CARITA EKLUND

Gene Polymorphisms Affecting the Production of C-reactive Protein

Clinical Implications

■

ACADEMIC DISSERTATION
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To the Eklund family

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List of original communications

This dissertation is based upon the following original communications, referred to in the text by their roman numerals (I-V)

I Eklund C, Jahan F, Pessi T, Lehtimäki T, Hurme M (2003): Interleukin 1B is associated with baseline C-reactive protein levels in healthy individuals. Eur Cytokine Netw 14:168-171.

II Eklund C, Lehtimäki T, Hurme M (2005): Epistatic effect of C-reactive protein (CRP) single nucleotide polymorphism (SNP) +1059 and interleukin-1B +3954 on CRP concentration in healthy male blood donors. Int J Immunogen 32: 229-232.

III Eklund C, Nenonen A, Kukkonen-Harjula K, Borg P, Fogelholm M, Laine S, Huhtala H, Lehtimäki T, Hurme M (2006): Association of the IL6-174(G/C) polymorphism with C-reactive protein concentration after weight loss in obese men. Eur Cytokine Netw 17: 131-135.

IV Eklund C, Huttunen R, Syrjänen J, Laine J, Vuento R, Hurme M (2006): Polymorphism of the C-reactive protein gene is associated with mortality in bacteraemia. Scand J Inf Dis 38:1069-1073.

V Eklund C, Kivimäki M, Islam M.S, Juonala M, Kähönen M, Marniemi J, Lehtimäki T, Viikari J, Raitakari O.T, Hurme M (2007): C-reactive protein genetics is associated with carotid artery compliance in men in the Cardiovascular Risk in Young Finns Study. Atherosclerosis (in press).

In addition, this dissertation contains unpublished data.

Abbreviations

ALL	acute lymphoblastic leukaemia
APR	acute phase reaction/reactants
BMI	body mass index
CAC	carotid artery compliance
CCR2	CC-chemokine receptor 2
CHD	coronary heart disease
CRP	C-reactive protein
CVD	cardiovascular disease
C1q	complement factor 1q
D	diameter
eNOS	endothelial nitric oxide synthase
ER	endoplastic reticulum
Fc	constant/crystal fragment
FcγR	constant/crystal fragment γ receptors
Glu	glutamate
HAEC	human aortic endothelial cell
HCAEC	human coronary artery endothelial cell
HDL	high-density lipoprotein
HR	hazard ratio
HUVEC	human umbilical vein endothelial cell
Ig	immunoglobulin
IL	interleukin, e.g. interleukin-1
IMT	intima-media thickness
INF-γ	interferon-γ
IQR	interquartile range
IS	ischemic stroke
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MAC	membrane attack complex
MBL	mannose-binding lectin
MCP-1	monocyte chemoattractant protein-1
MGB	minor groove binding
MI	myocardial infarction
mCRP	monomeric/modified CRP
mRNA	messenger RNA
nCRP	native CRP
OR	odds ratio
OS	osteogenic sarcoma

PAGE	polyacrylamid gel electrophoresis
PBMC	peripheral blood mononuclear cells
PCh	phosphocholine
PCR	polymerase chain reaction
Phe	phenylalanine
PMN	polymorphonuclear
RR	risk ratio
SAP	serum amyloid P
SLE	systemic lupus erythrematosus
SNP	single nucleotide polymorphism
Spp.	species
TB	tuberculosis
TF	tissue factor
TNF	tumor necrosis factor
USF1	upstream stimulating factor 1
UTR	untranslated region
UV	ultra violet
VCAM-1	vascular cell adhesion molecule-1
VL	visceral leishmaniasis
VLDL	very low-density lipoprotein
VNTR	variable number of tandem repeats
WR	weight reduction phase

Abbreviations are defined at first mention in the abstract and review of the literature and used only for concepts that occur more than twice.

Abstract

Elevated C-reactive protein (CRP), a marker of inflammation, is a newly recognized predictor of cardiovascular events. Family and twin studies have shown its concentration to be 35-50 % heritable, and therefore genetic variations in genes affecting CRP concentration are possible risk predictors for cardiovascular events. The main genes presumably involved in CRP concentration determination are *CRP* and interleukin (*IL*) genes.

This study sets out to explore the associations between pro- and anti inflammatory candidate gene genotypes (*IL1A+4845, IL1B-511, IL1B+3954, IL1RA VNTR, IL6-174* and *CRP)* and CRP concentration, bacteraemia mortality and vascular changes in atherosclerosis. CRP concentrations and genetic variations were determined in four different Finnish study populations comprising a total of 2840 individuals; healthy blood donors, obese weight reducing men, bacteraemia patients and healthy participants of the Cardiovascular Risk in Young Finns Study. Associations between genetic variants and CRP concentration, bacteraemia mortality and early vascular changes were analysed.

Several genetic variants were associated with CRP concentration. Alleles of *IL1B*+3954C>T (Studies I and II), *CRP*+1059G>C (Studies II, III, IV and V), *CRP*+1846G>A (Study V), *CRP*-286C>T>A (Study V) and *CRP*+1444C>T (Study V) were associated with differences in CRP concentration. In weight reducing men, *IL6*-174G>C was associated with CRP concentration after weight reduction, but no difference was seen before weight loss.

We detected an association between bacteraemia mortality and *CRP*-717A>G polymorphism (Study IV). Patients with GG genotype died 9.6 times more often of bacteraemia caused by *Streptococcus pneumoniae* than other genotypes (95% CI 1.3 – 72.5). An association between *CRP*-286C>T>A non allele C-carriers (TT and TA genotypes) and decreased increased carotid artery compliance (CAC) was detected in men participating in the Cardiovascular Risk in Young Finns Study.

As a conclusion, many variants in *CRP* gene are associated with CRP concentration. In addition, *CRP* variants may have an influence on mortality from bacteraemia caused by *S. pneumoniae* and on early vascular changes in atherosclerosis.

Tiivistelmä

C-reaktiivinen proteiini (CRP) on tunnettu tulehdusta kuvaava markkeri. Jo lievästi kohonneen veren CRP-pitoisuuden tiedetään ennustavan tulevia aivo- ja sydäntapahtumia. Perhe- ja kaksostutkimuksin on osoitettu CRP-pitoisuuden olevan noin 35-50 % periytyvä. Näin ollen niiden geenien variaatiot, joilla on osuus CRP-pitoisuuden määrittymisessä, voivat myös ennustaa tulevia sydäntapahtumia.

Tämän työn tarkoituksena on tutkia assosiaatiota sekä CRP geenin omien variaatiokohtien että muiden CRP-pitoisuuteen vaikuttavien geenien *(CRP, IL1A, IL1B, IL1RA* ja *IL6)* variaatiokohtien ja CRP-pitoisuuden välillä. Lisäksi tarkoituksena on selvittää assosiaatiot näiden geenivariaatiokohtien ja sepsiskuolleisuuden sekä varhaisten suonimuutosten välillä. CRP-pitoisuus ja geneettiset variaatiokohdat määritettiin yhteensä 2840 henkilöltä; terveiltä verenluovuttajilta, ylipainoisilta laihduttavilta miehiltä, sepsispotilailta sekä Lasten ja nuorten aikuisten sepelvaltimotaudin riskitekijät (LASERI) -tutkimukseen osallistuvilta nuorilta aikuisilta. Assosiaatiot geneettisten variaatiokohtien ja CRP-pitoisuuden, sepsiskuolleisuuden, ja varhaisten suonimuutosten välillä analysoitiin näiltä henkilöiltä.

