuse WP (2004) Toll-like receptor 2 stimulation decreases IFN-gamma receptor expression in mouse RAW264.7 macrophages. J Interferon Cytokine Res 24: 699-710

Dagar V, Chow CW, Ashley DM, Algar EM (2009) Rapid detection of SMARCB1 sequence variation using high resolution melting. BMC Cancer 9: 437

De Beer FC, Mallya RK, Fagan EA, Lanham JG, Hughes GR, Pepys MB (1982) Serum amyloid-A protein concentration in inflammatory diseases and its relationship to the incidence of reactive systemic amyloidosis. Lancet 2: 231-4

de Beer MC, Yuan T, Kindy MS, Asztalos BF, Roheim PS, de Beer FC (1995) Characterization of constitutive human serum amyloid A protein (SAA4) as an apolipoprotein. J Lipid Res 36: 526-34

De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA, Jr., Shin WS, Liao JK (1995) Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest 96: 60-8

Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67: 737-44

Dickinson JL, Bates EJ, Ferrante A, Antalis TM (1995) Plasminogen activator inhibitor type 2 inhibits tumor necrosis factor alpha-induced apoptosis. Evidence for an alternate biological function. J Biol Chem 270: 27894-904

Dragomir AC, Laskin JD, Laskin DL (2011) Macrophage activation by factors released from acetaminophen-injured hepatocytes: potential role of HMGB1. Toxicol Appl Pharmacol 253: 170-7

Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O (2010) Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. Circulation 122: 1837-45

Eccles KA, Sowden H, Porter KE, Parkin SM, Homer-Vanniasinkam S, Graham AM (2008) Simvastatin alters human endothelial cell adhesion molecule expression and inhibits leukocyte adhesion under flow. Atherosclerosis 200: 69-79

Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M, Goetinck P (2001) Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. J Clin Invest 107: R9-R14

Edbrooke MR, Foldi J, Cheshire JK, Li F, Faulkes DJ, Woo P (1991) Constitutive and NF-kappa B-like proteins in the regulation of the serum amyloid A gene by interleukin 1. Cytokine 3: 380-8

Edwards MR, Haas J, Panettieri RA, Jr., Johnson M, Johnston SL (2007) Corticosteroids and beta2 agonists differentially regulate rhinovirus-induced interleukin-6 via distinct Cis-acting elements. J Biol Chem 282: 15366-75

Erdman LK, Cosio G, Helmers AJ, Gowda DC, Grinstein S, Kain KC (2009) CD36 and TLR interactions in inflammation and phagocytosis: implications for malaria. J Immunol 183: 6452-9

Ernst S, Lange C, Wilbers A, Goebeler V, Gerke V, Rescher U (2004) An annexin 1 N-terminal peptide activates leukocytes by triggering different members of the formyl peptide receptor family. J Immunol 172: 7669-76

Forrest MJ, Bloomfield D, Briscoe RJ, Brown PN, Cumiskey AM, Ehrhart J, Hershey JC, Keller WJ, Ma X, McPherson HE, Messina E, Peterson LB, Sharif-Rodriguez W, Siegl PK, Sinclair PJ, Sparrow CP, Stevenson AS, Sun SY, Tsai C, Vargas H, Walker M, 3rd, West SH, White V, Woltmann RF (2008) Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone. Br J Pharmacol 154: 1465-73

Furman C, Copin C, Kandoussi M, Davidson R, Moreau M, McTaggiart F, Chapman MJ, Fruchart JC, Rouis M (2004) Rosuvastatin reduces MMP-7 secretion by human monocyte-derived macrophages: potential relevance to atherosclerotic plaque stability. Atherosclerosis 174: 93-8

Gabay C, Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 340: 448-54

Gallego C, Golenbock D, Gomez MA, Saravia NG (2011) Toll-like receptors participate in macrophage activation and intracellular control of Leishmania (Viannia) panamensis. Infect Immun 79: 2871-9

Gallo R, Kim C, Kokenyesi R, Adzick NS, Bernfield M (1996) Syndecans-1 and -4 are induced during wound repair of neonatal but not fetal skin. J Invest Dermatol 107: 676-83

Garbin U, Fratta Pasini A, Stranieri C, Cominacini M, Pasini A, Manfro S, Lugoboni F, Mozzini C, Guidi G, Faccini G, Cominacini L (2009) Cigarette smoking blocks the protective expression of Nrf2/ARE pathway in peripheral mononuclear cells of young heavy smokers favouring inflammation. PLoS One 4: e8225

Ghazouani L, Khalifa SB, Abboud N, Addad F, Khalfallah AB, Brahim N, Mediouni M, Almawi WY, Mahjoub T (2009) -308G>A and -1031T>C tumor necrosis factor gene polymorphisms in Tunisian patients with coronary artery disease. Clin Chem Lab Med 47: 1247-51

Gossl M, Herrmann J, Tang H, Versari D, Galili O, Mannheim D, Rajkumar SV, Lerman LO, Lerman A (2009) Prevention of vasa vasorum neovascularization attenuates early neointima formation in experimental hypercholesterolemia. Basic Res Cardiol 104: 695-706

Greenwel P, Tanaka S, Penkov D, Zhang W, Olive M, Moll J, Vinson C, Di Liberto M, Ramirez F (2000) Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. Mol Cell Biol 20: 912-8

Gross TJ, Sitrin RG (1990) The THP-1 cell line is a urokinase-secreting mononuclear phagocyte with a novel defect in the production of plasminogen activator inhibitor-2. J Immunol 144: 1873-9

Harris ED, Jr. (1990) Rheumatoid arthritis. Pathophysiology and implications for therapy. N Engl J Med 322: 1277-89

Hayashi K, Yandell DW (1993) How sensitive is PCR-SSCP? Hum Mutat 2: 338-46

He RL, Zhou J, Hanson CZ, Chen J, Cheng N, Ye RD (2009) Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. Blood 113: 429-37

Hu X, Dietz JD, Xia C, Knight DR, Loging WT, Smith AH, Yuan H, Perry DA, Keiser J (2009) Torcetrapib induces aldosterone and cortisol production by an intracellular calcium-mediated mechanism independently of cholesteryl ester transfer protein inhibition. Endocrinology 150: 2211-9

Humphries SE, Gudnason V, Whittall R, Day IN (1997) Single-strand conformation polymorphism analysis with high throughput modifications, and its use in mutation

detection in familial hypercholesterolemia. International Federation of Clinical Chemistry Scientific Division: Committee on Molecular Biology Techniques. Clin Chem 43: 427-35

Iacobellis G, Ribaudo MC, Assael F, Vecci E, Tiberti C, Zappaterreno A, Di Mario U, Leonetti F (2003) Echocardiographic epicardial adipose tissue is related to anthropometric and clinical parameters of metabolic syndrome: a new indicator of cardiovascular risk. J Clin Endocrinol Metab 88: 5163-8

Idel S, Dansky HM, Breslow JL (2003) A20, a regulator of NFkappaB, maps to an atherosclerosis locus and differs between parental sensitive C57BL/6J and resistant FVB/N strains. Proc Natl Acad Sci U S A 100: 14235-40

Ishikawa Y, Akasaka Y, Suzuki K, Fujiwara M, Ogawa T, Yamazaki K, Niino H, Tanaka M, Ogata K, Morinaga S, Ebihara Y, Kawahara Y, Sugiura H, Takimoto T, Komatsu A, Shinagawa T, Taki K, Satoh H, Yamada K, Yanagida-Iida M, Shimokawa R, Shimada K, Nishimura C, Ito K, Ishii T (2009) Anatomic properties of myocardial bridge predisposing to myocardial infarction. Circulation 120: 376-83

