Thesis for doctoral degree (Ph.D.) 2007

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Human C-Reactive Protein — Genetic and Hormonal Regulation and Role in Atherogenesis

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HUMAN C-REACTIVE PROTEIN — GENETIC AND HORMONAL REGULATION AND ROLE IN ATHEROGENESIS

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Stockholm 2007

Published by Karolinska Institutet. Printed by Larserics digital print.

ISBN [978-91-7357-160-9]

ABSTRACT

Although in decline in the developed world CHD is still the number one killer. The decline is thought to be due to better risk prevention and treatment. C-reactive protein (CRP) has emerged as a marker of CHD. It is associated with several of the major risk factors of CHD. *In vitro* studies have indicated that CRP is not merely a marker but involved in several stages of disease progression, such as lipid oxidation, thrombosis and tissue damage. Acute phase CRP expression is stimulated by foremost IL-6, acting in synergy with TNFα and IL1. Less is known about factors controlling basal levels but studies have suggested heritability as one factor. Several polymorphisms have been found in and around the CRP gene. However, none of these have proved to mutate the protein. Here, we report on new polymorphisms in the promoter region of the CRP gene (paper I) and possible hormonal regulation of unstimulated CRP levels (paper II). We also characterize a mouse model, developing atherosclerosis (paper III) and investigate the effects of CRP on atherogenesis *In vitro* (paper IV).

In short, 30 healthy individuals were screened for polymorphisms in the first 1.6 kb of the CRP 5' promoter using dHPLC. Five novel SNPs were found whereof two were frequent (-717 and –286). Four SNPs (-717, –286, +1059, +1444) were tested for associations with unstimulated (n=740) and stimulated (MI) (n=208) circulating CRP levels. One of the polymorphisms (–286 C>T>A) showed a significant association with unstimulated CRP levels. The A allele was associated to highest, T allele to intermediate and C allele to lowest CRP levels.

In paper II, 100 consecutive prostate cancer patients who were randomized to estrogen (n=53) or intervention by orchidectomy (n=47) were sampled for blood before and 6 month after treatment. IL-1, IL-6 and CRP were analyzed. Univariate analysis showed that orchidectomy tended to decrease circulating CRP concentrations whereas estrogen treatment tended to increase CRP. Treatment with estrogen resulted in higher circulating CRP levels than orchidectomy. Multivariate analysis including treatment, CRP concentrations before treatment and IL-6 levels after treatment showed that the difference in CRP concentration after treatment was highly significant indicating a role for estrogen in CRP expression.

When characterizing the mouse model in paper III, atherosclerosis progression develops slowly at first, expands rapidly after transformation of fatty streaks into plaques, and plateaus after advanced lesions form. This development was paralleled by the activity of 1259 genes forming four expression clusters. Genetic lowering of plasma cholesterol in mice with early lesions produced a distinct transcriptional response and prevented atherosclerosis development at 40 weeks. 37 cholesterol-responsive genes were identified. In THP-1 macrophages, inhibiting six of these genes with siRNA affected foam cell formation.

Human CRP was studied in relation to atherogenesis in atherosclerosis-prone mice in paper IV. Lesion development was studied at 15, 30, 40 and 50 weeks of age. At 40 and 50 weeks, atherosclerotic lesions of CRP transgenic mice were smaller and at 50 weeks had larger collagen deposits compared to littermate controls. Analysis of gene expression profiles from lesions of 40 weeks-old CRP transgenic and littermate controls revealed differentially expressed genes related to the proteasome.

In conclusion, the results show that genetic variation and estrogen are intimately involved in the regulation of unstimulated circulating CRP concentrations and that transgenic expression of CRP in our mouse model results in less atherosclerosis. Furthermore, some of the cholesterol-sensitive genes may be considered as future targets for atherosclerosis treatment.

LIST OF PUBLICATIONS

I. ***A novel common single nucleotide polymorphism in the promoter region of the C-reactive protein gene associated with the plasma concentration of C-reactive protein***
Alexander Kovacs MS, Fiona Green PhD, Lars-Olof Hansson MD PhD, Pia Lundman MD PhD, Ann Samnegård MD, Susanna Boquist MD PhD, Carl-Göran Ericsson MD PhD, Hugh Watkins MD PhD, Anders Hamsten MD PhD, Per Tornvall MD PhD.
Atherosclerosis 178 (2005) 193–198

II. ***Hormonal regulation of circulating C-reactive protein***
Alexander Kovacs MS, Peter Henriksson MD, PhD, Håkan Wallén MD, PhD, Johan Björkegren MD, PhD, Per Tornvall MD, PhD.
Clinical Chemistry 51 (2005) 911-913

III. ***Transcriptional profiling and genetic lowering of plasma cholesterol to identify cholesterol-responsive atherosclerosis target genes***
Josefin Skogsberg, Alexander Kovacs, Roland Nilsson, Peri Noori, Shohreh Maleki, Jesper Lundström, Marina Köhler, Björn Brinne, Anders Hamsten, Jesper Tegnér and Johan Björkegren.
Manuscript

IV. ***Atherosclerosis development in human C-reactive protein transgenic apoB100-only low density lipoprotein receptor deficient mice***
Alexander Kovacs, Anders Hamsten, Roland Nilsson, Jesper Tegnér, Per Tornvall, Johan Björkegren.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute Coronary Syndrome
AGE	Advanced Glycation Endproduct
AMI	Acute Myocardial Infarction
ANCOVA	Analysis of Co-Variance
ANOVA	Analysis of Variance
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ApoE-/-	Apolipoprotein E homozygote knock-out
APR	Acute Phase Response
BMI	Body Mass Index
C/EBP	CCAAT / Enhancer Binding Protein
C3	Complement factor 3
CAD	Coronary Artery Disease
CAM	Cell Adhesion Molecules
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
CRP	C-reactive protein
CVD	Cardio Vascular Disease
dHPLC	denaturing High Performance Liquid Chromatography
ECM	Extra Cellular Matrix
ELISA	Enzyme Linked Immuno Sorbent Assay
eNOS	endothelial NO Synthase
ESR	Erythrocyte Sedimentation Rate
FDR	False Discovery Rate
HDL	High Density Lipoprotein
HNF-1α	Hepatic Nuclear Factor 1α
HNF-3	Hepatic Nuclear Factor 3
HRP	Horse Radish Peroxidase
ICAM	Intercellular Adhesion Molecule
iNOS	inducible NO Synthase
LD	Linkage Disequilibrium
LDL	Low Density Lipoprotein
LDLr	Low Density Lipoprotein receptor
LDLr-/-	Low Density Lipoprotein receptor homozygous knock-out
LOX-1	Lectin-like OXidized low-density lipoprotein receptor 1
LPL	Lipoprotein Lipase
LRP	Low density lipoprotein receptor-Related Protein
MAF	Minor Allele Frequency
MBL	Mannan Binding Lectin
MCP-1	Macrophage Chemotactic Protein
MGD	Mouse Genome Database
MI	Myocardial Infarction
MMP	Matrix Metallo Proteinase
NFIL-6	Nuclear Factor IL-6

NFκB	Nuclear Factor kappa B
NO	Nitrogen Oxide
PPARα	Peroxisome Proliferator-Activated Receptor alfa
PPARδ	Peroxisome Proliferator-Activated Receptor delta
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
PSMA7	20s proteasome Core subunit A7
PSMB7	20s proteasome Core subunit B7
PSMB9	20s proteasome Core subunit B9 (immunoproteasome subunit)
PSMC6	19s proteasome cap subunit C6
PSME2	11s proteasome cap subunit E2
PTX3	Pentraxin 3
QTL	Quantitative Trait Locus
RA	Rheumatoid Arthritis
RFLP	Restriction Fragment Length Polymorphism
RI-Strain	Recombinant Inbred Strain
RMA	Robust Multichip Average
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAA	Serum Amyloid A protein
SAP	Serum Amyloid P protein
SLE	Systemic Lupus Erythematosis
SNP	Single Nucleotide Polymorphism
SREBF	Sterol Regulatory Element Binding Factor
STAT3	Signal Transducers and Activators of Transcription 3
TDT	Transmission Disequilibrium Test
TF	Tissue Factor / Transcription Factor
Ube2D3	E2 ubiquitin ligase D3
USF-1	Upstream Stimulating Factor 1
UTR	Untranslated Region
VCAM	Vascular Cellular Adhesion Molecule
VLDL	Very Low Density Lipoprotein

1 THESIS SUMMARY – MAIN SECTION

Although death from CVD has declined during the last decade CHD is still the leading cause of morbidity and mortality in the western world. Between 1960 and 1990 CVD mortality declined by 34-50% in Australia, Canada, Japan and the United States, with CHD being the major contributor to CVD. The decrease in mortality in CHD is attributed in part to decreasing prevalence of CVD risk factors and in part to better treatments of already manifest disease (2).
As reported by the World Health Organisation (WHO) an estimated 29.2% of total global deaths result from the various forms of CVD, including CHD, cerebrovascular disease, hypertension, heart failure and rheumatic heart disease. Of the 16.7 million deaths from CVD every year, 7.2 million are due to ischemic heart disease. From a society economic perspective maybe even more important at least 20 million people survive heart attacks and strokes every year, requiring costly clinical care, putting a huge burden on long-term care.
At the same time there is an epidemiological transition occurring in the developing world towards non-communicable diseases. Some 80% of all CVD deaths worldwide took place in developing countries. It is estimated that by 2010, CVD will be the leading cause of death in developing countries [1].
CRP has emerged as an important marker for CHD that together with LDL cholesterol predicts future cardiac events in both healthy and diseased men and women. CRP is a highly inducible protein with a serum concentration that has a strong genetic component. By studying regulation at the CRP gene locus knowledge can be gained that will improve risk prediction and prevention of CHD. *In vitro* studies have also pointed to molecular functions of CRP such as complement activation and LDL endocytosis, both at the heart of the atherosclerotic plaque. By studying the effects of CRP in a mouse model developing atherosclerosis *in vivo* knowledge can be gained on the importance and authenticity of these *in vitro* findings.

1.1 CRP AND DISEASE

1.1.1 Infection and inflammation

"The inflammatory response directs immune system components to injury or infection sites and is manifested by increased blood supply and vascular permeability, which allows chemotactic peptides, neutrophils, and mononuclear cells to leave the intravascular compartment. Oxidative products such as hydrogen peroxide are generated by the phagocytes and kill ingested microbes."
Inflammation can be caused by physical injury, infection or local immune response (ex. Autoimmunity or allergy). In cases of infection or local immune responses pattern recognition molecules such as mannose binding receptors, toll-like receptors (TLR) or certain scavenger receptors, but also complement components, are often responsible for triggering release of prostaglandins and leukotrienes. The release of these factors leads to vasodilatation, leakage of plasma proteins and upregulation of adhesion molecules, such as integrins, selectins and cellular adhesion molecules, on endothelial cells. In most cases phagocytic cells such as monocytes extravasate to the underlying tissue and may, upon ligand binding release cytokines such as IL1, IL6 and TNFα. Locally these cytokines stimulate generation of reactive oxygen species (ROS) to kill or degrade bacteriae and systemically they may eventually stimulate fever, production of leukocytes and acute phase reaction (APR). IL6 but also TNFα and IL1 are responsible for triggering the APR [2]. The APR protects the organisms from challenges such as infections, tissue trauma or bleedings. CRP is considered to be an early, positive acute

phase protein together with Mannan Binding Lectin and serum amyloid A protein (SAA) [2]. Interestingly, also the protein constitution of lipoproteins changes during an acute phase response and for example ApoAI on HDL particles is largely replaced by SAA [3], increasing affinity for macrophage binding and decreasing affinity for hepatocytes. Other systemic changes that occur are that the coagulative state of the blood increases through increased release of fibrinogen from the liver and the oxidaprotective effects of HDL decrease through downregulation of transferrin [3].

There is overwhelming *in vivo* and *in vitro* evidence that CRP is associated with inflammation. CRP activates complement and promotes uptake of microbes and particles (opsonisation) both in complement [4] dependent and independent ways. The general opinion seems to favor the idea that complement binding enhances opsonisation greatly and is more important [5]. However, there are different opinions whether opsonisation is mediated by FcRgI and FcRgII receptors or a CRP specific receptor [6-10]. CRP has also been shown to activate the endothelium and induce expression of chemotactic factors, such as monocyte chemotactic protein 1 (MCP-1) [11] and its receptor CCR-2 [12], resulting in recruitment of leukocytes. Stimulation of macrophages with CRP induces inducible NO synthase (iNOS) activity that generates reactive oxygen species (ROS) used by cells to break down pathogens [13]. CRP has also been shown to bind to bacterial constituents of several fungi and bacteria (ex. Chlamydia Pneumonae) and protect mice from endotoxic chock [14].

1.1.2 Autoimmune disease

"In autoimmune disorders, the immune system produces antibodies to an endogenous antigen. Antibody-coated cells, like any similarly coated foreign particle, activate the complement system resulting in tissue injury."

Autoimmune disease results when foremost the adaptive immune system misinterprets endogenous antigens for foreign. Genetic factors play a role. Relatives of patients with autoimmune disorders often have the same type of autoantibodies, and incidence of autoimmune disorders is higher in identical than fraternal twins. Women are affected more often than men. In genetically predisposed people, environmental factors (such as drugs) may provoke disease. Systemic autoimmune diseases such as SLE and RA have many similarities to the inflammatory conditions seen in atherosclerosis and cardiovascular disease [15]. There is also epidemiological data supporting a connection between autoimmune diseases and atherosclerosis [16]. Furthermore, autoantibodies to oxLDL are common in atherosclerosis and treatment of these may have beneficial effects, depending on Ig-subtype [17].

SLE may develop abruptly with fever or insidiously over months or years with episodes of arthralgias and malaise. It is often characterized by antiphospholipid antibodies, antinuclear antibodies, depressed complement serum levels, hypertension and increased atherothrombotic events. The disease is thought to be the consequence of impaired clearance of apoptotic and necrotic cells [18, 19]. CRP increase is absent or modest during disease, despite an Erythrocyte Sedimentation Rate (ESR) > 100 mm/h. Interestingly autoreactive antibodies to CRP are associated with worse prognosis and co-insides with relapse of disease [20, 21]. Since CRP has been shown to bind nuclear material and cell membranes of apoptotic and necrotic cells there are reasons to suspect CRP to act against autoimmunity in SLE. Supporting this notion, studies in a mouse models of SLE show CRP delaying or interfering with disease progress [22, 23].