Useat geneettiset variaatiokohdat olivat yhteydessä CRP-pitoisuuteen. Variaatiokohtien *IL1B*+3954C>T (osatyöt I ja II), *CRP*+1059G>C (osatyöt II, III, IV ja V), *CRP*+1846G>A (osatyö V), *CRP*-286C>T>A (osatyö V) ja *CRP*+1444C>T (osatyö V) genotyyppien välillä oli eroa CRP-pitoisuuksissa. *IL6*-174G>C variaatiokohta yhdistyi CRP-pitoisuuteen ylipainoisilla miehillä laihduttamisen jälkeen, mutta ennen laihdutusta eroa genotyyppien välillä ei ollut havaittavissa.

Sepsiskuolleisuuden ja *CRP*-717A>G GG genotyypin väliltä löytyi yhteys (osatyö V). Genotyypin GG omaavilla potilailla oli 9,6-kertainen riski kuolla *S. pneumoniaen* aiheuttamaan sepsikseen verrattuna muihin genotyyppeihin. *CRP*-286C>T>A TA/TT genotyyppien ja vähentyneen kaulavaltimon joustavuuden välillä havaittiin yhteys henkilöillä, jotka osallistuivat LASERI – tutkimukseen. Väitöskirjatutkimuksen löydösten mukaan useat *CRP* geenin variaatiokohdat ovat yhteydessä CRP-pitoisuuteen. Lisäksi *CRP* variaatiot voivat lisätä pneumokokin aiheuttamaa sepsiskuolleisuutta ja voivat vaikuttaa varhaisiin valtimokovettumataudin suonimuuttujiin.

Introduction

CRP is a long conserved acute phase molecule, which accurately reflects the level of inflammation. CRP concentration above 10 mg/l has traditionally been considered clinically important. However, more recent studies have shown the value of minor concentrations in the prediction of coronary events (Thompson et al. 1995, Ridker et al. 1997, Ridker et al. 1998, Danesh et al. 2004, Sabatine et al. 2007). Slightly elevated CRP concentration, but higher than those in most normal subjects, is now called low-grade inflammation. Subjects with concentration ≤ 1 mg/l are considered to be in the low risk group, those with concentrations between 1 to 3 mg/l in the moderate risk group and those with more than 3 mg/l in the high risk group. Thus, the subclinical state of atherosclerosis can be identified by an increase in circulating markers of inflammation before acute events occur, e.g. CRP (Ross 1999, Hansson 2005). Indeed, in 2003 the Centers for Disease Control and Prevention and the American Heart Association published the first set of guidelines to endorse the use of CRP as an adjunct to traditional risk factor screening in cardiovascular risk prediction/assessment (Pearson et al. 2003).

As a molecule, CRP consists of five non-covalently associated identical protomers arranged symmetrically in a cyclic configuration. Each protomer has a recognition face for ligand binding and an effector face for complement and FcγR binding. The known main functions of CRP are complement activation, enhancement of phagocytosis and induction of cytokine synthesis. The precise role of CRP in cardiovascular diseases, however, is still unknown. It could be merely an innocent bystander, a marker of arterial inflammation, or it could contribute to the atherosclerotic process by binding to lipoproteins in atherosclerotic plaques, activating complement and thus promoting inflammation and disease progression.

CRP is produced by the liver in response to microbial sensing and circulating inflammatory cytokines. The basal concentration of CRP has been estimated to be 35-52% heritable according to family and twin studies (Retterstol et al. 2003, MacGregor et al. 2004). Therefore, in the present study we investigated the association of *IL1A, IL1B, IL1RA, IL6* and *CRP* genotypes, the genotypes of candidate genes presumably involved in CRP level determination, with CRP concentration in different study populations. The dissertation is based on the results from four different study series, all representing different levels of inflammation. In the first series, the association between *IL1B* and *CRP* gene variants and low grade inflammation was studied in healthy middle-aged blood donors. In the second series, the association between CRP concentration and *IL1B, IL6* and *CRP* genotypes was studied in obese men before and after weight

reduction. The third series consisted of bacteraemia patients, where the association between outcome of bacteraemia and *CRP* genotypes was studied, as well as the association between the genotypes and acute and recovery phase CRP concentration. In the last series, the association of five *CRP* gene variations and the haplotypes they formed with CRP concentration was studied in a population-based investigation of young Finns as a part of the ongoing Cardiovascular Risk in Young Finns Study. Furthermore, the association between the genotypes/haplotypes and early vascular changes, i.e. carotid artery compliance was investigated.

Review of the literature

1. C-reactive protein

1.1. Discovery and phylogeny

C-reactive protein (CRP) was first discovered in Oswald Avery's laboratory at the Rockefeller Institute for Medical Research by Tillett and Francis in 1930 (Tillett and Francis 1930). The blood of patients with acute pneumococcal pneumonia was found to contain a substance that reacted with the cell wall C-polysaccharide of *Streptococcus pneumoniae*. The substance appeared in a very early phase of the infection and was present at high concentrations in the blood. After infection (if the patient survived) the substance became undetectable as the disease subsided. The substance was later named C-reactive protein (CRP). In the 1940s, the interaction of CRP with C-polysaccharide was found by Abernethy and Avery (1941) to be dependent on the presence of calcium in the media. Forty years after the original discovery of CRP, Volanakis and Kaplan identified the specified ligand for CRP in C-polysaccharide to be phosphocholine (PCh), a part of the techoic acid of the pneumococcal cell wall (1971).

CRP is an ancient molecule although discovered in humans only about 75 years ago. It belongs to a protein family called pentraxins (from the Greek words 'penta', five and 'ragos', berries) that constitutes a phylogenetically ancient family of proteins exhibiting a remarkable conservation of structure and binding reactivities. The pentraxins are, in turn, part of the lectin fold superfamily. The members of the pentraxin family include the short pentraxins, i.e. CRP and serum amyloid P (SAP), a constituent of all amyloid deposits, and the later discovered long pentraxins, e.g. pentraxin-3 and neuronal pentraxin (Garlanda et al. 2005). All short pentraxins consist of single polypeptide chain subunits arranged in pentagonal, hexagonal or decagonal cyclic symmetry. Members of the family have been found in the blood of the dogfish, *Mustelus canis* (Robey et al. 1983), the blood of certain marine teleosts (Baldo and Fletcher 1973) and of all vertebrates examined (Pepys et al. 1978). The horseshoe crab, *Limulus polyphemus*, has the most ancient CRP homologous protein and this species was present 70 million years before the dinosaurs appeared on earth (Robey and Liu 1981, Kilpatrick and Volanakis 1991). Mammalian CRP and SAP are thought to be products of an ancestral gene duplication event (Rubio et al. 1993). This hypothesis is supported by the facts that the pentraxins are structurally similar, with a disc-like arrangement of five non-covalently bound subunits, and their genes are within 250 kb from each other in human (Kingsmore et al. 1989) and

also remain tightly linked in mouse (Yunis and Whitehead 1990). Human CRP and SAP also share 51% amino acid and 59% nucleotide sequence identity (Woo et al. 1985). In humans, CRP is a major acute phase reactant, but by contrast, SAP is expressed constitutively at relatively constant serum levels. Contrary to expectations, the situation is reversed in mice (Pepys et al. 1979).

Besides belonging to the pentraxin family, CRP can also be classified as an acute phase protein according to its biological properties. The role of CRP as an acute phase protein is discussed below.