Jensen LE, Whitehead AS (1998) Regulation of serum amyloid A protein expression during the acute-phase response. Biochem J 334 (Pt 3): 489-503

Ji Y, Jian B, Wang N, Sun Y, Moya ML, Phillips MC, Rothblat GH, Swaney JB, Tall AR (1997) Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. J Biol Chem 272: 20982-5

Jimenez-Marin A, Collado-Romero M, Ramirez-Boo M, Arce C, Garrido JJ (2009) Biological pathway analysis by ArrayUnlock and Ingenuity Pathway Analysis. BMC Proc 3 Suppl 4: S6

Johnson BD, Kip KE, Marroquin OC, Ridker PM, Kelsey SF, Shaw LJ, Pepine CJ, Sharaf B, Bairey Merz CN, Sopko G, Olson MB, Reis SE (2004) Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: the National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE). Circulation 109: 726-32

Kagan HM, Raghavan J, Hollander W (1981) Changes in aortic lysyl oxidase activity in diet-induced atherosclerosis in the rabbit. Arteriosclerosis 1: 287-91

Kanehisa M (2009) Representation and analysis of molecular networks involving diseases and drugs. Genome Inform 23: 212-3

Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MP (2003) Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 112: 1176-85

Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML (2007) Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. N Engl J Med 356: 1620-30

Kelder T, Pico AR, Hanspers K, van Iersel MP, Evelo C, Conklin BR (2009) Mining biological pathways using WikiPathways web services. PLoS One 4: e6447

Kelkitli E, Bilgici B, Tokgoz B, Dilek M, Bedir A, Akpolat I, Utas C, Akpolat T (2006) SAA1 alpha/alpha alleles in amyloidosis. J Nephrol 19: 189-91

Kinkley SM, Bagshaw WL, Tam SP, Kisilevsky R (2006) The path of murine serum amyloid A through peritoneal macrophages. Amyloid 13: 123-34

Kisilevsky R, Subrahmanyan L (1992) Serum amyloid A changes high density lipoprotein's cellular affinity. A clue to serum amyloid A's principal function. Lab Invest 66: 778-85

Kivirikko KI, Helaakoski T, Tasanen K, Vuori K, Myllyla R, Parkkonen T, Pihlajaniemi T (1990) Molecular biology of prolyl 4-hydroxylase. Ann N Y Acad Sci 580: 132-42

Kluve-Beckerman B, Malle E, Vitt H, Pfeiffer C, Benson M, Steinmetz A (1991) Characterization of an isoelectric focusing variant of SAA1 (ASP-72) in a family of Turkish origin. Biochem Biophys Res Commun 181: 1097-102

Kluve-Beckerman B, Song M (1995) Genes encoding human serum amyloid A proteins SAA1 and SAA2 are located 18 kb apart in opposite transcriptional orientations. Gene 159: 289-90

Ko KW, Erickson B, Lehner R (2009) Es-x/Ces1 prevents triacylglycerol accumulation in McArdle-RH7777 hepatocytes. Biochim Biophys Acta 1791: 1133-43

Koj A (1996) Initiation of acute phase response and synthesis of cytokines. Biochim Biophys Acta 1317: 84-94

Kosuge M, Ebina T, Ishikawa T, Hibi K, Tsukahara K, Okuda J, Iwahashi N, Ozaki H, Yano H, Kusama I, Nakati T, Umemura S, Kimura K (2007) Serum amyloid A is a better predictor of clinical outcomes than C-reactive protein in non-ST-segment elevation acute coronary syndromes. Circ J 71: 186-90

Kotani K, Satoh N, Kato Y, Araki R, Koyama K, Okajima T, Tanabe M, Oishi M, Yamakage H, Yamada K, Hattori M, Shimatsu A (2009) A novel oxidized low-density lipoprotein marker, serum amyloid A-LDL, is associated with obesity and the metabolic syndrome. Atherosclerosis 204: 526-31

Kuhn C, 3rd, Boldt J, King TE, Jr., Crouch E, Vartio T, McDonald JA (1989) An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 140: 1693-703

Kumar S, Baglioni C (1991) Protection from tumor necrosis factor-mediated cytolysis by overexpression of plasminogen activator inhibitor type-2. J Biol Chem 266: 20960-4

Kumon Y, Loose LD, Birbara CA, Sipe JD (1997) Rheumatoid arthritis exhibits reduced acute phase and enhanced constitutive serum amyloid A protein in synovial fluid relative to serum. A comparison with C-reactive protein. J Rheumatol 24: 14-9

Kumon Y, Suehiro T, Hashimoto K, Nakatani K, Sipe JD (1999) Local expression of acute phase serum amyloid A mRNA in rheumatoid arthritis synovial tissue and cells. J Rheumatol 26: 785-90

Kwon HM, Sangiorgi G, Ritman EL, McKenna C, Holmes DR, Jr., Schwartz RS, Lerman A (1998) Enhanced coronary vasa vasorum neovascularization in experimental hypercholesterolemia. J Clin Invest 101: 1551-6

Ladenson PW, Kristensen JD, Ridgway EC, Olsson AG, Carlsson B, Klein I, Baxter JD, Angelin B (2010) Use of the thyroid hormone analogue eprotirome in statin-treated dyslipidemia. N Engl J Med 362: 906-16

Lappalainen T, Kolehmainen M, Schwab U, Pulkkinen L, Laaksonen DE, Rauramaa R, Uusitupa M, Gylling H (2008) Serum concentrations and expressions of serum amyloid A and leptin in adipose tissue are interrelated: the Genobin Study. Eur J Endocrinol 158: 333-41

LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J, Wenger NK (2005) Intensive lipid lowering with atorvastatin in patients with stable coronary disease. N Engl J Med 352: 1425-35

Laufs U, La Fata V, Plutzky J, Liao JK (1998) Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 97: 1129-35

Lee BC, Kim MS, Choi SH, Kim TS (2010) Involvement of capsular polysaccharide via a TLR2/NF-kappaB pathway in Vibrio vulnificus-induced IL-8 secretion of human intestinal epithelial cells. Int J Mol Med 25: 581-91

Lee HY, Kim SD, Shim JW, Lee SY, Yun J, Bae YS (2009a) LL-37 inhibits serum amyloid A-induced IL-8 production in human neutrophils. Exp Mol Med 41: 325-33

Lee HY, Kim SD, Shim JW, Yun J, Kim K, Bae YS (2009b) Activation of formyl peptide receptor like-1 by serum amyloid A induces CCL2 production in human umbilical vein endothelial cells. Biochem Biophys Res Commun 380: 313-7

Lee MS, Yoo SA, Cho CS, Suh PG, Kim WU, Ryu SH (2006) Serum amyloid A binding to formyl peptide receptor-like 1 induces synovial hyperplasia and angiogenesis. J Immunol 177: 5585-94

Lee RT, Libby P (1997) The unstable atheroma. Arterioscler Thromb Vasc Biol 17: 1859-67

Lewis KE, Kirk EA, McDonald TO, Wang S, Wight TN, O'Brien KD, Chait A (2004) Increase in serum amyloid a evoked by dietary cholesterol is associated with increased atherosclerosis in mice. Circulation 110: 540-5