1.1.3 Metabolic syndrome

"The metabolic syndrome is characterized by excess abdominal fat, insulin resistance, dyslipidemia, and hypertension."

The metabolic syndrome is very common, possibly affecting almost half of people aged > 50 yr in the US. In Europe the prevalence is slightly lower, affecting about 45% aged >50yr. The syndrome develops more often in people with accumulated abdominal fat and a high waist-to-hip ratio. It is less common among people whose fat accumulates around the hips and who have a low waist-to-hip ratio. This might be explained by abdominal fat causing excess free fatty acids in the portal vein, increasing fat accumulation in the liver and induce dyslipidemia. In parallel, insulin resistance, hyperinsulinemia and hypertension develop and ultimately also diabetes mellitus and CHD. Furthermore, serum uric acid levels are elevated and a prothrombotic state (inc. fibrinogen and PAI) develops [15]. The risk of CVD in subjects with the metabolic syndrome is 2- to 4-fold higher than in subjects without the syndrome. The risk of CVD associated with the metabolic syndrome is higher in women (about 4-fold) than in men (about 2-fold). The risk is particularly high when affected subjects have pre-existing diabetes and/or CVD and/or chronic mild inflammation (high CRP). However, since there are at least three different sets of criteria for diagnosis these numbers may vary [24].

ATPIII Criteria Used for Metabolic Syndrome

Parameter	*Value*
Waist circumf. (cm [in])	>102(40) men >88(35) women
Glucose (mg/dL [mmol/L])	≥100(5.6)
Blood pressure (mm Hg)	≥130/85
Tg, fasting (mg/dL [mmol/L])	≥150(1.7)
LDL-chol. (mg/dL [mmol/L])	≥100(2.59)
HDL-chol. (mg/dL [mmol/L])	≤40(0.9) men ≤50(1.1) women

Weight and dyslipidemia

Several large studies have shown that circulating CRP levels are increased in patients with high BMI [25]. In women, the influence of BMI on CRP concentrations appears to be stronger than in men [26] and indeed when circulating CRP in normal weight women and men was compared no difference was noted [27]. In contrast, comparing women and men with BMI 25-30 women had significantly higher CRP levels. This difference was further increased in obese women [28]. The mechanism behind the association between CRP and BMI is not clear but it could be that adipose tissues produce IL-6 [29] that stimulates liver production of CRP or that there is local production of CRP in adipose tissue [30]. In addition, some studies show a correlation between CRP and triglycerides and HDL cholesterol [31]. However, the correlation with triglycerides, has to be questioned since fibrates (significantly lowering triglycerides) do not appear to lower CRP [32]. Also, the correlation between CRP and total and LDL cholesterol remains equivocal [31].

Hypertension

High diastolic or systolic blood pressure is a risk factor for CVD. The CVD risk associated with hypertension is lower in societies with low average cholesterol levels. Treatment of patients with elevated blood pressure reduces stroke and overall mortality, but the effect on coronary events is less striking [15]. The pathophysiology behind hypertension is not clearly understood but some have speculated that the increased risk for CVD is mediated by vascular inflammation, possibly through the renin-angiotensin system. CRP has been shown to interact with endothelin-1 and the angiotensin-1 receptor, both important mediators of vascular tone [33, 34]. Furthermore, CRP is linked to vascular tonus control by inhibition of eNOS [35].

1.1.4 Diabetes mellitus

"Diabetes mellitus is impaired insulin secretion and variable degrees of peripheral insulin resistance leading to hyperglycemia. Later complications include vascular disease, peripheral neuropathy, and predisposition to infection."

Although primarily not belonging to the metabolic syndrome but closely linked to insulin resistance and hyperglycemia, diabetes mellitus is associated with earlier and more extensive development of atherosclerosis as part of a widespread metabolic derangement that includes dyslipidemia, glycosylation of connective tissue, growth factor promoted ECM production and cytokine release. Diabetes mellitus is a particularly strong risk factor in women and significantly negates the protective effect of female hormones. Although tight glycemic control reduces the risk of microvascular complications of diabetes, the effects on macrovascular disease and atherosclerosis are less clear. Hyperlipidemia and hypertension are more common in diabetics. These risk factors together with hyperinsulinemia may contribute to the increased CAD risk. In diabetes mellitus as much as 70% of the mortality is accounted for by atherosclerosis and its terminal stages MI and stroke [15] .

Several studies have linked CRP to diabetes [36, 37]. The mechanism is unknown but it has been suggested that obesity and AGEs are responsible for stimulating cytokine release and animal experiments have proved TNFα and IL-6 to increase in obese and diabetic settings [38]. However, one recent prospective population-based study supports a role of CRP in prediction of disease when corrected for obesity. This was supported by meta analysis of other studies [39].

1.1.5 Coronary artery disease

"Coronary artery disease involves impairment of blood flow through the coronary arteries, most commonly by atheromas. Clinical presentations include ischemia, angina pectoris, acute coronary syndromes (unstable angina, MI), and sudden cardiac death."

Although CAD has been associated with increased cholesterol concentrations in plasma, 40-50% of those diagnosed with CAD have normal or mildly elevated cholesterol levels [40]. Growing evidence points out that inflammation and possibly autoimmunity play an important role in atherosclerosis highlighting circulating CRP as an important marker of CHD. CRP and cholesterol levels in the highest quartile increase the relative risk 6 times compared with the lowest quartiles of both [40].

CRP has for many years been used to measure inflammation in patients suffering from bacterial infection. The "cut off" for what was considered normal was 10 mg/l. However, new more sensitive methods (hs; high sensitivity) have decreased the "cut off" to below 3 mg/l. Since the introduction of hsCRP, several studies have suggested that CRP is an independent prognostic marker for increased risk of AMI, stroke and death in apparently healthy men and women [25, 41-43] and in patients with stable and unstable angina pectoris [25]. However, recently a meta analysis of healthy people in prospective studies, including 7068 incident CHD events, by Dannesh and colleagues has decreased the strength of association from odds ratio 2.0 to 1.5, between bottom and upper third of CRP levels [48]. CRP is associated with many of the major risk factors of CHD such as age, gender, BMI, smoking, hypertension, and diabetes mellitus. For example, several studies have shown that circulating CRP is increased in smokers and that smokers with increased CRP levels also have higher risk of CVD events [44-47]. The reason for increased CRP in smokers is not known, but a possible link could be that IL-6 is increased in smokers.

CHD has also been shown to associate with antibodies against chronic infections. The

mechanism behind this is not known but infection with for example Chlamydia pneumonia or CMV is associated with endothelial injury and chronic inflammation in arteries. Serum CRP levels show association with total infectious burden, but not with any particular pathogen [49].

1.1.6 Atherosclerosis

"Atherosclerosis is patchy intimal plaques (atheromas) in medium and large arteries; the plaques contain lipids, inflammatory cells, smooth muscle cells, and connective tissue. Risk factors include dyslipidemia, diabetes, cigarette smoking, family history, sedentary lifestyle, obesity, and hypertension. Symptoms develop when growth or rupture of the plaque reduces or obstructs blood flow; symptoms vary by artery affected."

In the vast majority of cases CHD is due to atherosclerosis in large and medium-sized arteries. Atherosclerosis often manifests clinically late in life as angina pectoris (stable and unstable), AMI, heart failure, kidney failure, ischemic stroke, and peripheral arterial disease. Atherosclerosis is considered to be multifactorial, dependent on both genetic and environmental risk factors. Risk factors with a strong genetic component includes; elevated levels of VLDL/LDL, reduced levels of HDL, elevated levels of lipoprotein(a), hypertension, elevated homocysteine, diabetes mellitus, obesity, elevated haemostatic factors, systemic inflammation and the metabolic syndrome. Risk factors considered having a strong environmental component include; high-fat diet, smoking, low antioxidant levels, lack of exercise and infectious agents such as Chlamydia pneumonia [50-54]. The earliest stages of atherosclerosis are marked by fatty streak (lipid deposits in the arterial wall) formation during the first decade of life [55, 56]. The aorta is the first to be affected, followed by the coronary arteries and then also by the cerebral arteries in the third and fourth decades of life [56]. There are three main hypotheses that have been proposed to explain the pathogenesis of atherosclerosis. They exhibit similar characteristics and are not mutually exclusive. However, they differ in their view on what the primary mechanism driving disease is.

The lipid hypothesis postulates that an elevation in plasma LDL results in penetration of LDL into the arterial wall, leading to lipid accumulation in SMC and in macrophages (foam cells). This elicits an inflammatory reaction that recruits more inflammatory cells to the intima. Since the key event is the influx of LDL into the vascular wall small dense LDL cholesterol particles are considered to be more susceptible to penetration, modification and oxidation [57].

The endothelial injury hypothesis postulates that endothelial injury by various mechanisms produces loss of endothelium, which increases lipoprotein infiltration and chemotaxis of monocytes and T-cell lymphocytes. In addition, regenerating endothelial cells are functionally impaired and increase the uptake of LDL from plasma [57].

The retention hypothesis postulates that the diffusion/transport of lipoproteins to the intima and their subsequent binding to ECM has an evolutionary important role in making them more accessible to enzymes such as LPL. The longer LDL is trapped in the intima, the higher the possibility that cellular by-products or enzymes oxidize them. The hypothesis further postulates that when serum lipid levels rise the influx of lipoproteins into the intima increase, leading to more oxidized lipoproteins which activates the endothelium, recruiting leukocytes such as T-cells and macrophages [58].

1.2 PLAQUE BIOLOGY

1.2.1 Normal artery

The vascular system consists of five main types of vessels; arteries, arterioles, capillaries, venules and veins, all with specialised structures and functions. Specialised vascular beds also

exist such as the cerebrovascular and renal vessels. While the initial damages in diabetes are mostly taking place in the small vessels atherosclerosis is a disease of comparatively large arteries, such as the aorta, the carotides and the coronary vessels. The function of the largest arteries are to convert the pulsatile pressure generated by the heart to a continues pressure and flow in the rest of the vascular system. As compared to veins, arteries contain a relatively large proportion elastic fibres and smooth muscle cells. The normal artery consists of three distinct layers; tunica intima, tunica media and tunica adventitia. The intima is lined with endothelial cells preventing clotting and regulating vascular tonus through the release of substances such as NO. The endothelium also regulates transport of fluid and particles to the underlying intima, both through fenestrae and active vesicular transport through the endothelial cells. A layer of elastin marks the boundary between the intima and media. The media contains elastic fibers and matrix and is lined by layers of smooth muscle cells. The outer most layer, the adventitia consists of connective tissue and fibroblasts. Also, the vessels (vasa vasorum) responsible for supplying mainly the adventitia and media with oxygen and nutrition's stretch from here.

1.2.2 Early atherosclerotic lesion

Whether through factors such as infection, autoimmune disease, reactive oxygen species (ROS), advanced glycation endproducts (AGE), hypertension or flow disturbances the first disturbances seen in the vascular wall is often endothelial dysfunction. It is characterized by dysregulation of tonus and increased permeability of the vessel. At the earliest stages lipoprotein particles are deposited in the intima of the affected vessel. Lipoproteins subsequently become oxidized and taken up by macrophages and other phagocyting cells through scavenger receptors such as SR-A, CD36, CD68 and LOX-1. As the lipids accumulate, foam cells are formed (macrophages loaded with lipid droplets). Foam cells can be found scattered in the vascular intima at first but then start to cluster and accumulate. Clinically, at this stage fatty streaks start to get apparent. As a response to the excessive lipids and increased oxidative environment mast cells, macrophages and lymphocytes start to release cytokines that activates the endothelium. The activated endothelium expresses adhesion molecules such as selectins and CAMs and secretes chemotactic mediators such as MCP-1. This further stimulates recruitment of leukocytes to the plaque. In the early stages roughly 80% of the cells constitutes of macrophages and 10-20% of T-cells. CD8+ T-cells are more common at the early stages of disease while CD4+ memory T-cells are more common at late stages. In ApoE-/- and LDLr-/- mice the density of T-cells decrease over time, being more abundant in ApoE-/- mice at all investigated time points. [56, 59]

1.2.3 Intermediate atherosclerotic lesion

At progressive stages of the disease extracellular lipids and cellular debris start to occur. Soon a necrotic core forms, containing cell debris, cholesterol and its esters and tissue factor (TF). The release of growth factors mainly from T-cells and macrophages stimulate SMC to start producing matrix metallo proteinases (MMP), degrading the internal elastic lamina and migrates into the inflamed intima. The SMC start producing matrix components such as fibronectin and collagen, generating a fibrous cap. As the disease progresses more leukocytes are recruited to the site of inflammation. With increased inflammation in the vessel wall both the necrotic core and the fibrous cap grows and start bulging out into the lumen of the vessel. [56, 59]

1.2.4 Advanced atherosclerotic lesion

Plaques do not give symptoms until they occupy around 60% of the lumen. The plaque can now evolve either into a lipid laden plaque with a thin fibrous cap or a fibrous plaque with a small lipid core and an extensive fibrous cap. The factors regulating this process are not known. The lipid laden plaque is regarded as unstable and studies have shown that they are more prone to rupture than the stable fibrous plaques. If rupture occurs the inflammatory content (most importantly TF) of the plaque start coagulative processes leading to total occlusion or thrombus formation with potential of occluding down stream vasculature. The complexity of the plaque increases as disease progresses and may exhibit features such as focal endothelial loss, several plaque ruptures and fissures. During late stages of disease the plaque also becomes hypoxic and vasculogenesis occurs with subsequent intra-plaque thrombus as result. With age the plaque may become more calcified and stabilized [56, 59]