1.2. Structure

The structure of CRP has been revealed by X-ray crystallography (Shrive et al. 1996). It consists of five noncovalently associated identical protomers (Mr 115 135) arranged symmetrically in a cyclic, planar disc-like configuration around a central pore. The total molecular weight is 23 000 Da. Each protomer contains 206 amino acid residues and has a characteristic 'lectin fold' composed of two antiparallel β sheets with flattened jellyroll topology. A single disulphide bond links the two half-cystines at positions 36 and 97 (Oliveira et al. 1979) Each protomer has a 'recognition face' for ligand binding consisting of PCh and calcium-ion binding sites and an 'effector face' carrying a single α helix to which complement C1q binds and Fcγ receptors are presumed to bind. Because of the pentameric arrangement of its binding sites, a high repeat number of any of the varied ligands in a large array, as found on the surface of a pathogen, can bind CRP with high avidity. The co-crystal structure of CRP suggests that Phe^{66} and Glu^{81} are the two key residues mediating the binding of PCh to CRP. The choline moiety reacts with the negatively charged glutamic acid, whereas the phosphate moiety reacts with calcium ions bound to CRP (Thompson et al. 1999). The importance of these amino acids has been confirmed by mutagenesis studies (Agrawal et al. 2002, Black et al. 2003). Amino acids 175-185 mediate the binding of CRP to Fcγ receptors (Marnell et al. 1995, Bang et al. 2005). The noncovalent interprotomer interactions include three salt-bridges and involve mainly the 115-120 loop of one protomer and the 40-42 and 197-202 regions of the adjoining protomer. The protomers are not on the same plane but rotated by 15-20° about an axis almost parallel to the α-helix, thus allowing multivalent binding of the pentamer to cell surfaces with different distributions of PCh residues (Thompson et al. 1999). Due to the rotation, the α-helixes lie closer to the pentameric 5-fold axis, while the bound Ca^{2+} are carried away from it.

1.3. Production

1.3.1. Production of CRP by the liver in response to cytokines

The studies by Hurlimann and colleagues provided the first evidence that CRP was produced by the liver (1966). The liver produces CRP in response to microbial sensing and circulating inflammatory cytokines. The two main cytokine inducers are interleukin-6 (IL-6) and interleukin-1β (IL-1β). Most nucleated cells have been shown to produce IL-6 *in vitro*, but the most prominent source of IL-6 seems to be stimulated monocytes/macrophage lineage cells, fibroblasts and endothelial cells (Kato et al. 1990). IL-1β is also produced by monocytes/macrophages, endothelial cells and fibroblasts, likewise by mast cells (Tocci and Schmidt 1997). After synthesis these molecules enter the bloodstream and circulate via it, e.g. to the liver.

The IL-1β molecule is part of the IL1 complex family of proteins, which are all involved in the inflammatory response. The members of this family include e.g. IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). Functionally they can be divided into pro-inflammatory (IL-1α, IL-1β) and anti-inflammatory (IL-1Ra) molecules. The pro-inflammatory molecules are involved in the enhancement of inflammation and host defence, whereas the anti-inflammatory IL-1Ra counteracts the function of IL-1α and IL-1β, as they share a common receptor (Dinarello 1996).

The IL-6 molecule is a multifunctional cytokine involved in the immune response and inflammation. It was initially known as “hepatocytes stimulating factor” reflecting its relevant role in the regulation of the acute phase response.

1.3.1. Control of expression

The expression of CRP in cultured human hepatoma cells seems to be under different control in cell lines of different origin (Ganapathi et al. 1988). Data from *in vitro* studies using Hep3B cells show that induction of CRP is principally regulated at the transcriptional level by the cytokine IL-6, an effect which can be enhanced by IL-1β (Ganter et al. 1989). By contrast, studies using NPLC/PRF/5 cells show that IL-6 alone is capable of inducing full CRP production (Ganapathi et al. 1988). In addition, human CRP transgenic mice seem to have both IL-6 dependent and independent induction of CRP expression (Weinhold and Ruther 1997).

1.3.2. Intracellular synthesis of CRP

Under normal physiological conditions CRP is synthesized as a monomer at relatively low rates and the pentamer assembly occurs in the endoplasmic

reticulum (ER) (Macintyre et al. 1985). After synthesis CRP is largely retained in ER without being degraded, bound by two carboxylesterases, namely glycoprotein 60a (gp60a) and glycoprotein 60b (gp60b) (Macintyre et al. 1994). gp60a is an abundant ER protein with a relatively low affinity for CRP, whereas gp60b is a less abundant protein, but has a higher affinity for CRP (Macintyre et al. 1994). They are both 60 kDa in size. These esterases are restricted to the lumen of ER by virtue of their carboxyl-terminal sequences, which are effective ER retention signals (Korza and Ozols 1988, Ozols 1989). In a rabbit model the half-time for exit for pulse-labelled CRP from the normal rabbit's hepatocytes was more than 18 hours, whereas a half-time of only 75 min was observed in cells isolated from animals stimulated *in vivo* to undergo the acute phase response (Macintyre et al. 1985, Macintyre 1992). The observed reduction in retention time seems to be due to diminished affinity of gp60b for CRP and leads to reduced transit time from ER to secretion (Macintyre 1992). As a result of this enhanced secretion and synthesis rapidly increased levels of CRP are seen during the acute phase response. The half-life of the CRP molecule is about 19 hours, and there is no evidence showing accelerated CRP clearance or catabolism in any disease studied (Vigushin et al. 1993).

In general, CRP concentration correlates with inflammation and other markers of the acute phase response like the erythrocyte sedimentation rate. However, the ability of CRP to rise and fall more rapidly and dramatically than other acute phase molecules makes it an especially suitable marker for clinical disease course monitoring and treatment response follow-up. Because the plasma half-life of CRP is the same under all conditions (Vigushin et al. 1993), and the sole determinant of plasma concentration is therefore the synthesis rate of CRP, CRP accurately reflects the on-going inflammation.

1.3.3. Extra hepatic production of CRP

In addition to hepatic cells, extra hepatic production of CRP has been reported. CRP can also be produced by monocytes and T-cells (both $CD4^+$ and $CD8^+$ T-cells) (Haider et al. 2006), natural killer cells (NK cells) (Kuta and Baum 1986), alveolar macrophages (Dong and Wright 1996), coronary artery smooth muscle cells (Calabro et al. 2003), aortic endothelial cells (Venugopal et al. 2005), the epithelial cells of the upper respiratory tract (Gould and Weiser 2001) and thymic medullary epithelial cells for the purpose of inducing self-tolerance (Klein et al. 1998). Besides these cells, production has been observed in coronary plaque (Ishikawa et al. 2004), atherosclerotic lesions (by smooth muscle cells and macrophages) (Yasojima et al. 2001), atrial tissue (Wilson et al. 2007), vascular tissue (Wilson et al. 2007), adipose tissue (Ouchi et al. 2003, Calabro et al. 2005), the kidney (Jabs et al. 2003, Jabs et al. 2005) and in neurons (Yasojima et al. 2000). However, the contribution of extra hepatic CRP production to circulating CRP concentration remains to bedefined. Similarly, the effect of local

production of CRP on local CRP concentrations in different tissues is not yet well understood, although there is some evidence that vascular and atrial tissue CRP production could induce differences in the CRP concentration of the coronary circulation (Wilson et al. 2007).