Li WH, Sadler LA (1991) Low nucleotide diversity in man. Genetics 129: 513-23

Liao F, Lusis AJ, Berliner JA, Fogelman AM, Kindy M, de Beer MC, de Beer FC (1994) Serum amyloid A protein family. Differential induction by oxidized lipids in mouse strains. Arterioscler Thromb 14: 1475-9

Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C (2004) Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin Chem 50: 1156-64

Lindhorst E, Young D, Bagshaw W, Hyland M, Kisilevsky R (1997) Acute inflammation, acute phase serum amyloid A and cholesterol metabolism in the mouse. Biochim Biophys Acta 1339: 143-54

Linke RP, Bock V, Valet G, Rothe G (1991) Inhibition of the oxidative burst response of N-formyl peptide-stimulated neutrophils by serum amyloid-A protein. Biochem Biophys Res Commun 176: 1100-5

Liu Q, Feng J, Sommer SS (1996) Bi-directional dideoxy fingerprinting (Bi-ddF): a rapid method for quantitative detection of mutations in genomic regions of 300-600 bp. Hum Mol Genet 5: 107-14

Lorkowska B, Chlopicki S (2005) Statins as coronary vasodilators in isolated bovine coronary arteries--involvement of PGI2 and NO. Prostaglandins Leukot Essent Fatty Acids 72: 133-8

Lowell CA, Stearman RS, Morrow JF (1986) Transcriptional regulation of serum amyloid A gene expression. J Biol Chem 261: 8453-61

Lukacs NW, Ward PA (1996) Inflammatory mediators, cytokines, and adhesion molecules in pulmonary inflammation and injury. Adv Immunol 62: 257-304

Lupia E, Bosco O, Goffi A, Poletto C, Locatelli S, Spatola T, Cuccurullo A, Montrucchio G (2010) Thrombopoietin contributes to enhanced platelet activation in cigarette smokers. Atherosclerosis 210: 314-9

Lusis AJ (2000) Atherosclerosis. Nature 407: 233-41

Luyendyk JP, Piper JD, Tencati M, Reddy KV, Holscher T, Zhang R, Luchoomun J, Chen X, Min W, Kunsch C, Mackman N (2007) A novel class of antioxidants inhibit LPS induction of tissue factor by selective inhibition of the activation of ASK1 and MAP kinases. Arterioscler Thromb Vasc Biol 27: 1857-63

Malle E, Bollmann A, Steinmetz A, Gemsa D, Leis HJ, Sattler W (1997) Serum amyloid A (SAA) protein enhances formation of cyclooxygenase metabolites of activated human monocytes. FEBS Lett 419: 215-9

Malle E, Sodin-Semrl S, Kovacevic A (2009) Serum amyloid A: an acute-phase protein involved in tumour pathogenesis. Cell Mol Life Sci 66: 9-26

Malle E, Steinmetz A, Raynes JG (1993) Serum amyloid A (SAA): an acute phase protein and apolipoprotein. Atherosclerosis 102: 131-46

Manzi S, Meilahn EN, Rairie JE, Conte CG, Medsger TA, Jr., Jansen-McWilliams L, D'Agostino RB, Kuller LH (1997) Age-specific incidence rates of myocardial infarction and angina in women with systemic lupus erythematosus: comparison with the Framingham Study. Am J Epidemiol 145: 408-15

Marhaug G, Harklau L, Olsen B, Husby G, Husebekk A, Wang H (1986) Serum amyloid A protein in acute myocardial infarction. Acta Med Scand 220: 303-6

Marrades MP, Gonzalez-Muniesa P, Martinez JA, Moreno-Aliaga MJ (2010) A dysregulation in CES1, APOE and other lipid metabolism-related genes is associated to cardiovascular risk factors linked to obesity. Obes Facts 3: 312-8

Marsche G, Frank S, Raynes JG, Kozarsky KF, Sattler W, Malle E (2007) The lipidation status of acute-phase protein serum amyloid A determines cholesterol mobilization via scavenger receptor class B, type I. Biochem J 402: 117-24

McKenney JM, Davidson MH, Shear CL, Revkin JH (2006) Efficacy and safety of torcetrapib, a novel cholesteryl ester transfer protein inhibitor, in individuals with below-average high-density lipoprotein cholesterol levels on a background of atorvastatin. J Am Coll Cardiol 48: 1782-90

McNeill E (2010) RVX-208, a stimulator of apolipoprotein AI gene expression for the treatment of cardiovascular diseases. Curr Opin Investig Drugs 11: 357-64

Meek RL, Urieli-Shoval S, Benditt EP (1994) Expression of apolipoprotein serum amyloid A mRNA in human atherosclerotic lesions and cultured vascular cells: implications for serum amyloid A function. Proc Natl Acad Sci U S A 91: 3186-90

Meeker AK, Sack GH, Jr. (1998) A fusion protein between serum amyloid A and staphylococcal nuclease--synthesis, purification, and structural studies. Proteins 30: 381-7

Meng X, Ao L, Song Y, Babu A, Yang X, Wang M, Weyant MJ, Dinarello CA, Cleveland JC, Jr., Fullerton DA (2008) Expression of functional Toll-like receptors 2 and 4 in human aortic valve interstitial cells: potential roles in aortic valve inflammation and stenosis. Am J Physiol Cell Physiol 294: C29-35

Meuwese MC, de Groot E, Duivenvoorden R, Trip MD, Ose L, Maritz FJ, Basart DC, Kastelein JJ, Habib R, Davidson MH, Zwinderman AH, Schwocho LR, Stein EA (2009) ACAT inhibition and progression of carotid atherosclerosis in patients with familial hypercholesterolemia: the CAPTIVATE randomized trial. JAMA 301: 1131-9

Mizuno K, Nakaya N, Ohashi Y, Tajima N, Kushiro T, Teramoto T, Uchiyama S, Nakamura H (2008) Usefulness of pravastatin in primary prevention of cardiovascular events in women: analysis of the Management of Elevated Cholesterol in the Primary Prevention Group of Adult Japanese (MEGA study). Circulation 117: 494-502

Mogensen J, Bahl A, Kubo T, Elanko N, Taylor R, McKenna WJ (2003) Comparison of fluorescent SSCP and denaturing HPLC analysis with direct sequencing for mutation screening in hypertrophic cardiomyopathy. J Med Genet 40: e59

Mohler ER, 3rd, Ballantyne CM, Davidson MH, Hanefeld M, Ruilope LM, Johnson JL, Zalewski A (2008) The effect of darapladib on plasma lipoprotein-associated phospholipase A2 activity and cardiovascular biomarkers in patients with stable coronary heart disease or coronary heart disease risk equivalent: the results of a multicenter, randomized, double-blind, placebo-controlled study. J Am Coll Cardiol 51: 1632-41

Mora S, Glynn RJ, Hsia J, MacFadyen JG, Genest J, Ridker PM (2010) Statins for the primary prevention of cardiovascular events in women with elevated high-sensitivity C-reactive protein or dyslipidemia: results from the Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) and meta-analysis of women from primary prevention trials. Circulation 121: 1069-77

Moriguchi M, Kaneko H, Terai C, Koseki Y, Kajiyama H, Inada S, Kitamura Y, Kamatani N (2005) Relative transcriptional activities of SAA1 promoters polymorphic at position -13(T/C): potential association between increased transcription and amyloidosis. Amyloid 12: 26-32