1.2.5 CRP in the plaque

Studies show that CRP mediate uptake of LDL to macrophages [60, 61]. It has been shown that CRP binds to oxidized cholesterol of oxidized LDL [62] and binding of CRP to degraded non-oxidized LDL enhances complement activation [63]. Experimental studies have shown that CRP promotes atherosclerosis [61, 64] and enhance tissue damage [65]. CRP is 10 times more abundant in atherosclerotic than in normal arteries [66] and the intensity of CRP immunohistological staining of atherosclerotic plaques correlates with serum levels of CRP [67]. Deposited in atherosclerotic lesions CRP can be found in and around foam cells and areas heavily stained for complement activity [68]. Furthermore, CRP has been shown to co-localize with complement in plaques [69]. Several in vitro experiments have also shown CRP to induce endothelial cells to display adhesion molecules such as ICAM and VCAM and to secrete MCP-1 [11, 70]. CRP has also been shown to induce tissue factor expression in monocytes and macrophages, linking it to late events including thrombus formation [71]. CRP has also been linked to endothelial function through down-regulated eNOS expression in endothelial cells and to oxidative events by upregulating iNOS in inflammatory cells [72]. eNOS activity relaxes SMC but also inhibit platelet and leukocyte aggregation and adhesion to the endothelium, whereas iNOS on the other hand produces reactive oxygen species used by cells to breakdown or fight pathogens. Furthermore, CRP has been shown to predict progress of atherosclerosis. After adjustment of risk factors, ORs associated with CRP levels in the highest compared to the lowest quartile, were increased for progression of carotid (OR=1.9), aortic (OR=1.7), iliac (OR=2.0), lower extremity (OR=1.9) and composite (OR=4.5) atherosclerosis score. Geometric Mean levels of CRP also increased with the total number of sites showing progression of atherosclerosis [73]. This was supported by a smaller study [74]

1.3 CRP REGULATION, INTERACTIONS AND STRUCTURE

1.3.1 Regulation

The liver is thought to be the major source of CRP [2], but other cells, like macrophages, kidney epithelial cells and neurons have also been shown to produce CRP [75-77]. CRP increases during the acute-phase reaction together with other liver derived acute-phase proteins such as fibrinogen and SAA. During the acute-phase, levels of CRP can increase up to a 1000-fold [78]. Previous studies have shown that CRP to a large extent is regulated on the transcriptional level [79]. Both in vivo and in vitro data support the hypothesis that IL-6 is the main inducer of the acute phase CRP response but other factors such as IL-1β can potentiate IL-6 stimulation of

CRP [80-84]. Studies of hep3B and other hepatoma cell lines also show that corticosteroids enhance IL-6 induced expression [81], but no further investigations have been undertaken to verify these results.

Factors determining unstimulated CRP expression are less known. But importantly, studies have indicated the genetic influence of circulating CRP levels to be 40-60% [85-87]. Also, studies have shown that women have higher circulating CRP concentrations than men [88]. However, Rifai and co-workers [27] investigated the normal distribution of CRP in the US population without finding a gender difference among 22.000 apparently healthy women and men. In some of the studies the effect of oral contraceptive use has not been taken into account [89] and might therefore explain the different results. Data have shown both positive and negative associations between female sex hormones and circulating CRP concentrations [90]. Since contraceptives and hormone replacement therapy varied in dose, brand, formula and contraceptives were added on top of endogenous estrogen it is difficult to draw any firm conclusions from these studies. Less is known about the effects of male sex hormones on CRP. Androgen therapy in older men showed no effect on circulating CRP whereas androgens increased serum CRP concentrations in bodybuilders [79, 91]. Whether this is due to similar body mass index with different fat distribution or higher percentage body fat is not clear.

Studies of CRP expression in human CRP transgenic mice (where CRP is not naturally an acute-phase reactant) further support these in vitro findings, but also suggest that, in vivo, IL-6 is necessary but not sufficient for CRP expression [82]. It seems that IL-6 have to be accompanied by at least another factor, which in these transgenic mice was testosterone or a factor induced by testosterone [92].

The possibility of hormonal regulation of circulating CRP is of great interest since male gender is considered to be an independent risk factor for CHD and estrogen has been proven to have beneficial cardiovascular effects in women [93]. For example, studies have shown that estrogen improves endothelial function and lipid profile, and modulates the immune system. Similar positive cardiovascular effects of testosterone have in part been attributed to the conversion of testosterone to estrogen by aromatization locally in peripheral tissues. Testosterone per se has been shown to have negative cardiovascular effects for example by lowering plasma HDL cholesterol [94]. Two separate studies have also shown CRP to increase with age [25, 43].

1.3.2 Protein structure and interactions

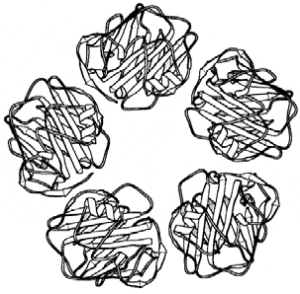
CRP was discovered through its capability to bind to phosphocholine residues of C-polysaccharide, a teichoic acid of Streptococcus Pneumonae [95], hence its name. It has later been found that CRP also binds to damaged cell membranes, histones, ribonuclear particles and many bacterial and fungal polysaccharides [96-103]. The binding to phosphocholine and other ligands mentioned above is Ca^{2+} dependent and two Ca^{2+} ions bind to each protomer [104]. It has also been reported that, in the absence of Ca^{2+}, CRP may also bind polycations such as poly-L-Lysine, poly-L-arginine and myelin basic protein [14, 105]. Furthermore, the binding to lipoproteins such as LDL and VLDL have also been shown [14, 62, 63, 106].

Studies suggest that binding of ligands introduce a conformational change, which enables complement factors to interact with CRP [79]. C1q binds to the ligand bound CRP effector side and recruit complement factors via the classical pathway [105, 107, 108]. Also complement inhibitory factors such a factor H and complement 4b binding protein have been shown to interact with CRP [109-111].

The protein is a 25 kDa protein protomer, 206 aa (amino acids) long. It is a member of the very well conserved family of proteins called pentraxins [112]. The pentraxin family has an

evolutionary history spanning at least 400 million years, and CRP can be found in the A horseshoe crab as well as in mammals (vertebrates and invertebrates) [113, 114 preservation throughout evolution emphasizes the importance of CRP.

The functional form of CRP, like the other members of the pentraxin family, is a penta homoprotomers. The protomers are arranged in a symmetrical configuration around a pore and are oriented at a 15-20 degree angle to an axis running through the central por protomers are noncovalently attached to each other and the main interaction is through salt bridges. Each protomer has an effector and a ligand binding side [115, 116].



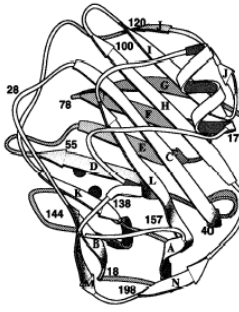


Figure 1

CRP pentamer (left) and protomer (right).

1.4 CRP AND GENETICS

1.4.1 Gene structure

The CRP gene has been mapped to chromosome 1 between q1.21 and q1.23. Only one of the gene and one copy of the pseudogene have been found [117]. The gene spans nucleotides and consists of two exons and one single intron. 104 bp downstream of the ca site there is a 54 bp sequence coding for a signal peptide, 18 amino acids long. The exon 1 codes for two further amino acids and is then followed by the intron. The intron is 2 long and among its features is a poly A sequence and a GT run that can adopt the unus form of DNA, to which certain proteins bind. The second exon codes for the remainin amino acids. It has a 1.2 kb long 3'UTR, which is thought to mediate the rapid breakd CRP mRNA [79]. Exon two also contains a polyA signal at 2154 bp downstream of the ca site [112] (Figure 1). Further downstream of the polyA signal there are putative regu elements, mainly of negative character[118].

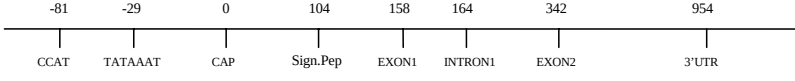


Figure1
CRP gene overview.

5' of the capping site at -29 bp a TATA-box and at -81 bp a CAAT-box have been Response elements for C/EBPβ/δ (NFIL-6), HNF-1α and HNF-3 have also been found

region (Figure 2) [119]. No hormone response elements have been found or mapped.

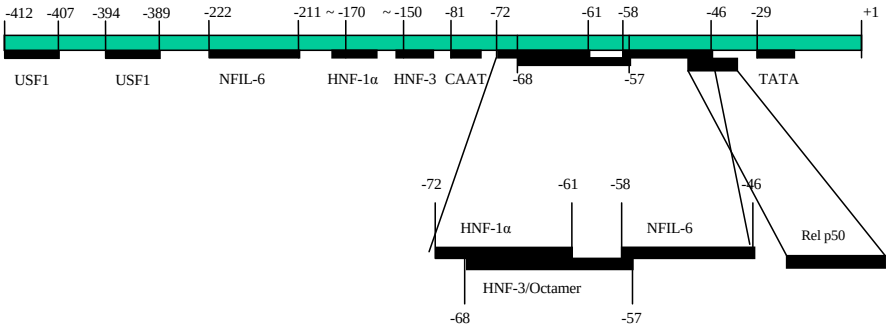


Figure 2
Known functional sequences in the CRP promoter region.

C/EBPβ/δ (NFIL-6) is an IL-6 inducible transcription factor and its response elements have proven to initiate expression of IL-6 inducible proteins such as CRP [119]. The HNF-1 (Hepatic Nuclear Factor) transcription factor is liver specific and may explain the liver expression of CRP. Unlike HNF-1, HNF-3 is not liver specific and it can be found in other tissues such as the intestine and pancreas. Also, cell studies in hepatoma cell lines have indicated that there is a functional NFκB (p50/p65) binding site at position –2652 and a Rel p50 binding site centered at position –46 proximal to the transcriptional start site. Agrawal and co-workers propose this to be how IL-1β influence CRP expression [120-122]. In two recent studies were the 5'-promoter regions have been reinvestigated both in silico and through functional studies 4 binding sites for the upstream stimulating factor (USF1) have been added to those already found [123]. As reported at least two of these are independent of IL6 stimulation. USF1 have been linked to cellular stress responses [123].

1.4.2 Polymorphisms

There are around 84 polymorphisms in the vicinity of the CRP gene as determined from repeated re-sequencings and databases (Seattle SNP, dbSNP, Celera). Use of a cut-off in the minor-allele-frequency (MAF) of >0.05, yields about 21 SNPs [124]. Initially, research focused on five SNPs [125-129], but with the development of LD-maps and increased use of haplotypes, tagSNPs are increasingly used [130]. As the MAF cut-off varies between different studies, the SNPs included also vary. Most studies concern cohorts of European descent. Due to the haploblocks identified 6-13 SNPs are used to represent the regions. The classical five are often included in these tagSNPs. A SNP located at -757 is often included but redundant due to complete linkage to the -286 SNP, also called -390 in some publications [124].

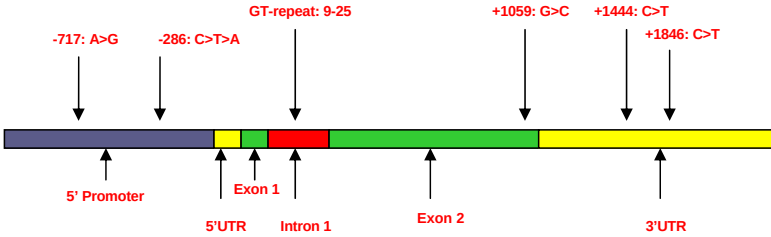


Figure 3 Polymorphisms in the CRP gene

Also, usually excluded a GT-repeat polymorphism in the intron has been investigat association to CRP. It was found that healthy subjects with GT-repeats of lengths 16 a exhibited an association with lower unstimulated CRP levels [131].

While the results from studies linking genotypes to disease conditions such as stro MI/ACS have been questionable, there seems to be a clear relationship between se polymorphisms and CRP levels [124, 130, 132, 133]. Most notably the -286 SNP has em as a relatively strong predictor of CRP levels in blood in the majority of studies, also considered in haplotypes. However, it should also be remembered that although the heri in numerous studies have been estimated to be strong, a recent study determined the ef genetic variation to account for ~1.4% of the CRP levels variations as opposed to 16% fo alone [130].

1.5 POPULATION AND DISEASE GENETICS

1.5.1 Genetic variation

A striking observation from the genome sequencing and comparative analysis of a number of organisms, including human, is the considerable degree of similarity obs between organisms and between individuals from the same species. Within a specie similarity is sufficiently large that we speak of a singular genome for each species, e human genome, but, on closer inspection, every individual genome is subtly different. Th of these differences constitute of the following main types; single nucleotide polymorp (SNP), microsatellites and sequence insertions, deletion and duplications. These differ are subject to forces, such as mutation, random genetic drift, natural selection recombination, acting on them. The pattern of genetic variation in a gene, and more ge the genome, are shaped by a multitude of factors, such as population size, immigratio migration patterns, selective effects, births and deaths of individuals, and mutation rates 135]. Mutation, gene flow and large population sizes tend to increase the amount of g variation. Population bottlenecks, selective events and no migration tend to decreas amount of genetic variation.

1.5.2 Linkage disequilibrium

Linkage disequilibrium also called allelic association is a measure of the extent to which several alleles occur together more frequently than what is predicted by their individua frequencies. An r^2 =1 means perfect co-occurrence and an r^2 = 0 means no co-occurre all.

Patterns of LD are created by a number of forces. LD arises when a variation on a chrom is transmitted along with other markers of that chromosomal background. This mean

demographic events such as isolation, migration, admixture and bottlenecks create LD, while recombination and gene conversion decrease LD. It is important to bear in mind that LD-tests are sensitive to small population sizes and rare allele frequencies, adjusting the LD upwards when population sizes are small or allele frequencies rare. For the same reason it is also sensitive to populations of unequal sizes.
Variations on a chromosome that exist together are said to constitute a haplotype. New haplotypes can be created by mutation or recombination. The fact that these haplotypes are inherited together makes them very useful in association studies, reducing multiple testing and genotyping costs [136, 137].