2. Functions of CRP in innate immunity

2.1. CRP as an acute phase reactant

The acute phase of the inflammatory response refers to the wide ranging physiological changes that are initiated immediately after an individual is threatened by stress, mental or physical; e.g. infection, trauma, surgical procedure, burns, tissue infarction, strenuous exercise or childbirth. Mammalian acute phase response involves the central nervous system, the hypothalamus and hypophysis, which via the so-called neuro-endocrine axis activates all the organs in the body. High temperature, changes in vascular permeability and changes in the biosynthetic, metabolic and catabolic profiles of many organs are all included in the symptoms of the reaction – all aimed to provide optimal protection against the progress of disease (Kushner 1982, Bengmark 2004).

During the acute phase reaction, many liver-derived proteins arise in concentration. Liver produces the so-called acute phase reactants (APRs); e.g. C-reactive protein and serum amyloid A protein, which are produced to provide protection against invading micro-organisms, limit tissue damage and promote a rapid return to homeostasis. Other cell types synthesize these proteins as well, e.g. monocytes, endothelial cells, fibroblasts and adipocytes. An APR has been defined as one whose plasma concentration increases (positive acute phase proteins) or decreases (negative acute phase proteins) by at least 25 percent during inflammatory disorders (Morley and Kushner 1982). The magnitude of the increases varies from about 50 % in the case of complement components to as much as 1000-fold increase in the case of CRP and serum amyloid A.

Under physiological circumstances, human CRP is a protein with a median serum concentration of ~ 0.8 mg/l (Shine et al. 1981). Following the onset of the inflammatory stimulus, CRP levels begin to rise within a few hours and peak within 48 hours. Depending on the type and chronicity of the stimulus or treatment, levels may fall rapidly or remain elevated. Moderate changes in concentration are seen after strenuous exercise and childbirth (Cicarelli et al. 2005) and small changes occur after psychological stress (Hapuarachchi et al. 2003) and in several psychiatric illnesses (Maes et al. 1997). For example, in myocardial infarction (MI) CRP concentration may rise from less than 2 mg/l to over 100 mg/l in 48 hours. The greatest changes in CRP concentration are seen in bacteraemia, where the concentration may rise over 500 mg/l (Morley and Kushner 1982). Viral infections usually cause elevation of CRP in the 10-40

mg/l range, and therefore CRP is widely used in the differential diagnosis of acute bacterial and viral diseases.

Several cytokines and other extracellular signalling molecules specially regulate the transcription of human APRs. These include IL-1, IL-6, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN- γ). The cytokines are the chief stimulators of the production of APRs, especially IL-6, which is capable of inducing most of the APRs (Gabay and Kushner 1999). Cytokines operate as a cascade and also as a network in stimulating the APRs. The most important sources of these cytokines are macrophages and monocytes at inflammatory sites.

2.2. CRP as a pattern recognition molecule

Innate immunity is a first line of resistance against pathogens, and has a key role in the activation and orientation of adaptive immunity and in the maintenance of tissue integrity and repair. CRP is part of the innate immunity system, where it plays an important role as a pattern recognition molecule (Janeway and Medzhitov 2002). Pattern recognition molecules discriminate self (host molecules) from non-self molecules (i.e. antigens) by recognizing pathogen associated molecular patterns. The patterns may, for example, be repeating structures on pathogen surfaces. As CRP is present in the plasma, it is part of the humoral arm of pattern recognition molecules, as opposed to the cellular arm of pattern recognition molecules, e.g. Toll-like receptors and scavenger receptors present on monocyte/macrophage cell surfaces (Janeway and Medzhitov 2002).

2.2.1. Phosphocholine as a ligand

In the case of CRP the recognised molecule is PCh (Volanakis and Kaplan 1971). PCh is a component of many prokaryotes and is almost universally present in eukaryotes. For example, it is present in the external components of a variety of pathogenic protozoa, fungi, nematodes and other intestinal parasites, as well as in plant products (Harnett and Harnett 1999). It has been found in a number of bacterial species, both in gram positive (*Streptococcus pneumoniae, Clostridium* spp., *Lactococcus* spp. and *Bacillus* spp.) (Gillespie et al. 1996) and gram negative bacteria (*Haemofilus influenzae, Neisseria meningitides, N. gonorrhoeae)* (Kolberg et al. 1997). CRP interaction with the PCh of the microorganism has an opsonic effect, which e.g. in the case of *Streptococcus pneumoniae* is protective against infections (Szalai et al. 1995). PCh binding to CRP requires presence of calcium ions. The PCh on pneumococci is assumed to be hidden underneath the capsule, meaning that CRP may bind more avidly to damaged pneumococci than to viable bacteria (de Beaufort et al. 1997).

In higher animals PCh is ubiquitous in the phospholipids of cellular membranes, and importantly also in the circulating plasma lipoproteins that are intimately involved in the pathogenesis of atherosclerosis, in particular low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (de Beer et al. 1982). In eukaryotic membranes it is a constituent of sphingomyelin and phosphatidylcholine, but in a form that cannot bind to CRP; the head groups of these phospholipids are normally inaccessible to CRP. However, CRP can bind these molecules on eukaryotic membranes in damaged and apoptotic cells (Volanakis and Wirtz 1979, Narkates and Volanakis 1982, Hack et al. 1997, Gershov et al. 2000, Chang et al. 2002). Similarly complement activation resulting in membrane damage can expose sites for CRP binding (Li et al. 1994), as well as damage due to phospholipases (Volanakis and Narkates 1981). Although micro-organisms express PCh and are likely to be important targets of CRP, the interaction of CRP with PCh in damaged membranes may even be biologically more important as clearance of host apoptotic and necrotic cells occurs via this route (Mold et al. 2002a).

As mentioned, PCh binding requires the presence of calcium ions. A highly conserved region in all pentraxins has been proposed to bind calcium (Kinoshita et al. 1989, Kinoshita et al. 1992).

2.2.2 Other ligands

In addition to PCh, CRP can bind nuclear material, like chromatin (Robey et al. 1984). CRP seems to be able to bind chromatin and chromatin fragments in a Ca^{2+} dependent manner, so that there is one CRP binding site for every 160 base pairs of DNA in chromatin (Robey et al. 1984). Later CRP-chromatin complexes were shown to activate complement, which led to the suggestion that CRP could mediate the removal of exposed nuclear DNA by complement dependent solubilization and subsequent removal of chromatin by phagocytes (Robey et al. 1985, Shephard et al. 1986). CRP is also able to bind other nuclear molecules, like histones and small nuclear ribonucleoprotein particles (Du Clos et al. 1988, Du Clos 1989). It is assumed that CRP reacts with these components and aids in their removal via interaction with the phagocytic cells and the complement system (Gershov et al. 2000). Transport of CRP into nucleus was reported to be mediated through a nuclear localization signal present in the primary structure of CRP (Du Clos et al. 1990). The ability of CRP to bind nuclear antigens as well as its ability to increase the clearance of host apoptotic and necrotic cells has led to the theory that CRP could prevent autoimmunity (Szalai 2004), as defects in the clearance of these cells have been suggested to be a source of autoantigens (Nauta et al. 2003). One of the suggested main functions of CRP, in fact, is waste management and the prevention of autoimmunity.