Morrow DA, Rifai N, Antman EM, Weiner DL, McCabe CH, Cannon CP, Braunwald E (2000) Serum amyloid A predicts early mortality in acute coronary syndromes: A TIMI 11A substudy. J Am Coll Cardiol 35: 358-62

Nakamura K, Sasaki T, Cheng XW, Iguchi A, Sato K, Kuzuya M (2009) Statin prevents plaque disruption in apoE-knockout mouse model through pleiotropic effect on acute inflammation. Atherosclerosis 206: 355-61

Nakamura T (2008) Clinical strategies for amyloid A amyloidosis secondary to rheumatoid arthritis. Mod Rheumatol 18: 109-18

Nakamura T, Higashi S, Tomoda K, Tsukano M, Baba S, Shono M (2006) Significance of SAA1.3 allele genotype in Japanese patients with amyloidosis secondary to rheumatoid arthritis. Rheumatology (Oxford) 45: 43-9

Navab M, Hama SY, Hough GP, Hedrick CC, Sorenson R, La Du BN, Kobashigawa JA, Fonarow GC, Berliner JA, Laks H, Fogelman AM (1998) High density associated enzymes: their role in vascular biology. Curr Opin Lipidol 9: 449-56

Nemes S, Jonasson JM, Genell A, Steineck G (2009) Bias in odds ratios by logistic regression modelling and sample size. BMC Med Res Methodol 9: 56

Niessner A, Steiner S, Speidl WS, Pleiner J, Seidinger D, Maurer G, Goronzy JJ, Weyand CM, Kopp CW, Huber K, Wolzt M, Wojta J (2006) Simvastatin suppresses endotoxin-induced upregulation of toll-like receptors 4 and 2 in vivo. Atherosclerosis 189: 408-13

Nishikawa H, Miura S, Zhang B, Shimomura H, Arai H, Tsuchiya Y, Matsuo K, Saku K (2004) Statins induce the regression of left ventricular mass in patients with angina. Circ J 68: 121-5

Nissen SE, Tuzcu EM, Schoenhagen P, Brown BG, Ganz P, Vogel RA, Crowe T, Howard G, Cooper CJ, Brodie B, Grines CL, DeMaria AN (2004) Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial. JAMA 291: 1071-80

Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE (2008) Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). J Biol Chem 283: 14542-51

O'Donovan MC, Oefner PJ, Roberts SC, Austin J, Hoogendoorn B, Guy C, Speight G, Upadhyaya M, Sommer SS, McGuffin P (1998) Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics 52: 44-9

Ogasawara K, Mashiba S, Wada Y, Sahara M, Uchida K, Aizawa T, Kodama T (2004) A serum amyloid A and LDL complex as a new prognostic marker in stable coronary artery disease. Atherosclerosis 174: 349-56

Patel R, Nagueh SF, Tsybouleva N, Abdellatif M, Lutucuta S, Kopelen HA, Quinones MA, Zoghbi WA, Entman ML, Roberts R, Marian AJ (2001) Simvastatin induces regression of cardiac hypertrophy and fibrosis and improves cardiac function in a transgenic rabbit model of human hypertrophic cardiomyopathy. Circulation 104: 317-24

Pico AR, Kelder T, van Iersel MP, Hanspers K, Conklin BR, Evelo C (2008) WikiPathways: pathway editing for the people. PLoS Biol 6: e184

Poitou C, Divoux A, Faty A, Tordjman J, Hugol D, Aissat A, Keophiphath M, Henegar C, Commans S, Clement K (2009) Role of serum amyloid a in adipocyte-macrophage cross talk and adipocyte cholesterol efflux. J Clin Endocrinol Metab 94: 1810-7

Poitou C, Viguerie N, Cancello R, De Matteis R, Cinti S, Stich V, Coussieu C, Gauthier E, Courtine M, Zucker JD, Barsh GS, Saris W, Bruneval P, Basdevant A, Langin D, Clement K (2005) Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. Diabetologia 48: 519-28

Preciado-Patt L, Hershkoviz R, Fridkin M, Lider O (1996a) Serum amyloid A binds specific extracellular matrix glycoproteins and induces the adhesion of resting CD4+ T cells. J Immunol 156: 1189-95

Preciado-Patt L, Pras M, Fridkin M (1996b) Binding of human serum amyloid A (hSAA) and its high-density lipoprotein3 complex (hSAA-HDL3) to human neutrophils. Possible

implication to the function of a protein of an unknown physiological role. Int J Pept Protein Res 48: 503-13

Pruzanski W, de Beer FC, de Beer MC, Stefanski E, Vadas P (1995) Serum amyloid A protein enhances the activity of secretory non-pancreatic phospholipase A2. Biochem J 309 (Pt 2): 461-4

Quinn KL, Henriques M, Tabuchi A, Han B, Yang H, Cheng WE, Tole S, Yu H, Luo A, Charbonney E, Tullis E, Lazarus A, Robinson LA, Ni H, Peterson BR, Kuebler WM, Slutsky AS, Zhang H (2011) Human neutrophil peptides mediate endothelial-monocyte interaction, foam cell formation, and platelet activation. Arterioscler Thromb Vasc Biol 31: 2070-9

Ragoussis J (2009) Genotyping technologies for genetic research. Annu Rev Genomics Hum Genet 10: 117-33

Ramankulov A, Lein M, Johannsen M, Schrader M, Miller K, Loening SA, Jung K (2008) Serum amyloid A as indicator of distant metastases but not as early tumor marker in patients with renal cell carcinoma. Cancer Lett 269: 85-92

Ridker PM, Hennekens CH, Buring JE, Rifai N (2000) C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 342: 836-43

Rios DL, Cerqueira CC, Bonfim-Silva R, Araujo LJ, Pereira JF, Gadelha SR, Barbosa AA (2010) Interleukin-1 beta and interleukin-6 gene polymorphism associations with angiographically assessed coronary artery disease in Brazilians. Cytokine 50: 292-6

Risch N, Merikangas K (1996) The future of genetic studies of complex human diseases. Science 273: 1516-7

Rizzello V, Liuzzo G, Trabetti E, Di Giannuario G, Brugaletta S, Santamaria M, Piro M, Boccanelli A, Pignatti PF, Biasucci LM, Crea F (2010) Role of the CD14 C(-260)T promoter polymorphism in determining the first clinical manifestation of coronary artery disease. J Cardiovasc Med (Hagerstown) 11: 20-5

Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340: 115-26

Salven P, Hattori K, Heissig B, Rafii S (2002) Interleukin-1alpha promotes angiogenesis in vivo via VEGFR-2 pathway by inducing inflammatory cell VEGF synthesis and secretion. FASEB J 16: 1471-3

Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A (2008) Is serum amyloid A an endogenous TLR4 agonist? J Leukoc Biol 83: 1174-80

Sarles H, Bernard JP, Johnson C (1989) Pathogenesis and epidemiology of chronic pancreatitis. Annu Rev Med 40: 453-68

Schafer G, Guler R, Murray G, Brombacher F, Brown GD (2009) The role of scavenger receptor B1 in infection with Mycobacterium tuberculosis in a murine model. PLoS One 4: e8448

Schreiber J, Jenner RG, Murray HL, Gerber GK, Gifford DK, Young RA (2006) Coordinated binding of NF-kappaB family members in the response of human cells to lipopolysaccharide. Proc Natl Acad Sci U S A 103: 5899-904