1.5.3 Genetics of disease

Pathologic conditions, or traits, are often a product of both environmental and genetic factors. A way of estimating the contribution of the genetic component is through twin, adoption or emigration studies. The contribution is given as a percentage of the total variance of the trait that is explained by inheritance and is called heritability. Heritabilities of 30-95% are considered as evidence of a strong genetic component.
The simplest trait to study in genetics is that of monogenic diseases. Assuming that environment plays a relatively minor role it is relatively easy to pick-out the pattern of inheritance in family pedigrees and apply the mendelian laws. Many of these condition have been collected in the online mendelian inheritance of man (OMIM) database. Although monogenic conditions such as abetalipoproteinemia and familial hypercholesterolemia exist in autoimmunity, hypertension, CAD and atherosclerosis the trait in the majority of patients is caused by many genes acting in concert and is said to be polygenic. Adding to that environmental factors seem to be important for complex traits. In complex traits, also called multifactorial, the phenotype may be continues or start at a certain level. In the latter the multitude of forces acting on the condition must reach a certain threshold in order for the patient to display the phenotype. The strength of the correlation between trait and allele is often referred to as the disease *penetrance* and is defined as the probability that an affected individual has the disease causing allele. Other important factors that may disturb the relationship between genotype and phenotype are locus and allele heterogeneity. In locus heterogeneity several locus give rise to the same phenotype. When allelic heterogeneity occurs several alleles give rise to the same phenotype. For all conditions there is also an uncertainty in correctly detecting/measuring the trait since several conditions might be similar, also called phenocopies. Diagnosis criteria and methodology are here essential.
Traits in relation to the genetic component can be studied in two fundamentally different ways, linkage and association. Linkage studies usually require large family pedigrees or some other information on the inheritance structure. Since large pedigrees are rare in complex traits this method is more suited for mendelian traits. To work around these difficulties non-parametric affected sibpair analysis may be used when suitable cohorts exist. Association studies build on the principle that alleles associated to a certain trait should be represented at a higher frequency in affected individuals than controls. It is common to construct association studies as case-control or Transmission Disequilibrium Tests (TDT).
To map the chromosomal region/regions responsible for the susceptibility of disease the researcher can choose to use positional cloning or the candidate gene approach. The positional cloning approach is performed on whole genome scale and has the advantage of not being hypothesis driven. The candidate gene approach on the other hand is usually performed on a single or a few genes and needs some preinformation generating a hypothesis that is being

tested. The major disadvantages of these methods are that the resolution of the mapping generated by positional cloning rarely produces a single gene and that disease pathology often is more complex so that candidate genes can not explain the complex phenotype seen.

Quantitative Trait Locus

In complex disease where the trait by definition does not exhibit mendelian inheritance and usually depend on multiple loci and environmental factors the trait is often non-dichotomous, that is continues or discrete. Quantitative Trait Locus analysis (QTL) may then be used. The advantage is that there is less loss of information in the analysis as compared to the dichotomous dataprocessing. However, the difficulty of correctly identify and estimate effect of each QTL rise as more QTLs are involved. The QTL approach have become popular in animal models because of the ease with which one can arrange breeding schemes to isolate genomic regions. Combined with gene expression arrays, the realization that expression data is actually very suited as quantitative traits and gene network modeling a new type of analysis has emerged called eQTL analysis [138]

1.6 MOUSE MODELS OF ATHEROSCLEROSIS

Although mice weigh about 2000 times less and have a heart rate about 10 times higher many studies on endothelial function in mice have yielded results also valid for humans [139]. When comparing QTLs in mouse models of atherosclerosis such as ApoE-/- and LDLr-/- mice about 67% of the mouse QTLs map to human QTLs [140]. Also, studies of recombinant inbred strains between atherosclerosis susceptible and resistant strains have yielded results that seems to predict risk also in subsequent human studies [141]. However, there are also important differences to have in mind. For example the lipoprotein profile differs; the predominant lipoprotein in normal mice is HDL whereas in humans the predominant lipoprotein is LDL. Other differences related to lipids are the non-existence of the CETP enzyme in mice and differences in apoB expression in liver and intestine (for a comprehensive list see www.jax.org). Related to the immune system it should be mentioned that mice are highly resistant to infections and inflammations as opposed to humans. Because of these and other differences mice do not naturally develop atherosclerosis. Given a high fat diet (50%) some strains such as C57BL6 develop fatty streaks in the aorta. Other strains such as the C3H however are highly resistant to this dietary challenge [142-145]. When comparing biological parameters the most striking differences can be seen in innate (eosinophile, basophile, neutrophile cells and monocytes) and adaptive immunity (lymphocytes), blood pressure, fat deposits, cholesterol and bone mineral density (For comparisons of phenotypic data see www.jax.org). Most interestingly the cholesterol levels are higher in C3H resistant, than C57BL6 susceptible mice. Several transgenic and knock-out mouse models of atherosclerosis have been constructed (www.jax.org). The most widely used models are the ApoE-/- and LDLr-/-, made 1992 and 1993, respectively. Countless modifiers of atherosclerosis have been introduced on these two background models. The ApoE3 Leiden model is included due to the published cross-breed with the human CRP transgenic mouse referred to in the fourth article. Despite these gross differences between humans and mice they are used because of the short reproduction periods, relatively low cost and knowledge about ES cells. The latter is important for production of transgenic or knock-out models.

1.6.1 ApoE knock-out

The apoE knock-out ($apoE^{-/-}$) mouse is to date probably the most widely used model of

atherosclerosis. The apoE protein was selected because of its role in lipoprotein particle uptake by foremost the LDL receptor in the liver but later also proven to be important for macrophages. The inability of ApoE knock-out mice to take up lipoproteins through this route renders it with extremely high cholesterol levels (>500mg/dl on chow diet) and a shift from HDL to VLDL being the predominant lipoprotein particle. This strain automatically develops atherosclerosis when fed a chow diet, resulting in lesions that are human like in appearance [146]. Foam cells can be detected in the proximal aorta at 10 weeks and after 15 weeks intermediate lesions with foam cells and SMC have developed. At 20 weeks fibrous lesions have developed and after 32 weeks the plaques becomes calcified and the wall is thinning [146]. Cholesterol levels are quadrupled when fed a western type diet and lesion expansion progresses faster, lesions becoming more lipid-rich. Correlation between whole aorta measurements (*en face*) and sinus sections is good up to 40 weeks, were after lesion progress becomes more and more heterogeneous [147]. Lately the model have been criticized because of the hugely unphysiological cholesterol levels but also for the apoE proteins participation in processes such as immunopresentation [148, 149].

1.6.2 ApoE3 Leiden

The apoE3 Leiden model is transgenic for the human apoE3 variant (www.jax.org). The model is hyperlipidemic with VLDL being the dominant lipoprotein particle [150, 151]. It is less susceptible to atherosclerosis than the apoE model and needs to be fed an atherogenic diet containing cholate. The drawback however is that cholate by itself causes inflammation in the liver. It is less studied than the LDL receptor knock-out (Ldlr $^{-/-}$) and apoE$^{-/-}$ mouse models.

1.6.3 LDL receptor knock-out

Because of the nature of the apoE$^{-/-}$ model it is not suited for dietary studies. The Ldlr $^{-/-}$ model has therefore emerged as the predominant model for dietary studies. Knocking out of the LDL receptor renders the mouse model with IDL/LDL as the predominant lipoprotein particle. Hence, this model has a lipoprotein profile similar to humans. On chow diet the cholesterol levels reaches around 2-300mg/dl. However, the lesions only become moderate in size and complexity due to apoB48 and apoE which binds to the LRP receptor and is taken up by the liver. On a high-fat diet the Ldlr $^{-/-}$ model develops cholesterol levels of around 1500mg/dl and the lesions are widely distributed and becomes complex with age. The western diet also leads to an increase in autoantibodies towards an epitope found on oxLDL and titers correlate with the atherosclerosis extent, this is similar to findings in humans [152]. It is also worth noting that mean T-lymphocyte density decreases over time. The T-lymphocyte density is at all times larger in apoE$^{-/-}$ than in Ldlr $^{-/-}$ mice [153].

1.6.4 LDL receptor knock-out ApoB100 only

To render the Ldlr $^{-/-}$ model more susceptible to atherosclerosis it was cross bred with a mouse strain with a mutated apoB splice site. The mutation gives exclusive expression of apoB100 (apoB100$^{only/only}$) in this model. Since apoB100 is not taken up by LRP in the mouse liver as efficiently as apoB48, it therefore promotes atherosclerosis [154]. ApoB100only expression also results in increased numbers of small dense lipoproteins as compared to the Ldlr $^{-/-}$ and apoE-/- model [148]. This mouse model has, like humans, LDL as the predominant plasma lipoprotein, in contrast to the apoE$^{-/-}$ atherosclerosis mouse model that have plasma containing predominately VLDL. When fed a chow diet the cholesterol levels reaches 300mg/dl and when

fed a western type diet 500mg/dl. In paper III this mouse model also contains an inducible transgene (Mx1-Cre) [155] that upon activation conditionally mediate the expression of Cre recombinase. The activation of Cre recombinase is induced by injecting interferon γ inducing agents such as polyinosinic-polycytidylic ribonucleic acid (pI-pC). The recombinase enzyme recognizes flox P-sites that in our mouse model are flanked around the promoter region and exon 1 of the Mttp gene (Mttpflox/flox) [155]. The flanked region is excised and the expression of Mttp is lost. The recombination of Mttp prevents the synthesis of lipoproteins in the liver and as a consequence circulating cholesterol levels is lowered by more than 70%. The $Ldlr^{-/-}Apob^{100/100}Mttp^{flox/flox}$Mx1-Cre have a mixed genetic background (~25% 129/SvJae and ~75% C57BL/6).

1.6.5 Human CRP transgene

The CRP transgenic mouse model cross-bred with our atherosclerosis model was originally created by Murphy and colleagues [118], using the pCOS-CRP1 cosmid characterized by Ciliberto and collegues [156]. Our hCRP transgenic mouse contains a 31kb Cla-fragment (construct #42), 17kb 5' and 13.6kb 3' of the transcriptional start site, containg both the CRP gene and its psuedogene. The basal and induced blood levels were as described by Murphy et al. (~1µg/ml and ~50µg/ml respectively). The CRP transgenic mice were back-crossed 8 generations on the $Ldlr^{-/-}Apob^{100/100}$ mice. When bred upon our mouse model of atherosclerosis, serum levels were comparable up to 15 weeks but then increased several fold and varied greatly over time (paper IV). The human CRP transgenic mouse have also been cross-bred with the $apoE^{-/-}$ [157, 158] and apoE3 Leiden [159] models, as described in paper IV and the discussion.

2 AIM OF THESIS

The principal aim of this thesis is to investigate and increase the understanding of the regulation and function of CRP in cardiovascular disease and atherosclerosis in man and mouse.

Specific aims:

1. To search for polymorphisms in the CRP regulatory regions and to investigate their relations to circulating CRP levels in humans.

2. To determine the relation between sex hormones and circulating CRP levels in men.

3. To establish atherosclerosis progression and associated changes in gene expression a mouse model of atherosclerosis with familial hypercholesterolemic phenotype.

4. To investigate the effect of transgenic expression of hCRP on atherosclerosis development in a mouse model.

3 METHODOLOGICAL CONSIDERATIONS

In this section methods used in the articles are briefly described or commented upon. For more information see papers in thesis.

3.1 DETECTION METHODS

3.1.1 Denaturing high performance liquid chromatography

Denaturing high performance liquid chromatography (dHPLC) was used to detect SNPs. PCR amplified DNA is first denatured and then cooled slowly in order for heteroduplexes to form. The method utilizes the fact that single strands of DNA with a base change, when annealed with the noncomplementary strand has slightly different physico-chemical properties than if perfectly matched. The perfectly matched strands will have a shorter retention time when run through a HPLC column as compared to mismatched strands. Each polymorphism present in the amplified DNA sequence will generate a specific duplex formation, which gives a specific retention time (examples shown in figure 6) The technique is able to detect and distinguish heterozygotes from homozygotes, since it is only in heterozygotes that heteroduplexes are formed. Since the technique does not give any information about the sequence variation and position, heterozygotes must be sequenced. The method is very useful for fast screening of relatively large pieces of genomic DNA.

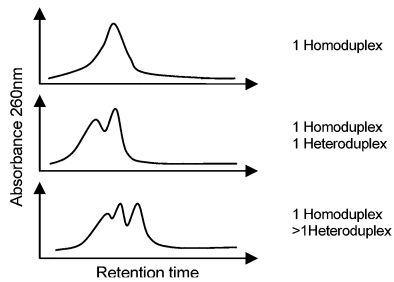


Figure 6.
Wave pattern generated by dHPLC

3.1.2 Restriction fragment length polymorphism analysis

RFLP was a commonly used technique to detect SNP's, but have now largely been overtaken by other techniques such as TaqMan, Pyrosequencing and Sequenom. Restriction endonucleases are used to recognize certain DNA sequences. The specificity of the technique is highly dependent on the specific enzyme used. It is an easy and inexpensive method, but results can, in some cases be hard to interpret. To find useful recognition sites including your polymorphism, the Insizer software (http://zeon.well.ox.ac.uk/git-bin/insizer) can be used.

3.1.3 Enzyme-linked immunosorbent assay

ELISA is a method using antibodies to detect antigens, such as interleukins, marker proteins or antibodies, in solution. The sandwich ELISA method uses two sets of antibodies. One attached to the surface of a microtiterplate, catching the antigen from the solution. Another used to attach an enzyme to the antigen, generating a color-change quantitative to the amount of antigen bound by the first antibody, reflecting the concentration of antigen in solution. Since the method is highly dependent on the specificity and strength of the antigen/antibody interaction, factors such as temperature, duration of incubation and mixing of samples are of great importance. The method is very sensitive and can detect minute differences pipetted to wells. Because of the operator and environment variation a standard is included in each experiment, generating a standard curve of known concentrations against which the unknown samples are checked. The samples are run in at least duplicate and must not have a coefficient of variation greater than

12%. Although widely used other methods such as flowcytometric analysis of cytokines or turbidity based methods may be used instead.

3.1.4 Immunohistochemistry and stainings

Immunohistochemistry uses antibodies, usually a primary and secondary, to detect antigens in tissue. The primary to bind to the antigen with great affinity and specificity, the secondary to bind directly to the primary in order to amplify the signal. Depending on the method of detection the secondary antibodies may be conjugated with gold, HRP or a fluorescent marker. While gold together with electron microscopy may be used for high resolution pictures and sub cellular localization, Horse Radish Peroxidase (HRP) and fluorescent markers may be used for gross localization in larger tissue samples. Due to the inherent auto flourescence of tissues HRP may have a better signal to noise ratio. Fluorescent conjugates however is the only choice when looking at co-localization using laser scanning confocal microscopy. The dynamic range is also greater with flourescent markers. Since the method is, as the ELISA, based on antibodies it is sensitive to the same factors but also to the method of fixation used. It is therefore important to use both an isotype control antibody as well as a control where the secondary antibody is omitted. The method is at best semi quantitative and other methods, such as western blot or flowcytometry, should complement this technique. In this thesis staining with Oil-red-O and Masson's Trichrome was also performed to visualize neutral lipids and collagen, respectively. The molecular function of these stains is often unknown but specificity has been determined empirically.