CRP is also able to bind fibronectin, an extracellular matrix protein (Salonen et al. 1984). Each CRP molecule is able to bind nine fibronectin molecules, when either CRP or fibronectin is in solid phase (Salonen et al. 1984, Tseng and Mortensen 1988). The interaction between these molecules may involve amino

acid Glu^{42} of CRP and the C-terminal region of fibronectin (Agrawal et al. 1992). Other CRP ligands include the basement membrane protein laminin, which binds CRP in the presence of Ca^{2+} (Swanson et al. 1989), and *Staphylococcus aureus* cell surface protein A, which probably binds to a different site in CRP than PCh (Das and Mandal 2004a).

2.3. Different forms

Modified CRP. In addition to the native pentameric structure, the protein can also exist in the form of subunits. It was originally noted in 1965 by Gotschlic and Edelman that if CRP was stored at low ionic strength in basic buffers, the multimeric form dissociated irreversibly into subunits (1965). Subsequently it was shown that it can dissociate into free subunits through chemical manipulations *in vitro*, e.g. by urea chelation, acid treatment, heating or direct immobilization onto polystyrene plates (Potempa et al. 1987). The free subunits have characteristics that are distinct from the parent native CRP (nCRP) molecule and are referred as modified CRP (mCRP). These two forms of CRP can be distinguished antigenically, electrophoretically, and by ligand binding reactivity (Potempa et al. 1983). Upon dissociation of the pentameric quarternary structure the CRP subunits undergo a spontaneous and presumably unidirectional conformational change to mCRP (Kresl et al. 1998), which has reduced aqueous solubility and a tendency to aggregate into a matrix-like lattice structure. Monoclonal antibodies have been described that recognise specific epitopes only expressed on the mCRP (Potempa et al. 1987); however, these are not commercially available. Immunohistochemical localization of mCRP has suggested that mCRP is a naturally occurring form of CRP and that it is a tissue-based rather than serum-based molecule (Diehl et al. 2000), but the existence of mCRP *in vivo* is still uncertain. A third form of CRP, called mCRPm, was recently described (Ji et al. 2006c). This variant seems to form when nCRP binds to membranes, including liposomes and cell membranes, and undergoes a rapid but partial structural change, and produces molecules that express CRP subunit antigenicity but retain native pentameric conformation. This form can detach from the membranes and form the well-recognized mCRP isoform converted in solution. It is a potent stimulator of endothelial cells.

Glycosylated CRP. Until 2003 it was assumed that CRP is a nonglycosylated protein. However, about four years ago Das and co-workers found glycosylated CRP in the serum of patients with six different pathological conditions; systemic lupus erythematosus (SLE), acute lymphoblastic leukaemia (ALL), tuberculosis (TB), visceral leishmaniasis (VL), Cushing's syndrome and osteogenic sarcoma (OS). The blood CRP concentration in these patients ranged from 22 to 342 mg/l. Small, but significant changes in electrophoretic mobilities on native polyacrylamide gel electrophoresis suggested differences in the molecular mass, charge and/or shape of the patient CRP. The authors reported the presence of different amounts of sialic acid residues, mannose, glucose and galactose in the CRP of the patients. Computer aided molecular modelling, based

on sequence data, suggested two potential glycosylation sites on the CRP cleft floor. The amino-acid composition of three patients' CRP also varied to a certain extent after tryptase treatment. There was an absence of two peptide fragments, one N-terminal fragment of six amino acids and another near the C-terminus of three amino acids (Das et al. 2003). In a subsequent paper by the same author, the binding characteristics of the patient CRPs were reported. First, BALB/c mice were immunized with a mixture of CRPs (from VL, ALL, TB, SLE and OS patients) and the IgG fractions were collected from the mice. Then each CRP was coated on a microtiter plate and IgG from the mice was added. As a result the antibodies showed different amounts of binding to specific CRPs, suggesting different immunodominant epitopes in different patient CRPs (Das and Mandal 2004b).

2.4. CRP and complement

2.4.1. Classical pathway activation

Activation of the classical complement pathway by ligand bound CRP was discovered in the 1970's by two independent groups (Kaplan and Volanakis 1974, Siegel et al. 1974). These and subsequent studies showed that ligand bound CRP binds to C1q and activates the classical complement pathway. The activation is similar to the antibody activation of the classical pathway. However, CRP binding to C1q differs from antibody binding in that CRP binds to the collagen-like regions rather than globular head groups of C1q (Jiang et al. 1991). Examination of individual complement components after CRP induced complement activation compared to antibody induced activation has shown that CRP is most efficient at early classical pathway activation (C1, C4, C2) and this selective activation of early components is done without the formation of the membrane attack complex (MAC) C5b-9 (Berman et al. 1986). This is due to the interaction of CRP with one of the complement regulatory proteins, factor H (Jarva et al. 1999).

Complement activation has been shown to contribute to the killing of micro-organisms and thereby protection against pathogenic organisms like *S. pneumoniae* (Mold et al. 1981) and *H. influenzae* (Weiser et al. 1998). Activation of complement may also be of importance at sites of tissue injury where CRP binds.

2.4.2. Inhibition of complement alternative pathway

The effect of CRP on complement alternative pathway (Mold et al. 1984) helped to clarify the limited classical pathway activation. As mentioned, the late events

of complement cascade forming the MAC are inhibited and this is due to factor H, an alternative pathway component, and CRP interaction.

Factor H is one of the factors interacting with C3b to prevent complement activation from proceeding when C3b binds to host cells instead of pathogens. This is done by either preventing the alternative pathway C3-convertase, C3bBb, from forming or promoting its rapid dissociation, thus reducing generation of MAC and the lysis of cells. Upon cell damage, however, factor H does not bind to host cells. The damaged structures are bound by CRP and consequently CRP binds factor H. Thus CRP may help to limit the inflammatory reaction (Mold et al. 1999). Because the alternative complement pathway serves as an amplification loop for the classical pathway, this may also explain the lack of C5-C9 consumption during the classical pathway activation of complement. Two specific binding sites for CRP on Factor H have been located onto its seventh short consensus repeat domain, labelled respectively site A and site B. These sites contain positively charged residues, Arg^{369} and Lys^{370} in site A and Arg^{386} and Lys^{387} in site B (Giannakis et al. 2003). The interaction is of ionic nature (Jarva et al. 1999). Recently a T>C polymorphisms in factor H gene at position 1277 (Tyr402His) was also found to affect CRP binding to factor H. His^{402} homozygotes showed reduced binding of factor H to CRP, compared to Tyr^{402} homozygotes (Laine et al. 2007).

2.4.3. Inhibition of complement lectin pathway

Complement can also be activated by a third pathway, the lectin pathway. This pathway uses a protein very similar to C1q to trigger the complement cascade, the mannose-binding lectin (MBL). The protein is a member of the collectin family of proteins, and is also an acute phase protein. MBL binds mannose and some other sugars on many pathogens, as well as lipopolysaccharide (LPS). On vertebrate cells, however, the sugars are covered by other sugar groups. Because of this, MBL is able to activate complement only on pathogen cells, not on host cells. This pathway is homologous to the classical pathway. After binding to a ligand, the MBL complex cleaves C2 and C4, forming a C3 convertase. The effect of CRP on the lectin pathway has been studied on erythrocytes coated with mannan and PCh-BSA to provide binding sites for both MBL and CRP. A dose-dependent inhibition of erythrocyte lysis after CRP addition was observed, indicating that haemolysis via lectin pathway requires alternative pathway amplification (Suankratay et al. 1998). Subsequent study showed that the lectin pathway is particularly susceptible to the regulatory effects of C4bp and factor H (Suankratay et al. 1999).