Schroder WA, Le TT, Major L, Street S, Gardner J, Lambley E, Markey K, MacDonald KP, Fish RJ, Thomas R, Suhrbier A (2010) A physiological function of inflammation-associated SerpinB2 is regulation of adaptive immunity. J Immunol 184: 2663-70

Selvatici R, Falzarano S, Mollica A, Spisani S (2006) Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. Eur J Pharmacol 534: 1-11

Serruys PW, Garcia-Garcia HM, Buszman P, Erne P, Verheye S, Aschermann M, Duckers H, Bleie O, Dudek D, Botker HE, von Birgelen C, D'Amico D, Hutchinson T, Zambanini A, Mastik F, van Es GA, van der Steen AF, Vince DG, Ganz P, Hamm CW, Wijns W, Zalewski A (2008) Effects of the direct lipoprotein-associated phospholipase A(2) inhibitor darapladib on human coronary atherosclerotic plaque. Circulation 118: 1172-82

Sewell KL, Trentham DE (1993) Pathogenesis of rheumatoid arthritis. Lancet 341: 283-6

Shah C, Hari-Dass R, Raynes JG (2006) Serum amyloid A is an innate immune opsonin for Gram-negative bacteria. Blood 108: 1751-7

Sheikh S, Rainger GE, Gale Z, Rahman M, Nash GB (2003) Exposure to fluid shear stress modulates the ability of endothelial cells to recruit neutrophils in response to tumor necrosis factor-alpha: a basis for local variations in vascular sensitivity to inflammation. Blood 102: 2828-34

Shim JW, Jo SH, Kim SD, Lee HY, Yun J, Bae YS (2009) Lysophosphatidylglycerol inhibits formyl peptide receptorlike-1-stimulated chemotactic migration and IL-1beta production from human phagocytes. Exp Mol Med 41: 584-91

Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ (2010) HMGB1 and RAGE in inflammation and cancer. Annu Rev Immunol 28: 367-88

Sirois-Gagnon D, Chamberland A, Perron S, Brisson D, Gaudet D, Laprise C (2011) Association of common polymorphisms in the fractalkine receptor (CX3CR1) with obesity. Obesity (Silver Spring) 19: 222-7

Smith JD, Peng DQ, Dansky HM, Settle M, Baglione J, Le Goff W, Chakrabarti E, Xu Y, Peng X (2006) Transcriptome profile of macrophages from atherosclerosis-sensitive and atherosclerosis-resistant mice. Mamm Genome 17: 220-9

Soh D, Dong D, Guo Y, Wong L (2010) Consistency, comprehensiveness, and compatibility of pathway databases. BMC Bioinformatics 11: 449

Song C, Hsu K, Yamen E, Yan W, Fock J, Witting PK, Geczy CL, Freedman SB (2009) Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. Atherosclerosis 207: 374-83

Stevens FJ (2004) Hypothetical structure of human serum amyloid A protein. Amyloid 11: 71-80

Strachan T, Reed P (1999) Human Molecular Genetics, 2nd edn. Wiley-Liss

Sundell CL, Somers PK, Meng CQ, Hoong LK, Suen KL, Hill RR, Landers LK, Chapman A, Butteiger D, Jones M, Edwards D, Daugherty A, Wasserman MA, Alexander RW, Medford RM, Saxena U (2003) AGI-1067: a multifunctional phenolic antioxidant, lipid modulator, anti-inflammatory and antiatherosclerotic agent. J Pharmacol Exp Ther 305: 1116-23

Tam SP, Ancsin JB, Tan R, Kisilevsky R (2005) Peptides derived from serum amyloid A prevent, and reverse, aortic lipid lesions in apoE-/- mice. J Lipid Res 46: 2091-101

Tam SP, Kisilevsky R, Ancsin JB (2008) Acute-phase-HDL remodeling by heparan sulfate generates a novel lipoprotein with exceptional cholesterol efflux activity from macrophages. PLoS One 3: e3867

Tang WC, Yap MK, Yip SP (2008) A review of current approaches to identifying human genes involved in myopia. Clin Exp Optom 91: 4-22

Tardif JC, Gregoire J, Schwartz L, Title L, Laramee L, Reeves F, Lesperance J, Bourassa MG, L'Allier PL, Glass M, Lambert J, Guertin MC (2003) Effects of AGI-1067 and probucol after percutaneous coronary interventions. Circulation 107: 552-8

Tardif JC, L'Allier P L, Ibrahim R, Gregoire JC, Nozza A, Cossette M, Kouz S, Lavoie MA, Paquin J, Brotz TM, Taub R, Pressacco J (2010) Treatment with 5-lipoxygenase inhibitor VIA-2291 (Atreleuton) in patients with recent acute coronary syndrome. Circ Cardiovasc Imaging 3: 298-307

Tardif JC, McMurray JJ, Klug E, Small R, Schumi J, Choi J, Cooper J, Scott R, Lewis EF, L'Allier PL, Pfeffer MA (2008) Effects of succinobucol (AGI-1067) after an acute coronary syndrome: a randomised, double-blind, placebo-controlled trial. Lancet 371: 1761-8

Thorn CF, Whitehead AS (2002) Differential glucocorticoid enhancement of the cytokine-driven transcriptional activation of the human acute phase serum amyloid A genes, SAA1 and SAA2. J Immunol 169: 399-406

Tobin JF, Celeste AJ (2006) Bone morphogenetic proteins and growth differentiation factors as drug targets in cardiovascular and metabolic disease. Drug Discov Today 11: 405-11

Topper JN, Gimbrone MA, Jr. (1999) Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. Mol Med Today 5: 40-6

Trager J, Ward MM (2001) Mortality and causes of death in systemic lupus erythematosus. Curr Opin Rheumatol 13: 345-51

Turnell W, Sarra R, Glover ID, Baum JO, Caspi D, Baltz ML, Pepys MB (1986) Secondary structure prediction of human SAA1. Presumptive identification of calcium and lipid binding sites. Mol Biol Med 3: 387-407

Tward A, Xia YR, Wang XP, Shi YS, Park C, Castellani LW, Lusis AJ, Shih DM (2002) Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. Circulation 106: 484-90

Twyman RM, Primrose SB (2003) Techniques patents for SNP genotyping. Pharmacogenomics 4: 67-79

Uhlar CM, Whitehead AS (1999) Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem 265: 501-23

Utku U, Dilek M, Akpolat I, Bedir A, Akpolat T (2007) SAA1 alpha/alpha alleles in Behcet's disease related amyloidosis. Clin Rheumatol 26: 927-9

van der Hilst JC, Drenth JP, Bodar EJ, Bijzet J, van der Meer JW, Simon A (2005) Serum amyloid A serum concentrations and genotype do not explain low incidence of amyloidosis in Hyper-IgD syndrome. Amyloid 12: 115-9

Van Lenten BJ, Hama SY, de Beer FC, Stafforini DM, McIntyre TM, Prescott SM, La Du BN, Fogelman AM, Navab M (1995) Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. J Clin Invest 96: 2758-67

Van Lenten BJ, Wagner AC, Navab M, Anantharamaiah GM, Hama S, Reddy ST, Fogelman AM (2007) Lipoprotein inflammatory properties and serum amyloid A levels but not cholesterol levels predict lesion area in cholesterol-fed rabbits. J Lipid Res 48: 2344-53