3.1.5 Fixation and tissue sectioning

Fixation of tissues/sections is used in order to stop degradation of structures, stop diffusion of antigens and in some cases as means to change epitopes for better antigen detection by antibodies. For the latter usually the opposite is true and antigen retrieval is necessary for antigen recognition.
Depending on the embedding material cryo sectioning or microtomes may be used for sectioning of tissue. If the tissue of choice is not homogenous and attempts to quantify is to be made it is highly important to choose anatomical guides to verify correct position. Even so, often due to great anatomical heterogeneity it is difficult to have an accurate quantitative measure.

3.1.6 En face analysis

As described in paper III and IV *en face* analysis was used to measure the fraction lesion surface area of the aorta (aortic root to iliac bifurcation). In brief, formalin fixed aortas were isolated and cleaned, cut-open longitudinally and pinned-out flat on black wax surfaces. Subsequently, the aortas were stained using Sudan IV to clearly distinguish lesions from healthy aortic wall. Aortas were photographed using a 12-bit digital color camera mounted on a microscope. Guided by an operator, software then scored each picture and calculated the fraction of lesional area (sudan IV stained) of the whole aorta and reported it as a percentage.
The operator was blinded to CRP genotype.

3.1.7 RNA extraction

In brief, after RNAlater perfusion and isolation of aorta (from root to third rib), excess fat and connective tissue was dissected away using forceps and micro-scissors under a microscope. As described by manufacturer fast-prep was used to shear tissue mechanically. Samples were

then purified on silica columns and submitted to a DNAse I step, as recommended by manufacturer (Qiagen). Eluted RNA was then used for Affymetrix Gene chip analysis or reverse transcribed and used for real time PCR analysis (TaqMan, Applied Biosystems).

3.1.8 Real time RT-PCR

The method is based on the Reverse Transcriptase and Polymerase Chain Reaction.
In brief, first the template RNA is reversely transcribed to cDNA and secondly real time detection of the PCR reaction is used to quantify the original transcript. While the method have a great dynamical range, on the order of 10^6, and can detect minute amounts it suffers from normalization problems. The underlying assumptions are that cell types and numbers in compared samples are equal, that there are no pipetting errors and that reverse and amplification reactions proceed with the same efficiency in all samples. Since this is utopic, the results must be normalized/corrected to one or several house keeping genes.
In this thesis a standardized sample, serially diluted, was also used for corrections between different runs of the same gene.

3.1.9 Affymetrix genechip technology

The Affymetrix Genechip technology consists of a high density array of DNA on a silica surface. Using lithographic techniques 25-mer oligonucleotides are sequentially build up on the surface. The array is divided into small squares containing multiple 25-mers of the same sequence. The fluorescently labeled, fractionated, RNA-sample is hybridized to the chip and by high stringency washes only those transcripts matching the 25-mers remain bound. A laser then scans the surface to excite the fluorophores. A high resolution CVD camera takes a picture of the chip which then is used in the following data processing procedures. The Mouse Genome 430 2.0 Gene Chips used in this thesis contains 11,979 Gene Chip probe sets.
There are a number of factors distinguishing the Affymetrix arrays from the RT-PCR procedure making them hard to directly compare. First, compared to the RT-PCR technology the dynamic range of the chip is only on the order of 10^2. Second, the RT-PCR method is more sensitive. Third, because of the ability of the camera to distinguish small intensity differences arrays has a greater ability to determine the exact number of transcripts of a certain gene. Fourth, some of the problems involved in using the house keeping gene approach are omitted because of the method of global normalization. Furthermore, pipetting errors are less of a problem since “all” genes are run in a single experiment. On the other hand it should be mentioned that although the stringency in the wash steps is high, due to the many parallel hybridizations specificity is hard to reach for all genes at one and the same condition. Because of this cross-hybridizations do occur. Another problem occurring when multiple transcripts are mapped on the same array is the probability that the same sequence occurs more than one time. As described in a later section we have handled this problem by remapping of probe sets.

3.1.10 Silencing interfering RNA technology

In paper III, monocytes of the human monocytic cell line THP-1 were plated in six-well culture dishes at 6 x 10^5 cells/well in 10% FCS-RPMI-1640 medium with L-glutamin (2mM) and HEPES buffer (25 mM) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) (PEST) and induced to differentiate into macrophages with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) for 72 h. For each gene, up to three siRNAs were transfected with Lipofectamine 2000 according to the manufacturer’s instructions in medium without FCS, PEST and PMA. Two

days after transfection, siRNA-targeted macrophages and siRNA-negative Lipofectamine controls were incubated with acetylated low-density lipoproteins (AcLDL, 50 μg/mL) for 48 h in 1% FCS medium with PEST. AcLDL was prepared as described. The samples were dialyzed against PBS at 4°C. AcLDL protein concentration was determined by Bradford. LDL was isolated from plasma of healthy donors by sequential ultracentrifugation.

3.2 STATISTICAL METHODS

3.2.1 Hardy-Weinberg equilibrium

In paper I the frequencies of the alleles were checked for Hardy-Weinberg equilibrium using a chi-square test. All the alleles were in Hardy-Weinberg equilibrium; this indicates that our genotyping was correct.

3.2.2 Student's t-test and ANOVA/ANCOVA

Student's t-test was used to detect the differences between the groups. When more than two groups were tested for differences ANOVA and ANCOVA (in the case of co-variates) was used. Since all three methods assume normal distribution, skewed variables were log-transformed.

3.2.3 Univariate and multivariate analysis

In paper II the correlations between treatment and CRP levels were tested by univariate analysis. Multivariate analysis was used to take into account multiple factors such as treatment, CRP concentrations before treatment and IL-6 concentrations after treatment that could partially explain the differences seen in univariate testing.

3.2.4 Array normalization and mapping

In order to describe biological meaning to the probe intensities they need to be mapped to transcripts and subsequently to genes. Prior to mapping the probes need to be normalized. Currently no standard exists. For example Affymetrix cuts away the top 1% and lowest 1% probe intensities and then fit the rest to a regression line of a standard chip.
In paper III the MAS 5.0 (Affymetrix) algorithm was used with default settings, log-transformed, and normalized to total intensities (Global Scaling). The probe sets where then mapped on the mouse genome database (MGD) and an average was calculated for each gene. Lowess normalization was used in a pair-wise fashion before differential testing [160].
In paper IV a procedure called Quantile normalization was used [161]. It is a non-parametric test which assumes that the distribution of probes on each chip is the same. The quantile normalization is part of a pre-processing procedure called robust multichip average (RMA) [162]. Following the RMA procedure, because of the cross-hybridization and non-exclusive probe sequences, the probes are mapped against a gene database, in the case of paper IV the RefSeq database and a weighted average is calculated for each gene [163], before differential testing.

3.2.5 Array data analysis and significance testing

There are two principle ways of analyzing array data; exploratory and tests for differential expression. The exploratory approach should be used for finding patterns in the data, without preconceived ideas. One way of doing this is by clustering. Four principle and arbitrary choices must be made; data scale to use, use all genes or not, what distance measure and what

clustering algorithm to use. The mostly used clustering technique by biologists is hierarchical clustering. Important to remember is that common to most clustering techniques is that they falsely imply descent. In paper III we used the FindCluster algorithm in Matematica 5.1 [164]. When testing for differential expression the scientist find himself in a situation of making thousands of comparisons. The usual t-test grossly underestimates the false positives due to multiple testing. One way of dealing with this is the Bonferroni correction. However, this is a very stringent correction that punishes the data. A better way of managing the multiple testing problem is to use the false discovery rate (FDR). Usually the FDR calculations start by choosing an appropriate statistic, e.g. t-statistics for two groups. The statistics is then computed for all the genes. The labels are then permutated and the test is run again (ex. 1000 times). Lastly, the fraction of cases from the permutations that exceeds the real statistics is computed.
In paper IV we have chosen to use a regularized t-statistic since its better suited for small sample groups. However, since the distribution of this regularized t-statistics is unknown we adopted an empirical bayes method to calculate the FDR [165].

3.2.6 Expression mapping

To ascribe functional meaning to the datasets annotation software such as EASE and GSEA were used. EASE uses differentially expressed genes and report over representations in pathway databases such as KEGG and GO [166]. The GSEA instead uses the whole dataset to look for overrepresentations [167, 168]. The GO database is maintained by the gene ontology consortium and comprises of three independent ontologies; biological processes, cellular components and molecular functions. They all describe relationships between genes and eukaryotic functions and processes. The KEGG database comprises of curated biological pathways extracted from literature.

4 STUDY COHORTS

4.1 STUDY GROUP I

A total of 357 consecutive patients under the age of 60 years from the northern Stockholm Metropolitan Area, diagnosed to have a first myocardial infarction, according to national criteria, were enrolled in a clinical research programme targeting mechanisms underlying premature CHD. All patients had been admitted to the coronary care units of the Danderyd, Karolinska and Norrtälje Hospitals. Patients with insulin-dependent diabetes mellitus, renal insufficiency, chronic inflammatory disease, malignancy and unwillingness to participate were excluded. Age- and sex-matched controls were recruited from the same catchment area using a registry kept by the Stockholm County Council.

4.2 STUDY GROUP II

A total of 222 patients aged 75 years or under, with typical symptoms of myocardial infarction with a duration of less than 12 h on admission to hospital and with an electrocardiogram showing ST-segment elevation in at least one standard or two adjacent precordial leads or bundle branch block treated with thrombolysis, were recruited to the second cohort. They were all admitted to the coronary care unit of one hospital (Danderyd Hospital, Stockholm, Sweden). Exclusion criteria and representativeness of the patients have been reported (113). All patients and controls gave their informed consent before entering the research programmes, which were approved by the ethics committee at the Karolinska Hospital.

4.3 STUDY GROUP III

One hundred consecutive patients with prostate cancer suitable for hormonal therapy were enrolled in the study. Exclusion criteria were: Cardiovascular disease such as myocardial infarction, cardiac decompensation, severe angina pectoris, severe intermittent claudication, cerebrovascular or thromboembolic disease. Patients were randomized to either orchidectomy or estrogen treatment. The estrogen regimen was: 160 mg polyestradiol phosphate i.m. every month the first three months, then 80 mg monthly, plus 1 mg ethinylestradiol orally for two weeks, followed by 150 µg daily. For different reasons only 83 patients finally participated in the study (orchidectomy; n=41, estrogen; n=42). Blood samples were taken before and 6 months after intervention or start of estrogen treatment. The study cohort has been characterized previously (114).

4.4 MOUSE HOUSING

All experiments were reviewed, commented upon and approved by the local ethics committee. The mice were maintained with a 12h light/dark cycle and had food and water *ad libitum*. In paper III both male and female mice were used to establish the atherosclerosis progression curve but only female mice for expression analysis and cholesterol lowering experiments. Only male mice were used in paper IV due to higher blood levels of human CRP. Mice were housed 4-6 per cage. Blood was collected from tail veins at time points described in the papers III and IV. The mice were sacrificed by CO2 inhalation, perfused with PBS/formalin or RNAlater and tissues were excised. Correct genotypes were controlled using PCR as described in papers III and IV. Also, CRP phenotypes were verified by ELISA of plasma CRP levels and apoB100 by western blot.

5 SUMMARY OF RESULTS

5.1 GENETIC AND HORMONAL REGULATION

CRP (PAPER I AND II)

5.1.1 Genetic regulation

Using dHPLC and sequencing we found two novel common SNPs in the promoter region
CRP gene at positions –286 and –717, in relation to the transcriptional start site. Two no
286, -717) and two published (+1059, +1444) polymorphisms were investigated in at lea
of two study cohorts.

At position –286 (C>T>A) we found the allele frequencies of the two less common T
variants to be 34% and 5%, respectively, in study group I (patients and controls taken tog
No difference in allele frequency was noted between patients (T allele 33%; A allele 5%
controls (T allele 34%; A allele 4%). The corresponding allele frequencies in study group
43% and 7%. The polymorphism at position -717 was identified as an A > G substitution w
allele frequency of the less common G variant of 28% in patients and controls taken to
(study group I). No difference in allele frequency was seen between patients and control
allele frequencies of the less common T and C, respectively for the previously des
polymorphisms at positions +1059 and +1444 were 7% and 32% (study group
differences in allele frequencies were seen between patients and controls. The frequen
previously known SNPs were similar to previously published results. The polymorph
position -286 was associated with the CRP concentration in patients and controls an
together (Table 3, Figure 7A, $p < 0.01$). When data were analyzed in patients and c
separately, a statistically significant difference in CRP levels between genotypes was see
in patients (Figure 7B, $p < 0.02$). The + 1444 C > T polymorphism showed a bor
significant difference in plasma CRP levels between the alleles ($p = 0.08$), but neither the
A > G, nor the +1059 G > C polymorphisms were associated with the plasma
concentration.

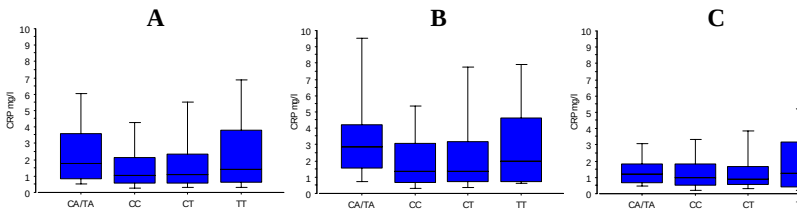


Figure 7. Genotype distribution in study group I
A; Patients and controls together, B; Patients only, C; Controls only.

CRP levels stimulated by ST-elevation myocardial infarction did not differ accord
genotypes of the -286 SNP. CRP levels 3 months after the acute event tended to be asso
with the -286 SNP but this association failed to reach statistical significance ($p < 0.11$).