2.5. Binding to receptors on phagocytic cells

CRP binds to Fcγ receptors (FcγR) on phagocytic cells by its effector-binding face. The FcγRs are also the receptors for IgG. There are four families of these

receptors, FcγRI, FcγRII (classes a and b), and FcγRIII and FcγIV, only the first two of which probably bind CRP. FcγRI and FcγRIIa are found on the surface of neutrophils, monocytes and macrophages, and FcγRIII on neutrophils, macrophages, mast cells and natural killer cells. The FcγIV is a recently identified receptor expressed on neutrophils, monocytes, macrophages and dendritic cells (Nimmerjahn and Ravetch 2006). The differences in the expression and function of the FcγR explain many of the diverse activities of CRP.

The specific interaction of CRP with FcγRI was first established in 1991 (Crowell et al. 1991). Later it was found that the affinity of CRP for FcγRI actually exceeded the affinity of IgG for this receptor (Bodman-Smith et al. 2002). In spite of this, the affinity of CRP for this receptor is still lower than that for FcγRIIa receptor. CRP binding activates phospholipase D, a typical component through this receptor, and stimulates the release of TNF (Bodman-Smith et al. 2002). In resting monocytes and PMN the expression of FcγRI is low, but can be enhanced/induced by exposure to INFγ (Guyre et al. 1983, Stein et al. 2000). Two regions in IgG have been identified as important for binding to FcγRI, ^{234}Leu-Leu-Gly-Gly-Pro-Ser^{239} for human IgG1 and a second region ^{327}Ala-Leu-Leu-Pro-Ala-Pro-Ile^{333} (Marnell et al. 2005). CRP region ^{176}Leu-Gly-Gly-Pro^{179} has sequency homology with these regions. CRP amino acids 175-185 form part of the edge of a long deep cleft in each protomer on the effector face of the molecule (Marnell et al. 1995, Bang et al. 2005). The residues around this cleft are important for both FcγR (Bang et al. 2005) and C1q (Agrawal et al. 2001b) binding and the binding sites for these molecules in CRP overlap (Bang et al. 2005). Analysis of the binding sites by site-directed mutagenesis showed the Thr^{173} and Asn^{186} amino-acids of CRP to be important in FcRγI binding (Bang et al. 2005).

The role of FcγRIIa as a second receptor for CRP was reported in 1999 (Bharadwaj et al. 1999). Later it was found that this receptor was a functional high-affinity receptor for CRP, and that binding of CRP to this receptor was allele-specific, with stronger binding to the FγRIIa allele with an arginine at amino acid 131 than to the allele with histidine 131 (Stein et al. 2000). The binding avidity of CRP for FcγRIIa in Arg^{131}/His^{131} heterozygotic cells was decreased by 65-70% compared to Arg^{131} homozygotic cells. The functionality of the alleles was analysed by measurement of intracellular Ca^{2+} concentration in PMN cells after CRP binding and showed a rapid and transient increase in Ca^{2+} in PMN homozygotic for Arg^{131}, but no increase in PMN homozygotic for His^{131}. This confirmed the allele-specific nature of this receptor (Stein et al. 2000). However, other researchers did not find a difference in responses to CRP between donors homozygous for either Arg^{131} or His^{131} allele, but this is probably due to differences in experiments; this study investigated neutrophil IL-8 release, phagocytosis and respiratory burst to CRP-opsonized *S. pneumoniae* (Rodriguez et al. 2004).

Interestingly, human IgG2 is known to bind well to the alternate form H131 (Parren et al. 1992). As FcγRIIa, b and c have similar extracellular domains, it seems reasonable to assume that CRP binds all three of them, allowing it to bind

to a wide variety of leukocytes. Thus both the cytokine environment (INFγ up-regulates FcγRI expression) and allelic variations in receptor structure seem to be important in determining the host response to CRP. There is also evidence that receptor types I and II could act together, namely FcγRI could recruit FcγRIIa after CRP binding to initiate signal transduction and phagocytosis, rather than use its 'normal' signalling through γ-chain (Bodman-Smith et al. 2004).

It has been proposed that CRP glycosylation state may affect the interaction between FcγR and CRP. The carbohydrates are known to attach to the floor of the protein's C1q/FcγR binding cleft at the effector face of the molecule (Das et al. 2003). This prediction is not farfetched, because glycosylation is known to affect the ability of immunoglobulin to bind FcγR and to activate complement (Jefferis and Lund 2002).

2.6. Enhancement of phagocytosis and induction of cytokine synthesis

The responses of neutrophils and monocytes to CRP binding are phagocytosis of CRP-opsonized particles and production of cytokines. The cytokine response to CRP is similar to the response of aggregated IgG and includes the production of IL-1α, IL-6 and TNF-α (Ballou and Lozanski 1992). Initially it was thought that CRP induces pro-inflammatory cytokines and enhances the inflammatory response at the site of injury. However, some studies showed that CRP also induced the anti-inflammatory response, as it was found that peripheral blood mononuclear cells could produce an excess of IL-1RA over IL-1 in response to LPS (Tilg et al. 1993). Synthesis of IL-10, an anti-inflammatory molecule, was also found to be upregulated after CRP binding (Mold et al. 2002b). In 2006, Mold and Du Clos clarified this issue by showing that the cytokine response after CRP binding was probably dependent on the ligand bound state of CRP. In the absence of the ligand CRP produced relatively low levels of TNF-α, IL-1β and IL-6 from peripheral blood mononuclear cells, but after ligand binding (in this case *S. pneumoniae*) the levels of these cytokines increased significantly. This was due to CRP interactions with FcγRs, resulting in IL-1β and TNF-α production (Mold and Du Clos 2006). Thus it may be that optimal interaction of CRP with FcγR to induce cytokine responses requires interactions with multiple receptors and therefore requires binding to a multivalent ligand. Because these cytokine responses could be inhibited by specific antibodies to both FcγRI and FcγRII, involvement of both of these receptors was likely (Mold and Du Clos 2006). The regulation of FcγRI expression by IFN-γ could also be important in determining the nature of the response of cells to CRP.

3. CRP as a subclinical low-grade inflammation marker

CRP concentration above 10 mg/l has traditionally been considered clinically important (Morley and Kushner 1982). However, more recent studies have shown the value of minor concentrations in the prediction of coronary artery disease events. Indeed, slightly elevated CRP concentration, but higher than those in most normal subjects, is now called low-grade inflammation. Ridker and colleagues have suggested clinical categories for baseline CRP values in predicting the risk for future cardiovascular events. Subjects with concentration ≤1 mg/l are considered to be in the low risk group, those with concentration between 1 to 3 mg/l in the moderate risk group and those with more than 3 mg/l in the high risk group (Ridker et al. 2003). Thus CRP is now also to be considered as a low grade inflammation marker alongside with its traditional role as an acute phase protein. Low-grade inflammation is now recognized to play a role in many diseases, e.g. obesity, metabolic syndrome, diabetes and coronary heart disease (CHD).