Vergeer M, Bots ML, van Leuven SI, Basart DC, Sijbrands EJ, Evans GW, Grobbee DE, Visseren FL, Stalenhoef AF, Stroes ES, Kastelein JJ (2008) Cholesteryl ester transfer protein inhibitor torcetrapib and off-target toxicity: a pooled analysis of the rating atherosclerotic disease change by imaging with a new CETP inhibitor (RADIANCE) trials. Circulation 118: 2515-22

Verrecchia F, Wagner EF, Mauviel A (2002) Distinct involvement of the Jun-N-terminal kinase and NF-kappaB pathways in the repression of the human COL1A2 gene by TNF-alpha. EMBO Rep 3: 1069-74

Verstak B, Nagpal K, Bottomley SP, Golenbock DT, Hertzog PJ, Mansell A (2009) MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-mediated NF-kappaB proinflammatory responses. J Biol Chem 284: 24192-203

Vossen RH, Aten E, Roos A, den Dunnen JT (2009) High-resolution melting analysis (HRMA): more than just sequence variant screening. Hum Mutat 30: 860-6

Wang L, Lashuel HA, Walz T, Colon W (2002) Murine apolipoprotein serum amyloid A in solution forms a hexamer containing a central channel. Proc Natl Acad Sci U S A 99: 15947-52

Wang TD, Lee WJ, Shih FY, Huang CH, Chang YC, Chen WJ, Lee YT, Chen MF (2009) Relations of epicardial adipose tissue measured by multidetector computed tomography to components of the metabolic syndrome are region-specific and independent of anthropometric indexes and intraabdominal visceral fat. J Clin Endocrinol Metab 94: 662-9

Wang X, Chai H, Wang Z, Lin PH, Yao Q, Chen C (2008a) Serum amyloid A induces endothelial dysfunction in porcine coronary arteries and human coronary artery endothelial cells. Am J Physiol Heart Circ Physiol 295: H2399-408

Wang Y, Couture OP, Qu L, Uthe JJ, Bearson SM, Kuhar D, Lunney JK, Nettleton D, Dekkers JC, Tuggle CK (2008b) Analysis of porcine transcriptional response to Salmonella enterica serovar Choleraesuis suggests novel targets of NFkappaB are activated in the mesenteric lymph node. BMC Genomics 9: 437

Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL (2001) Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab 280: E685-94

Wu J, Wang Y, Zhang Y, Li L (2011) Association between interleukin-16 polymorphisms and risk of coronary artery disease. DNA Cell Biol 30: 305-8

Xie X, Ma Y-T, Yang Y-N, Fu Z-Y, Li X-M, Huang D, Ma X, Chen B-D, Liu F (2010) Polymorphisms in the <italic>SAA</italic>1/2 Gene Are Associated with Carotid Intima Media Thickness in Healthy Han Chinese Subjects: The Cardiovascular Risk Survey. PLoS One 5: e13997

Xu L, Badolato R, Murphy WJ, Longo DL, Anver M, Hale S, Oppenheim JJ, Wang JM (1995) A novel biologic function of serum amyloid A. Induction of T lymphocyte migration and adhesion. J Immunol 155: 1184-90

Xu R, Ogino S, Lip V, Fang H, Wu BL (2003) Comparison of PCR-RFLP with allele-specific PCR in genetic testing for spinal muscular atrophy. Genet Test 7: 277-81

Xu WY, Wang L, Wang HM, Wang YQ, Liang YF, Zhao TT, Wu YZ (2007) TLR2 and TLR4 agonists synergistically up-regulate SR-A in RAW264.7 through p38. Mol Immunol 44: 2315-23

Yamada T, Okuda Y, Takasugi K, Itoh K, Igari J (2001) Relative serum amyloid A (SAA) values: the influence of SAA1 genotypes and corticosteroid treatment in Japanese patients with rheumatoid arthritis. Ann Rheum Dis 60: 124-7

Yamada T, Wada A, Itoh Y, Itoh K (1999) Serum amyloid A1 alleles and plasma concentrations of serum amyloid A. Amyloid 6: 199-204

Yanbaeva DG, Dentener MA, Creutzberg EC, Wesseling G, Wouters EF (2007) Systemic effects of smoking. Chest 131: 1557-66

Yang RZ, Lee MJ, Hu H, Pollin TI, Ryan AS, Nicklas BJ, Snitker S, Horenstein RB, Hull K, Goldberg NH, Goldberg AP, Shuldiner AR, Fried SK, Gong DW (2006) Acute-phase serum

amyloid A: an inflammatory adipokine and potential link between obesity and its metabolic complications. PLoS Med 3: e287

Yilmaz E, Balci B, Kutlay S, Ozen S, Erturk S, Oner A, Besbas N, Bakkaloglu A (2003) Analysis of the modifying effects of SAA1, SAA2 and TNF-alpha gene polymorphisms on development of amyloidosis in FMF patients. Turk J Pediatr 45: 198-202

Yu H, Maurer F, Medcalf RL (2002a) Plasminogen activator inhibitor type 2: a regulator of monocyte proliferation and differentiation. Blood 99: 2810-8

Yu WH, Woessner JF, Jr., McNeish JD, Stamenkovic I (2002b) CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev 16: 307-23

Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L (2004) Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 364: 937-52

Zairis MN, Adamopoulou EN, Manousakis SJ, Lyras AG, Bibis GP, Ampartzidou OS, Apostolatos CS, Anastassiadis FA, Hatzisavvas JJ, Argyrakis SK, Foussas SG (2007) The impact of hs C-reactive protein and other inflammatory biomarkers on long-term cardiovascular mortality in patients with acute coronary syndromes. Atherosclerosis 194: 397-402

Zhao Y, Zhou S, Heng CK (2007) Impact of serum amyloid A on tissue factor and tissue factor pathway inhibitor expression and activity in endothelial cells. Arterioscler Thromb Vasc Biol 27: 1645-50

Zhou L, Myers AN, Vandersteen JG, Wang L, Wittwer CT (2004) Closed-tube genotyping with unlabeled oligonucleotide probes and a saturating DNA dye. Clin Chem 50: 1328-35

Zhu X, Feng T, Li Y, Lu Q, Elston RC (2010) Detecting rare variants for complex traits using family and unrelated data. Genet Epidemiol 34: 171-87

Zimlichman S, Danon A, Nathan I, Mozes G, Shainkin-Kestenbaum R (1990) Serum amyloid A, an acute phase protein, inhibits platelet activation. J Lab Clin Med 116: 180-6

APPENDIX 6-1 - Differential gene expression in THP-1 macrophages upon treatment with either wild-type or variant SAA1 for 24 h