The common allele of the -286 SNP was associated with the common allele of the +1444 SNP and the two less common -286 alleles were associated with the rare +1444 allele; for all other combinations, the rare allele of one SNP was found on the common allele of the other. Three common haplotypes were noted as follows (SNPs are listed in the order -717, -286, +1059, +1444): A, T, G, T with frequency 30%; G, C, G, C, 27%; A, C, G, C, 27%. None of these haplotypes were associated with the plasma CRP concentration (data not shown).

5.1.2 Hormonal regulation

To investigate regulatory effects of sex hormones on CRP in men, we analyzed serum of 100 consecutive prostate cancer patients for CRP and its main regulators IL-1β and IL-6. Blood was collected before and 6 month after intervention. One half of the patients received estrogen hormone therapy and the other half was subjected to orchidectomy.

Univariate analysis showed that orchidectomy tended to decrease circulating CRP concentrations whereas estrogen treatment tended to increase CRP. There was no difference in circulating CRP concentrations before treatment, whereas treatment with estrogen resulted in higher circulating CRP levels than orchidectomy (Fig. 8). Multivariate analysis including treatment, CRP concentrations before treatment and IL-6 concentrations after treatment showed that the difference in CRP concentration after treatment was highly significant (Figure 8). Furthermore, analyses showed that there were no differences between the orchidectomy and estrogen groups regarding serum IL-1β and IL-6 concentrations before and after treatment, respectively (Data not shown).

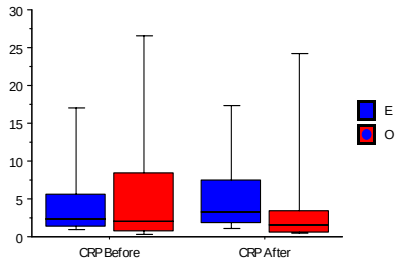


Figure 8. CRP concentrations divided by treatment and time. 95% are within the bars and 50% within the boxes. E=estrogen treatment, O=orchidectomy.

5.2 ATHEROSCLEROSIS AND CRP (PAPER III AND IV)

5.2.1 Atherosclerosis progression:

We performed transcriptional profiling and determined lesion development at 10-week intervals in atherosclerosis-prone mice with human-like hypercholesterolemia and a genetic switch to turn off hepatic lipoprotein production (*Ldlr*$^{-/-}$*Apob*$^{100/100}$ *Mttp*$^{flox/flox}$Mx1-*Cre* mice[155]). In a subgroup of mice, the lipoprotein synthesis was turned off at 30 weeks to quantify the effects on transcriptional regulation and lesion status. Six of the identified genes where then tested in siRNA experiments in a macrophage cell line (THP-1).

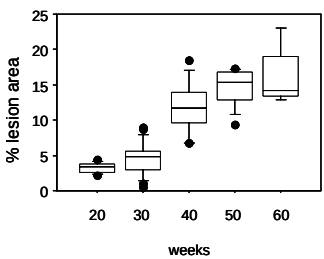


Figure 9 Atherosclerosis progression; at 20 (n=12), 30 (n=25), 40 (n=15), 50 (n=15), and 60 weeks (n=10). *P*<0.05, 20 *vs.* 30 weeks; *P*<0.0001, 30 *vs.* 40 weeks; *P*<0.02, 40 *vs.* 50 weeks. Values are surface lesion areas as a percentage of the entire aorta.

From 10–60 weeks of age, plasma cholesterol increased slightly; triglyceride and glucose were unchanged. Morphological analysis (Fig. 9) showed occasional spots of Sudan IV s at 10 weeks (n=10), whereas all mice had lesions at 20 weeks. Lesion size increased from weeks 20-30 and ~7.2% from weeks 30-40. At 20 weeks, only fatty streaks were p but at 30 weeks, all mice had small plaques in the aortic arch that expanded substantially weeks. Thereafter, plaque growth was restrained.

1259 (6.3%) genes were differentially expressed (FDR <0.05) at one or more comparisons. Cluster analysis of differentially expressed genes generated four distinct cl Cluster 1 genes were activated during rapid lesion expansion, remained activated throu 60 weeks, and had the highest percentage of genes previously related to atherosclero atherosclerosis related cell types (Fig. 10). Of cluster 1 genes, 89% were rela inflammatory cells, including the macrophage-marker CD68, which increased fivefold (v by IMH) and appeared to reflect macrophage recruitment and activation. Cluster 3 peaked at 30 weeks and was suppressed at 40 weeks (Fig. 10), coinciding with rapid lesion expansion. Re-occurring themes in the functional annotations were related to amino acid and lipid metabolism. Exclusive for cluster 3 was angiogenesis related GO categories. In cluster 3 transcription factors (TFs) established in lipid and energy metabolism, such as the peroxisome proliferator activator receptors PPARα, PPARδ, and PPARγ and sterol regulatory element-binding protein (SREBF)-2 were activated. Cluster 2 peaked at week 30 and cluster 4 at week 40, but were suppressed at late stages of progression (Fig. 10). The functional annotations of clusters 2 and 4 were harder to interpret. Cluster 2 was distinguished by activities such as sorting and degradation and protein folding, while cluster 4 contained no exclusive or over represented themes, as compared to the other clusters. Apoptosis a death genes could be found in all clusters but was most pronounced in cluster 2 an determined by GO. Apart from foam cells, which increased from 20-30 weeks, other lesion cell types were relatively stable as judge by cell type specific markers.

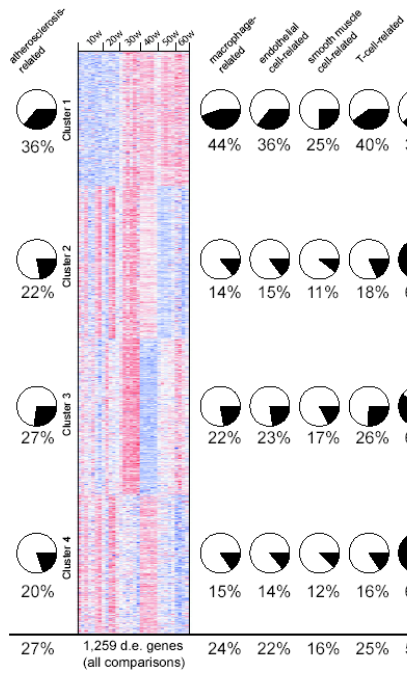


Figure 10 Heatmap of clustered mRNA levels (red=high ; blue=low Genes differentially expressed in at least one pair-wise time-p comparison (FDR<0.05). Each column represents mRNA levels one mouse (n=5 to 7 per time point); each row represents an M gene. Pie charts show percentages of genes related atherosclerosis.

To estimate gene activity per foam cell, we normalized mRNA levels of foam cell-related to those of macrophage markers and identified differentially expressed genes (FDR < Cluster analysis generated three clusters. Genes in *foam-cell cluster 1* were active i stages (10-20 weeks) and suppressed at late phases (30-60 weeks). These genes involved in processes such as growth, maintenance, homeostasis and biosynthesis. Ge

foam-cell cluster 2 were expressed at low levels in the early phases but were activated in the late phases and included genes involved in immune defense, inflammatory responses, and chemotaxis. *Foam-cell cluster 3* was harder to interpret but genes found exclusively in this cluster were related to angiogenesis and blood vessel development. Expression levels were high in the early phases (as in cluster 1) and remained active until week 30.

Cholesterol lowering

To genetically lower plasma LDL cholesterol, we induced recombination of *Mttp* (*Ldlr*$^{-/-}$ *Apob*$^{100/100}$*Mttp*$^{\Delta/\Delta}$) in 30-week-old mice. Plasma cholesterol levels were reduced >80% (from 427 to 54 ± 31 mg/L, n=6) and remained at this level for 10 weeks until sacrifice. At sacrifice, the lesion size had not increased and was significantly less than in controls with high cholesterol. To also identify transcriptional changes induced by cholesterol lowering, we recombined hepatic *Mttp* in 28-week-old mice and sacrificed them 1 week after cholesterol levels had been lowered (at 30 weeks). First, we established that lesion size and the relative numbers of the four major cell types (Macrophages, SMC, EC and T-cells) did not differ in mice with lowered cholesterol and high-cholesterol controls. These observations were supported by Oil-Red-O and CD68 protein staining.

Since the rapid plaque expansion was preceded by macrophage accumulation in the arterial wall at 30 weeks, lesion cluster 3 (intracellular lipid metabolism) was transiently activated at 30 weeks and TFs with established roles in inflammation and lipid homeostasis in foam cells were deactivated at 40 weeks (PPARs and SREBF-2), we speculated that cholesterol responsive genes could be important for foam cell formation. To confirm the importance of the cholesterol-responsive genes in their contribution to foam cell formation, 10 of the 37 cholesterol-responsive genes expressed in THP-1 macrophages were targeted using silencing interfering RNA (siRNA). The transfections were followed by incubation with acetylated LDL. Six of ten siRNA targeted genes affected the formation of foam cells (table 2).

Table 2 - siRNA targeted cholesterol-responsive genes

Gene symbol	Gene name	Degree of knock-down	%CE content relative control	p-value[a]	Visualization of ORO staining
1. AGL	amylo-1,6-glucosidase, 4-alpha-glucanotransferase	63%	-37%	p<0.001	decrease
2. AGPAT3	1-acyl-sn-glycerol 3-phosphate acyltransferases	69%	-14%	ns	decrease
3. CD36	CD36 antigen	66%	-17%	p<0.001	degrease
4. GPR81	G protein-coupled receptor 81	30%	-14%	ns	decrease
5. GPR120	G protein-coupled receptor 120	67%	+17%	p=0.04	increase
6. GYPC	gypc, glycophorin C	52%	nd	-	-
7. HMGB3	high mobility group box 3	65%	-18%	ns	decrease
8. PRKAR2B	protein kinase, cAMP dependent regulatory, beta II	49%	-55%	p=0.001	decrease
9. PVRL2	poliovirus receptor-related 2	65%	-30%	p=0.002	decrease
10. SOX6	SRY-box containing gene 6	72%	-27%	p<0.001	decrease

[a] P-value indicate CE content in the foam-cells for targeted genes (n=6) compared with lipofectamine transfected parallel controls (n=6)
CE indicates cholesterol ester; ORO, Oil-Red-O; nd, no difference; ns, not significant.

5.2.2 CRP and atherosclerosis progression

We performed transcriptional profiling and determined lesion development at 15, 30, 40 a
weeks in a mouse model of familial hyper cholesterolemia (Paper III) transgenic with a 31
fragment of the human C-reactive protein gene (*CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$). Lesions were st
en face and by staining aortic root sections. Transcriptional phenotypes were determined
arrays at 40 weeks an verified with RT-PCR at 30 and 40 weeks.
With the exception of plasma levels of hCRP, the basic characteristics of the *CRP*$^{+/0}$*Ldlr*$^{-/-}$
Apob$^{100/100}$ mice did not differ from those of littermate controls at any time point. Weight
increased in both groups between weeks 15 and 30. Plasma cholesterol, triglycerides and
glucose levels were stable in both groups except for a statistically nonsignificant peak at 4
weeks. SAA serum levels increased until week 40 and thereafter remained stable at 50 w
As expected, hCRP was only detected in *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice. The mean plasma
hCRP level was 28.1 mg/l at 15 weeks and peaked at 50.5 mg/l at 30 weeks (P<0.01). Th
differences in hCRP levels between 15 and 50 weeks (P<0.01) and 30 and 40 weeks (P<
were significant. Only occasional atherosclerotic lesions were observed in 15-week-old m
30 weeks, both fatty lesions and focal plaques were present in the aortic arch of both
CRP$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice and littermate controls. Scattered fatty lesions were also fou
the abdominal region. At 40 weeks, however, the lesions were more extensive in the cont
= 0.027, Fig. 11). Although the extent of plaques had increased in both groups at 50 week
lesion surface area was still smaller in *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice than in littermate cont
(P=0.041, Fig. 11).

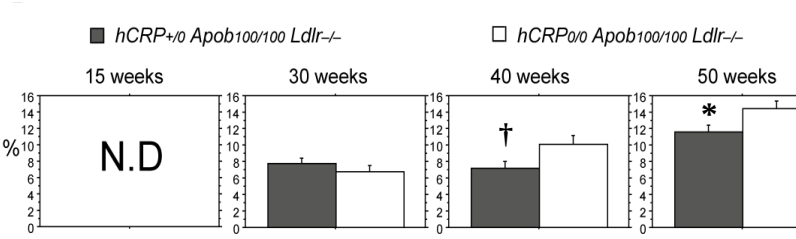


Figure 11 Lesion surface area is reported as the area of Sudan IV staining, expressed as percentage of the entire surface area the aorta from the iliac bifurcation to the aortic root. n = 17 *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice (black bars) and 19 controls (white bars at 30 weeks; n = 13 and 10 at 40 weeks; n = 11 and 15 at 50 weeks. Values are mean ± SEM. †P=0.027 at 40 weeks and *P=0.041 at 50 weeks versus control mice.

As expected, lesion histology showed that hCRP was detected only in lesions from *CRP*$^{+/}$
$^{-/-}$*Apob*$^{100/100}$ mice. At 15 weeks, hCRP could not be detected whereas it was clearly visible
weeks, increased markedly at 40 weeks, and then remained stable at 50 weeks. Complem
factor 3 (C3) followed a similar pattern in both groups except that the staining appeared m
focal in the controls than in the *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice. In both groups, CD68 staini
occasionally detected in 15-week lesions and then increased with lesion size over time; th
extent of staining did not differ between groups. The same pattern was observed for Oil R
and collagen staining, except that the area of collagen staining was ~10% larger in *CRP*$^{+/}$
Apob$^{100/100}$ mice at 50 weeks (n = 5 and 5, P<0.05).