3.1. CRP in obesity

Obesity increases the risk of cardiovascular disease and premature death. Adipose tissue releases many bioactive mediators that influence alterations in lipids, blood pressure, coagulation, fibrinolysis and inflammation, leading to endothelial dysfunction and atherosclerosis. Adipose tissue is actually an active endocrine and paracrine organ and releases cytokines and other bioactive molecules, such as leptin, adiponectin, IL-6 and TNF. It has been estimated that adipocytes may produce almost one third of the total IL-6 in circulation. IL-6, in turn, activates *CRP* expression from hepatic cells. As a consequence, overweight and obese individuals tend to have slightly elevated CRP levels (Visser et al. 1999). Adiposity is, indeed, one of the most important correlates of CRP. However, weight loss induces favourable changes in CRP (Bastard et al. 2000, Heilbronn et al. 2001, Tchernof et al. 2002), blood lipoprotein and insulin concentrations, as well as in blood pressure. A systematic review of the literature of the effects of weight loss on CRP was recently conducted. Thirty-three studies were included, where the study size ranged from 13 individuals per study to 199 individuals per study. The authors concluded that regardless of the type of intervention imposed, CRP concentrations declined, on average, when weight loss was achieved and the relationship was roughly linear. For each kg of weight loss, the mean change in CRP concentration was -0.13 mg/l (Selvin et al. 2007).

3.2. CRP in cardiovascular diseases –A current topic

3.2.1. Atherosclerosis

Atherosclerosis is a disease characterized by chronic arterial inflammation and is now widely accepted to be an inflammatory disease (Ross 1999). Indeed, at present the most intense research of CRP is done on cardiovascular diseases. The early events in atherosclerosis are primarily altered function of the endothelia of the arterial walls, resulting in endothelial dysfunction and the recruitment and accumulation of leukocytes and LDL in the intima. The endothelial cells of the intima have a number of important functions; forming a non-thrombotic, non-adherent surface, acting as a semi-permeable membrane, synthesising and releasing chemical mediators and maintaining the basement membrane. Initially, damage causes the endothelial cells to express adhesion molecules (vascular cell adhesion molecule 1, intercellular adhesion molecule 1, E-selectin), cytokines (IL-1, TNF-α) and chemokines (monocyte chemoattractant factor 1, interleukin-8). This 'sticky' endothelial surface then encourages inflammatory cells such as monocytes and T-cells to attach to the endothelial surface and migrate through it into the subendothelial space. Here many of the monocytes differentiate into macrophages, internalize accumulated oxidised LDL and subsequently transform into foam cells. These events lead to the formation of silent fatty streaks, which can develop further and be the precursors of more complex lesions, ultimately leading to coronary narrowing and clinical manifestations. Clinical manifestations of atherosclerosis include CHD (angina pectoris, MI, and sudden cardiac death), cerebrovascular disease (transient ischemic attacks, stroke) and peripheral vascular disease.

3.2.2. Association between CRP and cardiovascular disease

After the discovery of CRP in 1930, increased production of CRP was recognized as a characteristic feature of the response to acute MI and was also used for the monitoring of acute rheumatic fever. In 1963, deposition of rabbit CRP in experimental MI lesions was demonstrated by immunofluoresence (Kushner et al. 1963). The association between CRP and cardiovascular disease thus has quite a long history. However, the current phase of interest in CRP and cardiovascular disease began in the early 1990's, when observations were made of increased CRP in patients with acute MI tested very soon after the onset of pain, before the APR to infarction could have started. At the same time a large prospective European study of patients with stable and unstable angina unexpectedly revealed that the baseline level of CRP significantly predicted future coronary events (Thompson et al. 1995). Subsequently several groups showed independently that baseline CRP was associated with increased risk for

future coronary events in general population without known pre-existing coronary artery disease (Ridker et al. 1997, Ridker et al. 1998, Danesh et al. 2000). Thus, the subclinical state of atherosclerosis can be identified by an increase in circulating markers of inflammation before acute events occur, e.g. CRP (Ross 1999, Hansson 2005). In 2003, a statement from the Centers for Disease Control and Prevention and the American Heart Association concluded that it is reasonable to measure CRP, a sensitive circulating marker of inflammation, as an adjunct to the measurement of established risk factors in order to assess the risk of CHD (Pearson et al. 2003). A recent meta-analysis involving over 7000 patients with coronary events showed that subjects with CRP in the upper tertile have 50 % increased risk of developing acute cardiovascular events (Danesh et al. 2004). Moreover, the ability of elevated CRP also to predict cardiovascular death, MI or stroke in patients with stable coronary artery disease, has now been recognised (Sabatine et al. 2007).

3.2.3. The proatherogenic effects of CRP

The direct action of CRP in developing atherosclerosis is still uncertain. However, CRP has been shown to have some proatherogenic effects. Reports on these effects are introduced below.

LDL. One of the first discoveries linking CRP with atherogenesis was the finding that artificially aggregated CRP bound native LDL in the presence of calcium (de Beer et al. 1982). Later it was shown that native CRP was capable of binding entzymatically modified LDL (E-LDL) by either exposed phosphorylcholine (Bhakdi et al. 1999) or by non-esterified cholesterol present on the lipoprotein surface (Taskinen et al. 2002). Subsequently it was shown that CRP could bind also oxidized LDL (ox-LDL) via phosphorylcholine (Chang et al. 2002). The uptake of CRP-LDL complexes by macrophages has been controversial. Zwaka and associates showed that CRP could bind native LDL (in the liquid phase) and facilitate its uptake by macrophages via FcγRII (Zwaka et al. 2001), but this was not confirmed by Taskinen and associates (2002). Later Fu and co-workers showed that immobilized CRP-LDL complex was taken up by macrophages, not via FcγRII, but some other FcγII independent way (Fu and Borensztajn 2002). It has been demonstrated that the modified form of CRP can bind native LDL, whereas native CRP can bind modified LDL (de Beer et al. 1982, Taskinen et al. 2002, Ji et al. 2006b). Ji and colleagues also suggested that modified CRP is also able to bind ox-LDL (2006b). It therefore seems that both the form of CRP (nCRP, mCRP) and the form of LDL (nLDL, E-LDL, ox-LDL) play a part in the recognition process and may alter the interaction outcome.

Tissue factor. In 1993, monocytes were shown to have increased tissue factor (TF) expression after incubation with CRP (Cermak et al. 1993). TF is a membrane-bound glycoprotein that initiates the extrinsic pathway of coagulation. It is expressed on macrophages in atherosclerotic plaques and may contribute to acute thrombotic events associated with plaque rupture in unstable syndromes. In

1999, monocyte TF induction by CRP was verified by another study, which also found that the induction was potentiated by IFN-γ and LPS, and seemed to be influenced by age and sex. Tissue factor inductivity was higher in older (>50 years) than in younger individuals (<50 years), and post-menopausal women showed the most dramatic increase in activity (Nakagomi et al. 2000). This finding was intriguing because deposits of CRP near foam cells in atherosclerotic plaques together with CD4+ T cells producing IFN-γ could collectively induce high levels of tissue factor, which might initiate thrombus formation.

Adhesion molecules and endothelin. In 2000, Pasceri and associates showed increased adhesion molecule expression in human coronary artery endothelial cells (HCAECs) after incubation with CRP. This expression was found to be dependent on the presence of human or bovine serum in the culture media. Increased expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecules was observed at CRP concentrations of 5 μg/ml and was maximal at 50 μg/ml, whereas increase in E-selectin was found only at concentrations > 10 μg/ml (Pasceri et al. 2000). Similar increased adhesion molecule expression from sapheous vein endothelial cells were subsequently reported by Verma and colleagues, who also found that endothelin-1 antagonist as well as anti-IL6-antibodies attenuated the proatherogenic effects of CRP on these cells (2002a). Endothelin-1 is one of the most potent constrictors of human vessels to be discovered and is continuously released from the endothelial cells. It produces intense constriction of the underlying smooth muscle and contributes to the maintenance of endogenous vascular tone. In 2006, it was reported that CRP induced VCAM-1 expression in HUVECs and HAECs through binding of CD32 receptor (FcγRII) and activation of the nuclear factor – κB pathway (Liang et al. 2006).