Gene Symbol	Gene	Fold Difference (Wild-type/Variant)
CD63	CD63 molecule	0.43
CLOCK	Clock homolog (mouse)	0.43
P704P	Prostate-specific P704P	0.44
LOC389286	Similar to FKSG62	0.47
TERF2IP	Telomeric repeat binding factor 2, interacting protein	0.47
LST1	Leukocyte specific transcript 1	0.49
NUPR1	Nuclear protein 1	0.49
MED24	Mediator complex subunit 24	0.49
RASSF4	Ras association (RalGDS/AF-6) domain family member 4	0.49
NPTXR	Neuronal pentraxin receptor	0.49
IFT122	Intraflagellar transport 122 homolog (Chlamydomonas)	0.49
OSGEP	O-sialoglycoprotein endopeptidase	0.49
LOC402221	Similar to actin alpha 1 skeletal muscle protein	0.49
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	0.50
MRPL28	Mitochondrial ribosomal protein L28	0.51
PPIL1	Peptidylprolyl isomerase (cyclophilin)-like 1	0.51
BAT3	HLA-B associated transcript 3	0.51
CINP	Cyclin-dependent kinase 2-interacting protein	0.51
HS.523127	hd35c03.x1 Soares_NFL_T_GBC_S1 Homo sapiens cDNA clone	0.51
IFI30	Interferon, gamma-inducible protein 30	0.52
LOC644029	Similar to 60S ribosomal protein L7a	0.52
LOC644934	Similar to 40S ribosomal protein S26	0.52
OXA1L	Oxidase (cytochrome c) assembly 1-like	0.53
MYO1F	Myosin IF	0.53
SPTY2D1	Suppressor of Ty, domain containing 1 (S. cerevisiae)	0.53
CPNE2	Copine II	0.53
MEMO1	Mediator of cell motility 1	0.53
C9ORF98	Chromosome 9 open reading frame 98	0.53
LOC651894	Similar to ribosomal protein S12	0.53
RIOK2	RIO kinase 2	0.53
INHBE	Inhibin, beta E	0.53
AP2B1	Adaptor-related protein complex 2, beta 1 subunit	0.53
RPL14	Ribosomal protein L14	0.54
C20ORF149	Chromosome 20 open reading frame 149	0.54

Gene Symbol	Gene	Fold Difference
TUBB6	Tubulin, beta 6	0.54
MRAS	Muscle RAS oncogene homolog	0.54
MTHFS	5,10-methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase)	0.54
LOC651816	Similar to Ubiquitin-conjugating enzyme E2S	0.54
CKS1B	CDC28 protein kinase regulatory subunit 1B	0.54
WDR55	WD repeat domain 55	0.54
CLTA	Clathrin, light chain (Lca)	0.54
FTO	Fat mass and obesity associated	0.54
SRFBP1	Serum response factor binding protein 1	0.54
TMEM97	Transmembrane protein 97	0.54
ATXN3	Ataxin 3	0.55
CD37	CD37 molecule	0.55
SREBF1	Sterol regulatory element binding transcription factor 1	0.55
NUDT16L1	Nudix (nucleoside diphosphate linked moiety X)-type motif 16-like 1	0.55
C11ORF48	Chromosome 11 open reading frame 48	0.55
MYL9	Myosin, light chain 9, regulatory	0.55

APPENDIX 6-2 - Raw data for real-time PCR

		Crossing point value for THP-1 (untreated)		
Gene symbol	Efficiency	1	2	3
B2M	0.684	20.53	20.71	20.58
BIRC3[*]	0.841	28.75	28.945	28.72
CCL1[**]	0.689	29.825	30.38	30.22
CCL3[*]	0.849	25.655	25.77	25.645
CCL4[*]	0.647	30.255	30.71	30.28
FXYD5	0.849	22.285	22.455	22.505
IL23A[*]	0.693	32.945	33.40	32.90
ITGA1[*]	0.735	27.65	28.21	27.89
MARCKS[*]	0.878	31.75	31.75	31.595
OLR1[*]	0.767	25.305	25.785	25.29

	Crossing point value for THP-1 (with wild-type SAA1 treatment)		
Gene symbol	1	2	3
ACOT8[***]	28.04	27.87	28.38
B2M[1]	20.9	20.725	20.38
B2M[2]	23.07	23.52	23.95
BIRC3[*]	27.045	26.94	26.835
BMPR2[**]	28.13	28.27	27.94
CCL1[**]	22.685	24.8	25.285
CCL3[*]	22.095	22.035	21.98
CCL4[*]	25.235	25.54	25.045
CD63[***]	24.58	24.18	25.03
CES1[***]	28.30	28.01	28.56
CXCL6[*]	28.27	28.29	28.08
FXYD5[3]	22.815	22.345	22.675
FXYD5[4]	22.96	23.05	22.53
IL23A[*]	27.13	27.315	26.785
ITGA1[*]	26.21	26.585	26.505
ITGB1BP1[***]	23.74	23.55	24.16
LASP1	22.18	22.11	22.20
LOXL3[***]	31.73	31.05	31.33
MARCKS[*]	29.09	28.78	28.85
*MYO1F***	23.93	23.93	24.25
OLR1[*]	23.98	23.925	23.715
P4HA1[**]	26.00	26.04	25.72
PRKAG1[***]	27.57	27.54	27.95
PTGS2[*]	28.04	28.46	28.25
SERPINB2[**]	28.13	28.66	28.19
SREBF1[***]	27.92	27.54	28.41

	Crossing point value for THP-1 (with variant SAA1 treatment)		
Gene symbol	1	2	3
ACOT8[***]	27.51	27.77	27.61
B2M[2]	23.51	23.44	23.22
BMPR2[**]	27.91	28.38	28.09
CD63[***]	24.23	24.29	23.90
CES1[***]	28.13	28.09	28.06
CXCL6[*]	28.13	28.62	28.87
FXYD5[4]	22.68	22.92	22.80
ITGB1BP1[***]	23.27	23.23	23.37
LASP1	21.92	21.89	21.71
LOXL3[***]	31.61	30.82	31.18
MYO1F	23.72	23.80	23.64
P4HA1[**]	25.92	26.09	25.95
PRKAG1[***]	27.57	27.26	27.17
PTGS2[*]	28.56	28.69	28.8
SERPINB2	28.74	29.56	29.36
SREBF1	27.75	27.78	27.74

[*] B2M was used as internal control.
[**] FXYD5 was used as internal control.
[***] LASP3 was used as internal control.
[1] Used as internal control for BIRC3, CCL1, CCL3, CCL4, IL23A, ITGA1, MARCKS, OLR1.
[2] Used as internal control for CXCL6 and PTGS2.
[3] Used as internal control for CCL1.
[4] Used as internal control for BMPR2, P4HA1 and SERPINB2.

APPENDIX 6-3 - ELISA raw data for the quantification of cytokines secreted by macrophages upon induction by either wild-type SAA1 or variant SAA1

Wild-type vs variant SAA1 treatment (3 μg) on THP1 derived macrophages

	Medium			Wild-type SAA1			Variant SAA1		
IL-8 (μg/ml)				2.7	2.92	2.35	1.25	0.987	1.23
MCP-1 (ng/ml)	16.1	18.3	15.5	29.25	28.5	27	15.75	13.875	12.825
TNF-α (ng/ml)	0.25	0.28	0.2	21.35	18.5	19.45	9.38	13.6	11.9

Wild-type vs variant SAA1 treatment (15 μg) on THP1 derived macrophages

	Medium			Wild-type SAA1			Variant SAA1		
IL-8 (μg/ml)				3.33	3.64	3.06	1.47	1.74	1.22
MCP-1	11.85	12.3	9.675	26.1	18	24.4	10.35	10.05	11.33
TNF-α (ng/ml)	0.244	0.27	0.268	28.25	30	31.5	19.88	21.98	17.93

Wild-type vs variant SAA1 treatment (3 μg) on HL-60 derived neutrophils

	Medium			Wild-type SAA1			Variant SAA1		
IL-8 (ng/ml)	15.3	7.9	11.4	101	92	107.5	87	90	93
MCP-1 (ng/ml)	0.13	0.206	0.210	3	3.55	3.625	2.325	2.675	2.75