To identify differences in lesion mRNA levels that could explain the slower plaque development in *hCRP*$^{+/0}$*Apob*$^{100/100}$*Ldlr*$^{-/-}$ mice than in nontransgenic controls, we performed microarray studies in four mice from each group using Affymetrix Mouse Genome 430 2.0 Gene Chips. 742 differentially expressed atherosclerosis genes were identified (FDR<0.25). Among the most interesting findings GO analysis of these genes revealed significant activity in the biological processes of *Protein catabolism* (*P*= 2.00E-05), *Cellular macromolecule catabolism* (*P*= 5.00E-06), and *Biopolymer catabolism* (*P*= 1.00E-05); in the molecular functions *Proteasome endopeptidase activity* (*P*= 4.00E-16); and in the cellular compartment *Proteasome core complex* (*P*= 8.00E-16) and *Nuclear ubiquitination complex*, *P*= 2.00E-02). These findings, together with the KEGG pathway analysis (*Proteasome*, *P*= 4.00E-12), indicated an altered activity in the proteasome degradation pathway.

GSEA analysis (Fig. 13) — a statistical approach that considers the entire Gene Chip dataset (unlike the GO analyses performed) — confirmed that the mRNA levels of genes in the proteasome degradation pathway were consistently higher in *hCRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice than in littermate controls. Gene expression changes identified by Gene Chip analysis were validated using real-time PCR to examine mRNA isolated from atherosclerotic lesions in 13 *hCRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ and 15 littermate control mice. Specifically, we examined six genes central to proteasome degradation: the *20s proteasome subunits* PSMA7, PSMB7, and PSMB9 (immunoproteasome subunit), the *19s proteasome cap unit* PSMC6, the *11s proteasome cap unit* PSME2, and *E2 ubiquitin ligase* (UBE2D3). Between 30 and 40 weeks, the mRNA levels of these genes were stable in *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice whereas they were significantly decreased in littermate control mice. The levels were similar at 30 weeks in both groups (*P*>0.22 for all genes). At 40 weeks, however, the levels were 1.3- to 2-fold higher in lesions from *hCRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ than in littermate controls, reaching borderline significance (Fig. 12).

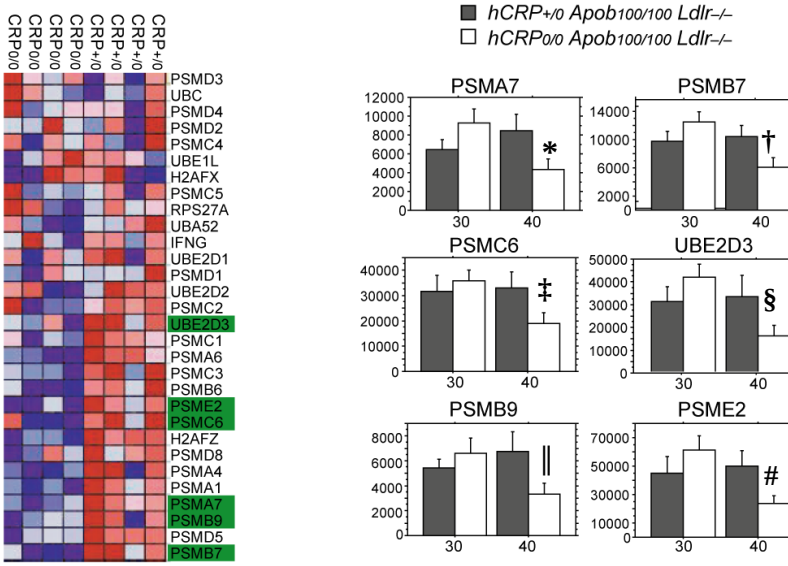


Figure 12 Global gene expression analysis and real-time PCR of atherosclerotic lesions.

Heatmap showing mRNA levels (blue, low; red, high) of genes (rows) in the proteasome degradation pathway, which was highly active in the *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice compared to the controls (FDR<0.09), as shown by GSEA analysis of the same GeneChips. Gene names marked in green were chosen for real-time PCR analysis. Also, mRNA levels of six genes central to proteasome degradation (PSMA7, PSMB7, PSMB9, PSMC6, PSME2, and UBE2D3) determined by real-time PCR of total RNA from aortic arch lesions in *CRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice (black bars) and *CRP*$^{-/-}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ controls (white bars) at 30 weeks (n=5 and n=7, respectively) and 40 weeks (n=8/group). Values are mean ± SEM. $^{*}P<0.02$, $^{\dagger}P<0.006$, $^{\ddagger}P<0.02$, $^{\S}P<0.05$, $^{\|}P<0.006$, $^{\#}P<0.004$ versus 30week controls.

6 DISCUSSION AND CONCLUSIONS

6.1 TO REGULATE OR NOT REGULATE

Despite the fact that the first 3000 bp of the 5' CRP promoter has been mapped and IL-6, USF-1, HNF and NFkB response elements found, little is known about regulatory elements upstream of the 5' first 3000 bp, in the intron and in the 3' region of the gene. Studies have suggested repressive regulatory elements 3' of the gene. Our results in paper I suggest that the –286 position of the CRP promoter has a regulatory effect on unstimulated circulating CRP levels. Another explanation could be linkage to a regulatory SNP elsewhere. However, a recent studies have suggested USF-1 binding elements to overlap with the -286 SNP both by in silico methods and functional in vitro studies [133]. Furthermore, none of the other SNPs investigated in our study were as highly associated with CRP and none of the three most common haplotypes were associated with CRP. The lack of associations regarding haplotypes could have other explanations. One could be that two or several SNPs are functional but are associated inversely to each other, pulling in different directions. Although regulatory elements could be found several kilobases 5' or 3' from a gene, variances found in the proximal promoter are more interesting since the likelihood of interference with the core regulatory machinery is higher. Since the publication of paper I, other prospective studies have shown the -286 tri-allelic SNP to be associated to CRP levels [124, 130, 132, 133]. Despite this re-occurring association the contribution from genetic variation to variation of CRP levels have suggested to only be 1.4% when adjusted for other factors, such as BMI [130]. Although highly interesting for diagnostic purposes none of the undertaken studies have been powered to test the predictive value of CRP SNPs to atherothrombotic events such as MI. With this in mind it should be mentioned that almost all studies have failed to detect any association to atherothrombotic events.

Steroid hormones have profound effects in humans by regulating important events such as growth, differentiation and fitness of cells. There is also strong evidence that, cortisol, estrogen and testosterone are important modulators of the immune system [169]. Estrogen has also been shown to modulate CVD, directly by interacting with the vascular wall and affecting NO production [93], and indirectly by redistribution of adipose tissue. It does not seem unlikely that estrogen either directly or through pathways such as NFkB, C/EBP or STAT3 would modulate CRP expression. Talking against direct interaction between CRP and estrogen is that studies have shown that the association between estrogen and CRP is dependent on route of administration [90]. There is a possibility that the different routes of administration have different pharmacokinetics. This could very well be the case since it has been shown that there is a dose-dependent effect on circulating CRP levels [170].

Although our results are suggestive there is always a risk of confounding factors. The limitations of our studies are several. First, medical treatment such as statins have been shown to affect circulating CRP levels. Second, there is a wide range of environmental factors such as smoking, fitness and alcohol use that have been shown to alter CRP levels. We have tried to circumvent these problems in paper I by using matched controls and verify our results in two cohorts and in paper II by using a randomized design and also checking for known inflammatory regulators of CRP expression. However, there are probably always factors that cannot be corrected for or that are unknown [171]. In our studies examples could be failure to survey the true number of smokers in paper I or that the prostate cancer has interacted in paper

II.

With this in mind the conclusions are that the –286 SNP in the promoter region of the CRP gene associate strongly with CRP concentrations, supporting a genetic influence on circulating CRP. Furthermore, estrogen associate with serum CRP concentrations indicating a role for estrogen in the regulation of unstimulated CRP levels. Although not proven directly, from our results, we believe that testosterone is not likely to be a major determinant of circulating CRP concentrations in humans.

6.2 ATHEROGENIC OR NOT ATHEROGENIC

As mentioned in the introduction RI-strains and transgenic mice have been extensively used in atherosclerotic research for at least 20 years. Due to improvements in both statistics and measurement technology, more research has focused on plaque biology and the relation between genetics and the gene transcriptional network. However, few standards have been implemented which makes comparisons across species, strains and even different measurement technologies hard. Due to cost issues most studies have just considered a single time point.

In order to explore new ways of handling data but also to investigate a relatively novel mouse model of atherosclerosis we have undertaken phenotypic characterization and global gene expression profiling of the *Ldlr*$^{-/-}$*Apob*$^{100/100}$ *Mttp*$^{flox/flox}$Mx1-*Cre* mouse model at multiple time points.

The finding of an S-shaped curve parallels findings in aortic root section in the apoE $^{-/-}$ model [146, 152, 172]. Although expected from biology, the late phases characterized by slowed growth have, to our knowledge, never been explored systematically in the Ldlr $^{-/-}$ or apoE $^{-/-}$ models and hard to investigate in humans due to the nature of human studies. Whether these differences are related to differences in lipid profiles or immunity remains to be investigated. On the subject of lipid profiles one study showed *Ldlr*$^{-/-}$*Apob*$^{100/100}$ *Mttp*$^{flox/flox}$Mx1-*Cre* mice to have increased numbers of small dense lipoproteins than both the Ldlr $^{-/-}$ and apoE $^{-/-}$ model [148].

Data in paper III suggest that the large transcriptional changes that occur are correlated with a massive infiltration of macrophages before 30 weeks. It is interesting to note that 3 out of 4 clusters identified experience a sharp in crease co-inciding with plaque expansion. Furthermore, after 30 weeks the total number of macrophages did not appear to change drastically, as judged by immunohistochemistry (IHM) and transcriptional markers. The usage of transcriptional data on cell markers to look for changes in the cell populations may be questioned. However, since few individual marker is neither truly cell type specific nor constant per cell, the transcriptional approach offers a way to simultaneously measure several markers at the same time and thereby averaging out individual differences. Again, it could be argued that this renders the measurement insensitive to small fluctuation. Probably the most useful approach is to verify a subset of the transcriptional findings with IHM. Another promising approach is using flow cytometry to probe the cell populations of the plaque.

Looking at transcription in lesional cluster 1 and foam cell cluster 2, data suggests that not only the general inflammation in the plaque but also the inflammatory phenotype per foam cell unit increases simultaneously. When regarding lesion cluster 3 and foam cell cluster 1, both with high metabolic, maintenance and biosynthesis activities, it is interesting to note that although lesion activities as a whole peak at 30 weeks the activities per foam cell unit actually decreases at 30 weeks and remain so. It is interesting to speculate whether these changes are related to a priming of the adaptive immune system and that at 30 week the adaptive immune system catalyzes an immunologic event related to a phenotypic switch in macrophages, from

scavenging/cholesterol re-transport to host defence. It is apparent that the metabolic situation in the plaque has not changed. Hence, something must drive inflammation and immune related processes. It remains to be proven but the apparent switch between savaging/cholesterol re-transport to host defence inspires to thoughts on antigen presenting processes and processes related to adaptive immunity.
However, it is important to keep in mind that the transcriptional measurements are truly average measurements. It is not inconceivable that the changes seen mark a change in macrophage subpopulations rather than a phenotypic switch. This is not unreasonable regarding the extensive data on macrophage heterogeneity that has been published lately [173].
The timing and themes of lesion cluster 2 and 4 were harder to interpret.
Investigating the lipid axis through a cholesterol perturbation yielded interesting results.
From a therapeutic angle it is interesting to note that rapid lowering of apoB containing particles have direct transcriptional effects on the plaque. This might model the lipid side of transcriptional changes during aggressive statin treatment leading to lesion regress in human studies [174, 175]. Results from the cell studies indicate that our results are also valid at the cellular level.

Through the inbreeding of a 31kb Cla fragment of the human CRP gene the impact of a perturbation of the inflammatory axis in the *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mouse model was studied. While studied in other mouse models previous studies of CRP in atherogenesis have been inconclusive. For instance, one study reported the atherosclerotic lesion area of aortic root sections in *Apoe*$^{-/-}$ mice to be larger in *hCRP* transgenic mice than in controls[157]. In contrast, two other studies of *Apoe*$^{-/-}$ mice [158, 176] and one study of *APOE*3-Leiden* mice [159] and ourselves in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice did not detect a difference in the lesion area in the aortic root. However, lesion areas in the entire aortic tree developed slower in h*CRP*$^{+/0}$*Ldlr*$^{-/-}$ *Apob*$^{100/100}$ mice than in nontransgenic controls, as judged by *en face* analysis. In our study, collagen was more abundant in the lesions of *hCRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice than in controls at late time points (e.g., 50 weeks), consistent with the findings of Paul and co-workers in CRP transgenic mice[157]. The CRP mice in that study also had increases in C3 deposits and macrophages (i.e., Mac-3). However, in our study, neither C3 deposits nor CD68 staining differed between lesions of *hCRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice and controls, although the C3 deposits appeared to be more focal in controls at 40 weeks and even more so at 50 weeks. In agreement with our data Hirschfield and colleagues [158] neither showed a difference in lesion C3 deposits, nor in CD68 staining. Complement factor 3, collagen, and macrophage stainings of the lesions might be related to the levels of CRP in the plaque. We detected hCRP protein and transcripts in the lesions of *hCRP*$^{+/0}$*Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice. Paul and colleagues [157] and Hirschfield and colleagues [158] detected only protein. Trion and colleagues [159] could not detect CRP protein in lesions. The conflicting results seen in the different mouse studies suggest that the effects of CRP in atherosclerosis are dependent on the mouse model used. In support of this notion, *Apoe*$^{-/-}$ mice have altered immune responses, including phagocytosis of apoptotic bodies, altered macrophage dynamics,[177] and altered antigen presentation efficiency,[149] possibly directly involving Apoe. Also, the effects of CRP on lesion development may be influenced by differences in the degree and type of lipoproteinemia in the mouse models. For instance, hCRP has a binding preference for LDL rather than VLDL.[106] Thus, the atheroprotective role of hCRP we observed in this study may be masked in *Apoe*$^{-/-}$ mice because they are VLDL animals and have altered immune functions.