Wild-type vs variant SAA1 treatment (15 μg) on HL-60 derived neutrophils

	Medium			Wild-type SAA1			Variant SAA1		
IL-8 (ng/ml)	14.4	11.5	11.2	266	236	221	219	171	181
MCP-1 (ng/ml)	0.2515	0.24	0.24	10.1	9.56	8.85	5.275	6.325	5.55

APPENDIX 6-4 - Raw data for microarray

Wild-type vs untreated at 8 h

Gene symbol	Wild-type SAA1		Untreated	
BIRC3	174.9	144	77.4	55.2
CCL1	2079	2595.1	130.8	154.8
CCL3	16584.7	19155.6	1746.1	1707.2
CCL4	4107.2	3642.4	179.1	179.2
IL23A	437.5	339.5	51.2	48.3
ITGA1	154.2	145.2	66.8	58.6
MARCKS	1292	1121.5	266.5	252.9
OLR1	3496.8	2820.4	910.2	926.2
SERPINB2	196.4	234.4	47.5	49.3

Wild type vs variant at 8 h

Gene symbol	Wild-type SAA1		Variant	
BMPR2	277.2	263.5	188.2	162.5
CXCL6	175.7	181.3	117.5	105.7
P4HA1	522.2	404.4	354.1	262.7
PTGS2	328.6	194.8	214.2	126.8
SERPINB2	234.4	196.4	152.5	118.3

Wild type vs variant at 24 h

Gene symbol	Wild-type SAA1		Untreated	
ACOT8	64.6	54.8	111	102.8
CD63	341.3	665	1072	1287.4
CES1	116.4	95.3	193.3	172.7
ITGB1BP1	310.1	214.9	398.6	459
LOXL3	175.6	151.5	301.6	261.4
MYO1F	386.1	296.8	691.7	603
PRKAG1	238.3	215.2	415.9	384
SREBF1	72.7	61.3	115.9	129.4

APPENDIX 7-1 - Upregulated genes upon wild-type SAA1 treatment at 8 h

Gene Symbol	Gene	Fold Difference
CCL4	Chemokine (C-C motif) ligand 4	21.6
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	17.6
CCL1	Chemokine (C-C motif) ligand 1	16.4
CCL3	Chemokine (C-C motif) ligand 3	10.3
SOD2	Superoxide dismutase 2, mitochondrial	8.4
LAMP3	Lysosomal-associated membrane protein 3	8.3
IL23A	Interleukin 23, alpha subunit p19	7.8
IL-8	Interleukin 8	6.6
SLC2A6	Solute carrier family 2 (facilitated glucose transporter), member 6	5.9
MCOLN2	Mucolipin 2	5.3
EBI3	Epstein-Barr virus induced 3	5.1
CD40	TNF receptor superfamily member 5	4.9
MARCKS	Myristoylated alanine-rich protein kinase C substrate	4.6
SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	4.5
DKFZP564-O0823	DKFZP564O0823 protein	4.4
TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	3.7
GBP2	Guanylate binding protein 2, interferon-inducible	3.7
LEPREL1	Leprecan-like 1	3.6
OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1	3.4
HS.551128	MSTP131	3.4
STAT4	Signal transducer and activator of transcription 4	3.3
CXCL6	Granulocyte chemotactic protein 2	3.2
ADORA2A	Adenosine A2a receptor	3.1
KYNU	Kynureninase	3.1
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	3.0
IL1B	Interleukin 1, beta	2.9
CD82	CD82 molecule	2.9
CCL2	Chemokine (C-C motif) ligand 2	2.9
CXCL10	Chemokine (C-X-C motif) ligand 10	2.9
HCK	Hemopoietic cell kinase	2.8
NCF1	Neutrophil cytosolic factor 1	2.8
IL18R1	Interleukin 18 receptor 1	2.8
NCF1C	Neutrophil cytosolic factor 1C pseudogene	2.7
PTGS2	Prostaglandin-endoperoxide synthase 2	2.7
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	2.7
IER3	Immediate early response 3	2.7
EPB41L3	Erythrocyte membrane protein band 4.1-like 3	2.7

Gene Symbol	Gene	Fold Difference
TNF	Tumor necrosis factor (TNF superfamily, member 2)	2.7
LOC647650	Hypothetical protein LOC647650	2.6
ITGA1	Integrin, alpha 1	2.4
HS.575038	FLJ21027 fis, clone CAE07110	2.4
SVIL	Supervillin	2.4
IL4I1	interleukin 4 induced 1	2.4
CD44	CD44 molecule	2.4
IGFBP3	Insulin-like growth factor binding protein 3	2.4
PTPRK	Protein tyrosine phosphatase, receptor type, K	2.3
LRFN5	Leucine rich repeat and fibronectin type III domain containing 5	2.3
ZC3H12A	Zinc finger CCCH-type containing 12A	2.3
CCL20	Chemokine (C-C motif) ligand 20	2.2
EHD1	EH-domain containing 1	2.1
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	2.1
BCL3	B-cell CLL/lymphoma 3	2.1
FKBP5	FK506 binding protein 5	2.1
HS.374023	cDNA DKFZp686N1644	2.1
SDC4	Syndecan 4	2.1
CT45-4	Cancer/testis antigen CT45-4	2.1
TRAF1	TNF receptor-associated factor 1	2.1
ZNF394	Zinc finger protein 394	2.1
CD83	CD83 molecule	2.1
NKX3-1	NK3 homeobox 1	2.1
CD97	CD97 molecule	2.1
SLC7A2	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	2.1
HS.10862	cDNA: FLJ23313 fis, clone HEP11919	2.1
HS.523127	hd35c03.x1 Soares_NFL_T_GBC_S1 Homo sapiens cDNA clone	2.1
HBEGF	Heparin-binding EGF-like growth factor	2.1
SLCO3A1	Solute carrier organic anion transporter family, member 3A1	2.1
CYP26A1	Cytochrome P450, family 26, subfamily A, polypeptide 1	2.0
CXCR7	Chemokine (C-X-C motif) receptor 7	2.0
CSF2	Colony stimulating factor 2	2.0
PIM2	Pim-2 oncogene	2.0
CYP26B1	Cytochrome P450, family 26, subfamily B, polypeptide 1	2.0
MS4A14	membrane-spanning 4-domains, subfamily A, member 14	2.0

APPENDIX 7-2 - ELISA raw data for the quantification of chemokines upon treatment with SAA1

	Medium			1 µg/ml SAA1			3 µg/ml SAA1		
CCL1 (ng/ml)	24.5	24.1	23.4	327	316	404	443	456	504
CCL3 (µg/ml)	0.07	0.106	0.112	1.87	2.08	2.07	2.5	2.463	2.462
CCL4 (ng/ml)	12.6	14	14	358	356	403	442	442	461

APPENDIX 7-3 - ELISA raw data for the quantification of cytokines upon antibody and SAA1 treatment

Inhibition of TLR2 cell surface receptor

	Medium			1 μg/ml SAA1			1 μg/ml SAA1 + antibody		
MCP-1 (pg/ml)	343	272	371	666	600	778	371	290	420
TNF-α (pg/ml)	350	355	396	1398	1601	1527	812	779	998

Inhibition of CLA-1 cell surface receptor

	Medium			1 μg/ml SAA1			1 μg/ml SAA1 + antibody		
MCP-1 (pg/ml)	343	272	371	650	770	633	1200	1310	1520
TNF-α (pg/ml)	350	355	396	1428	1485	1294	913	870	956