The delay in development of atherosclerosis in our *hCRP* transgenic mice may be related to

immunological events, as suggested by the timing and data from paper III. As processes like scavenging/cholesterol re-transport and antigen presentation is probably competing in the plaque it is not surprising to find differences in proteasome related pathways. The proteasome degradation pathway is a central component of the ubiquitin proteasome system, which is responsible for dealing with oxidized and misfolded proteins in the cell [178, 179] and regulates pathways of inflammation, cell proliferation, and apoptosis [179]. Decreased activity has also been linked to worse outcome in a study of carotid plaques [180]. Furthermore, proteasome activity is also important in immuno-presentation to T cells. Whether the transcriptional differences seen in proteasome degradation pathway is directly linked to CRP or not remains to be proven, but it has been suggested that hCRP acts as an extracellular chaperone [181] for modified or misfolded proteins. Furthermore, a recent study on primary dendritic cells show CRP inhibits differentiation of dendritic cells and proliferation of T-cells [182].
The transcriptional data also point to increased vesicular transport, linked to endocytosis. This seems to be in line with previous *in vitro* data were CRP binds to nuclear constituents and, importantly, modified LDL [106] and promote their uptake.

In conclusion, four major expression patterns of lesion development included 80% of established atherosclerosis genes, but these genes represented less than 9% of all the genes identified by this study. As judged from lesion and transcriptional phenotypes, the 30-week time point seems particularly interesting for interventions. Genetic lowering of plasma LDL cholesterol at 30 weeks prevented the rapid expansion of atherosclerotic lesions seen at 40 weeks. This effect involved a group of genes, most of which had little or no previously known relation to atherosclerosis. As indicated by the genes validated in our *in vitro* foam-cell model, these cholesterol-responsive genes merit attention as targets for future therapies to prevent the development of atherosclerotic lesions. We have also shown that *hCRP* is associated with slower development of atherosclerosis in an LDL-cholesterol mouse model of atherosclerosis, possibly related to proteasome levels. Based on our results, the fact that SAP [183] and CRP [22, 23, 184] act against autoimmune diseases in mouse models, that CRP promotes uptake of apoptotic cells [62, 99], nuclear material [62] and modified LDL [62, 98], only activates complement to the level of C3 [99, 109-111, 185] and inhibits DC differentiation [182] seems to support the notion of it not being pro-atherosclerotic but possibly anti-atherosclerotic. Recently, it has also been claimed that some or all of the pro-inflammatory reactions seen in *vitro* studies is due to bacterial (recombinant proteins) or azide (antibacterial agent) contaminations [186-191]. There have been some attempts to challenge these findings but more data is needed to decide on the matter. In the human setting, after adjustment of risk factors, geometric mean levels of CRP increased with the total number of sites showing progression of atherosclerosis [73]. These results might point to an association between lesion burden and CRP. However, it does not resolve the issue of whether CRP is pro- or anti-atherosclerotic.
Thus, hCRP might act as an extracellular chaperon [106], targeting ligands for proteasomal degradation and inhibiting antigen presentation and thereby slowing the progression of atherosclerosis in these mice. Weighing in all data it does not seem impossible that CRP may be anti- rather than pro- atherogenic and that it acts through immunoregulatory mechanisms.

7 FUTURE PERSPECTIVES

Recent studies have shown the huge complexity of the transcriptional machinery with at least a hundred factors and co-factors acting in concert and redundantly for transcription to take place. The importance of co-factors not binding to the DNA directly has also been shown. Since they are not represented by binding elements in the DNA, their co-operation in transcription cannot be investigated by purely by studying the DNA sequence. Also, the finding of duplications of large stretches of genomic sequences might be an additional factor perturbing genetic analysis and merit more investigation. Tiling array experiments [192] and the discovery of RNAi further adds complexity that needs to be taken into account when analyzing data. Hence, new approaches must be adopted to investigate the emerging complexity. The proposed usage of eQTLs combining information from transcription as well as genetic analysis is one way of accounting for some aspects of this complexity [138, 193].

For the future there is a need to test whether the -286 C > T > A polymorphism influences the risk of CHD in a prospective study of sufficient size to reliably detect differences in allele frequency between subjects who contract CHD and subjects who do not.

Furthermore, the mechanisms behind the estrogen effect must be determined in order to clearly show the relationship between CRP and estrogen. It is also important to remember that few studies have attempted to show which factors are responsible for the expression seen in various non-hepatic cells. The finding of proteasome related pathways associated to CRP expression in our mouse model and the results from studying primary dendritic cells [182] point to a need for investigating, more specifically, the regulation, expression and interactions in antigen presenting cells such as dendritic cells. Because of the recently reported contaminations of bacterial toxins and azide in many *in vitro* studies there is a great need to repeat these experiments. The report of a peptide blocking the function of CRP [194] also lend promise to further exploration of the results already gathered. In the *in vivo* setting it would be interesting to explore our mouse model concerning CRP's relation to scavenging, antigen presentation and the proteasome machinery.

Last but not least the contradictory results from mouse studies highlight the importance for human *in vivo* studies.

8 ACKNOWLEDGEMENTS

This research was conducted at the Atherosclerosis Research Unit, King Gustaf V Research Institute, Karolinska Hospital through the years 2002-2004. The research was funded by Anders Hamsten, head of the atherosclerosis research unit at the institution for medicine; Per Tornvall head of the Cardiology clinic at Karolinska Hospital; The Swedish heart and lung foundation and Pfizer. The atherosclerosis prone mice were kind gift from Steven Young's lab and the CRP transgenic mice a gift from Dr. Kyewski. Without these contributions paper III and IV wouldn't have been possible.

Anders Hamsten for granting me the opportunity to work and conduct interesting research at GV. For being one step ahead of us all and always being strategic. I have much to learn in this respect.

Per Tornvall for always taking his time to have interesting discussions about science and other things and for inspiration and a fantastic mentorship. For not giving me answers but questions that I can grow with, for being truly my main supervisor. For giving me space to grow.

Johan Björkegren for his challenging ideas and ways.

The people at the animal facility for inviting manners, help and fun chit-chats. You have made the looooong hours at the animal facility much shorter.

I want to thank everyone in the GV research team and the Procardis team for their support during these years. **Karin;** for help with SCARF material and chit-chat about Haninge, **Anita;** for initial help on ELISAs and chit-chats about Petra and Lina, **Karin D-T;** for being an invaluable resource when ordering, keeping track of whom, when, what, how long time and how much. **Barbro** for taking so good care of me during my first genotyping inc. days. **Camilla Berg**; What would the PhD education be without you. For all the help, advice, friendly chit-chat and for listening. And last but not least for all the good sandwiches for staying sharp during the meetings. **Ami;** for taking so good care and being so friendly. Don't worry he will survive the training. ☺

All the senior researches that have advised or helped me in other ways; **Ferdinand** for having a sharp mind and for always asking sharp questions that cut to core of things, that's the only way to learn. **Rachel** for the lovely contribution to our yearly Lucia program and kind questions and comments during the GV meetings, **Ewa** for her never tiring work with the biomed education and her pedagogical skills and company during late evenings at GV, **Jesper Tegnér** for being able to always find new angles on problems and asking the right questions. For being thought-inspiring. **Angela** for being knowledge full about ELISAs, **Per** for always having an open door, being humble despite his immense knowledge and when most needed offering advice regarding career, science and pointing to the importance of balance in life.

Dick for all the fun both on and of work. Think we should aim for "beer fest 2007". What do you think? ☺. Always having a thousand projects at a time but still being humble about it. For daring and always going your own way. **Pelle** and **Kalle;** for all the good beers and setting the standard during the Wallenberg course. **Pelle and Josefin**; Still think we should have had real sand. Rumours say there will be a beach party 2007! ☺ **Jousto;** I have no doubt you will one day write "The fantastic tales of Joustos interesting life, on and of work". ☺ Keep up the good work. **Vincent;** I'm looking forward to the french lessons. **Massimiliano**; Hope you will come to a good decision regarding what to do next. **Sergey**; You have given me insight in russian sayings. There must be at least a hundred for each situation. For educating me on areas such

as history and literature. **Sigge;** Hope everything goes according to plan in Japan. A big step even for a bold man. **Maria Nastase-Mannila**; My (epi-) genetics Wiz. So humble but knowledge full. With a sparkle in the eye. Just as it should be. You will make a fantastic researcher and cardiologist. **Petra;** for being lively and making a positive contribution to the GV PhD community. A terrific sense of humour. Up for another GO-Cart race? **Anna;** A winner at heart. I dare you in the next GV poker tournament. **Stina;** The fighter among fighters. Keep it up! I'm still waiting to see your renovated apartment. **Karin;** I really enjoy the verbal sparring. ☺ Humour and positive attitude have brought you a long way. Some strategic planning will make good perfect. You're on the right track, don't give up!!!! ☺ **Roland;** If I was half the knowledge full in biology that you are in mathematics I would be double the knowledge full that I am today. How knowledge full am I? ☺ Good luck in the US. **Shori;** A truly remarkable life story. I have plenty to learn from your immense pool of wisdom. **Peri;** You are the methodological rock in the group which we all lean on. Thank you for all the chats about family, science and KI business. **Sara, Jesper, Olivia and José** and the rest of the computational medicine group. The way have been bumpy as always when breaking new ground but I think that something really new is created in the group. I wish you all good luck, what ever you decide to do. **Marie Bennermo;** Genotyping inc. ☺ Someone with GO and energy. I'm confident we will stay in-touch. **Mattias and Martin;** Perfect examples of clinicians making good science, from PCR to Proteins. Always on the run. **Ann;** Giving a clinical touch to the journal clubs. I envy your sailing adventures. **Zhongpei;** For bringing home interesting chines candy through the years. **Mattias;** from tutor to colleagues. Who could have guessed. Need to try sparring some day.
See you in the karaoke bar. **Cecilia;** The constantly happy face and with witty humour. Good luck with your ideas. I'll invest and buy for sure. ☺ **Maria Kolak;** For your genuine ways and inviting manners. It is easy to feel at home in your company. **Magnus Mossfeldt;** Your house must be a castle by now. For all interesting discussions about all style, sparring and gossip in the Stockholm martial art community. **Katja;** You must be the only one at GV having a more advanced cell phone than I have. I new I should have aimed higher. ☺ **Lise-lotte;** The best student one can ask for. You're always welcome to come back. I'm confident that you will succeed with what ever you chose to do. Believe in yourself !!! ☺
Mike; Somebody that is truly self-made. Living under the banner "you are what you accomplish". I am confident you will find your way, whether it be Harvard, Oxford or somewhere else. Always having time for a friend. Not being politically correct and getting away with it. ☺
Peter o Oskar; The strategic masterminds of KI. KI is only the first step….. ☺.
For educating me about business in theory and practise. Thanks for all the graduate pubs and some insight into diving.
David; For always being on top of things and having witty manners. For being a good friend and sharing without even thinking about it. For inviting me into the Dr-community and dragging me along on ice adventures and fun in Båstad. For taking good care of me when most needed.
Charlotta Hjerpe; Interesting lunches about science and other things. For being great to bounce ideas against and taking your time despite being Mrs multitasking. By the way, I won!!! I'm having my dissertation first….. not that it was ever a competition….. but still! ☺
Nina; The one among us that have her head a little higher above the potato land. I have a few things to learn from you regarding organisation. Always a thousand projects at a time. A sophisticated and intelligent sense of humour that forces one to stay sharp.
Tomas;. You have a truly amazing job. It is refreshing to hear about the multitude of problems you have to solve and interesting persons that you get to meet. Hope your stay in Africa will be fun. I believe there will be some good concerts coming up at the end of the summer. Keep in-touch!

Sarah; Always on your way to a new destination in the world, exploring. Open minded and as close to being without prejudice as one can be. I miss the verbal sparring, enlightening me about feminism, politics and different cultures. ☺ For expanding my horizons.
Daniel, Olle and Katja; Thank you for adopting me into your gang. Seems like we are all going separate ways in the world. Keep in touch!
My old study buddies; Andreas and Jessica, Agne and Lotta, Stefan and Jenny, Johanna and David, Katarina and Ola; For having patience with my south european notion about time. I still blame my heritage (Lets not be to scientific about this). ☺ For all the past and coming fun and dinners, telling tales about interesting travelling destinations. I know we will always stay in-touch. I believe it's our turn to host the next wine tasting.
Nacka Kickers; Maja, Magnus, Annika, Carina, Mats, Fredrik, Karin, Martin, Dava and all the others. A great gang with bachelor parties, nights out, Gotland adventures, weddings and dinners. Thank you for still calling despite my workaholic manners sometimes. ☺
Tobias; For all the fun and lifting me up when I fell. I wouldn't have made it without you. For daring to make a "handbrake" manoeuvre in front of the Captain and Lieutenant. I hope you feel at home in Belgium and France. After the dissertation I promise I'll make some time to come visit.
Lage; for helping me understand the workings of the human mind, educating me about career and work related issues and for all the fun together with "grabbarna grus".
Gustavo and Ana; A true and warm friendship. Bull chasing, Spanish dinners with schamon and gombas and speaking across language barriers. I hope your stay in US will be up to expectations.
Stina; A buddy in all situations. You have a killer instinct as few, I hope it will rub of on me. Resistance is futile when your sitting on the spin-bike, I haven't given up the hope of beating you yet though. For interesting discussions about human actions, in-actions and inter-actions.
Peter; My french-speaking friend. ☺ I'm looking forward to more of "Alex and Peters adventures". Were are we heading next? Always keeping track of good concerts and spoken word performances. For always choosing your own way in life.
Linn; Finally somebody that is worse than I'm about *keeping in-touch*, although were just a few buildings apart. ☺ I believe it's harder to see you for a lunch than the king. I'm still gathering courage to go to your spinning sessions, but I believe I'm running out of excuses soon. Keep up the good management of the KI death network.
Johan Dahl; What ever I do, I know you can do it better. Always nearby and never budging for a beer. For daring to have a politically incorrect sense of humour and a good portion of realism. I can sense you're softening with age though. ☺

My family; To my amazing family. Always there, supporting and coming with good advise. Always understanding and adapting to my crazy working schedules and trying to be as interested and excited about my research as I am. Also for taking my mind of research when needed with interesting and passionate political, cultural and ethical discussions.

Malin my dear;
My princess. I'm truly lucky to have found you, or was it the other way around. I think we will never settle on that issue. ☺ I believe we have a shared interest in everything big and small. For sharing the habit of long and strange working hours. I admire you for putting up with thousands of half "nutty" ideas from me and actually encouraging me to follow them through. To sometimes see potential were I do not. Whether it be in a castle or a caravan I know we will be happy and supportive of each other. Together we will travel the globe.

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