Monocyte chemoattractant protein-1. Subsequently, Pasceri and co-workers showed increased expression of monocyte chemoattractant protein-1 (MCP-1) in human umbilical vein endothelial cells (HUVECs) after incubation with CRP. Induction was already apparent at CRP concentration of 5 ug/mL, but only in the presence of human serum (Pasceri et al. 2001). However, in a later study no MCP-1 expression from human aortic endothelial cells (HAECs) after CRP incubation was detected (Devaraj et al. 2004).

Plasminogen activator inhibitor-1. Devaraj and co-workers reported increased expression and activity of plasminogen activator inhibitor-1, a marker of atherothrombosis and impaired fibrinolysis, in HAECs after CRP incubation (2003). Later, decreased activity of tissue plasminogen activator was reported in the same cells after CRP incubation (Singh et al. 2005).

Endothelial nitric oxide. Soon two separate studies showed that CRP is able to decrease endothelial nitric oxide synthase (eNOS) expression and activity in endothelial cells. eNOS is an enzyme of the endothelial cells which produces nitric oxide. Nitric oxide promotes arterial vasodilatation and inhibits SMC proliferation, LDL oxidation, platelet adhesion and aggregation and monocyte adhesion to endothelium. Thus active eNOS is one important prerequisite for functional endothelium. Incubation of cultured HAECs, HUVECs or sapheous

vein endothelial cells with CRP decreased eNOS mRNA, protein abundance and enzyme activity (Venugopal et al. 2002, Verma et al. 2002b).

Other. In 2003, the release of another potent vasodilator and inhibitor of platelet aggregation, prostacyclin, was found to be decreased in endothelial cells after CRP incubation (Venugopal et al. 2003). In human monocytes, CRP was found to promote MCP-1 mediated chemotaxis by upregulating CC-chemokine receptor 2 (CCR2) expression (Han et al. 2004). CCR2 is the most dominant chemotaxis receptor in monocytes. The CRP-induced upregulation of CCR2 expression involved CRP binding to FcγR, most notably FcγRI. In another study CRP was shown to induce IL-8 expression from HAECs. IL-8 is a member of the CXC chemokines and promotes monocyte-endothelial cell adhesion and arrest (Devaraj et al. 2004).

3.2.4. mCRP versus nCRP in atherosclerosis

There are several reports on the impact of a modified form of CRP in atherosclerosis compared to pentameric CRP. In 2006, Ji and colleagues studied the effect of mCRP in activating complement, and reported that mCRP can both inhibit and activate the classical complement pathway by binding C1q, depending on whether it is in the fluid phase (inhibition) or surface-bound state (activation) (Ji et al. 2006a). mCRP was also found to be a more potent binder of native LDL and ox-LDL than pentameric CRP, whereas binding of E-LDL seemed to be equal in both isoforms (Ji et al. 2006b). Ji and colleagues also showed more potent activation of HAECs after mCRP challenge than after pentameric CRP challenge. The activation was determined as the amount of IL-8 and MCP-1 expression from HAECs after challenge (Ji et al. 2006c). However, contradictory on HAEC activation were reported soon after by Devaraj and associates, who showed nCRP to display more potent pro-atherogenic activities than mCRP (Devaraj et al. 2006).

3.2.5. Doubts about in vitro experiment validity

However, grave suspicions about the actual role of CRP in endothelial cell activation soon arose. *In vitro* studies using commercial CRP were criticized for the lack of robust controls performed to assess whether the effects observed were not attributable to contaminants in the preparations, e.g. LPS or sodium azide (Hirschfield and Pepys 2003). Because most of the studies used human CRP produced in *E. coli* by recombinant technology, concern about the presence of LPS or other bacterial products in CRP preparations arose. Azide, for its part, is a commonly used bacteriostatic preservative in commercial reagents. LPS is

known to activate ECs, but the mechanism of azide activation is not clear. It is an inhibitor of cytochrome oxidase and affects the metabolic functions of cells, inducing cell death, thus being generally toxic for cells. In 2005, Taylor and co-workers analysed the existing literature and found only two studies where azidic free CRP was mentioned as being used. The first of these studies showed a modulatory effect of CRP on monocyte adhesion to human endothelial cells (Woollard et al. 2002), and the other pro-inflammatory action of mCRP on HCAECs (Khreiss et al. 2004). The LPS contamination aspect was more controlled; at least four studies used a detoxigel column for CRP purification and then a Limulus assay for endotoxin level estimation (Pasceri et al. 2001, Venugopal et al. 2002, Verma et al. 2002b, Venugopal et al. 2003). The remainder of the published studies were potentially affected either by LPS or azide contamination. However, recently Dasu and associates reported the biological properties of in-house-purified CRP preparations in Toll-like receptor 4 small interfering RNA -transfected endothelial cells, i.e. in Toll-like receptor 4 knockdown endothelial cells. CRP incubation induced secretion of IL-8, IL-6, IL-1β, and plasminogen activator inhibitor-1 from knockdown endothelial cells and inhibited eNOS activity. The authors concluded that these effects are due to native pentameric protein and not to endotoxin or other contamination, as none of the reagents used contained azide as a preservative (Dasu et al. 2007). Taken together, it seems that at present the proatherogenic effects of CRP are carefully scrutinized and future research will shed more light on this topic.

3.2.6. CRP in the pathology of atherosclerosis

The role of CRP in atherosclerosis pathology is still controversial, and there are several theories as to how the association between CRP and cardiovascular disease is possible.

Theory I. Firstly, inflammation in arterial tissue results the in release of cytokines into the circulation which activate the expression of CRP in the liver. In this scenario the elevated CRP levels would then reflect the severity of the atherosclerosis as well as the risk of clinical events.

Theory II. According to another theory CRP is a marker for metabolic disturbances associated with an increased risk of cardiovascular disease. CRP is known to be associated with several metabolic changes that characterise insulin resistance and type 2 diabetes, e.g. high body mass index, hypertension, hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol (Fredrikson et al. 2004). This theory emphasizes the release of cytokines from adipose tissue but the full biological explanation behind the association of metabolic changes and increased risk for cardiovascular disease (CVD) remains to be elucidated. In this theory CRP is merely a marker of metabolic disturbances but is not itself associated with the actual cardiovascular disease process.

Theory III. The third theory is intriguing. According to this theory CRP actively contributes to the atherosclerosis progression. CRP is claimed to bind lipoproteins and damaged cells in atherosclerotic plaques, induce complement activation and thus promote inflammation and disease progression (Pasceri et al. 2000).

Theory IV. The fourth theory involves local CRP production from the arteries/myocardial muscle. In atherosclerotic lesions, the endothelial cells are in close proximity to both smooth muscle cells and macrophages. In these microenvironments macrophages can secrete both IL-6 and IL-1, thereby inducing local production of CRP from endothelial cells. As macrophages and smooth muscle cells themselves are also able to secrete CRP, both autocrine/paracrine loops among the the cells in the atherosclerotic lesions could arise. In these circumstances the microdomains in the intima could lead to higher CRP levels than in the circulation, and induce vascular dysfunction (Venugopal et al. 2005). However, the importance of local synthesis remains uncertain, because CRP is expected to be deposited in these lesions due to the presence of necrotic cells, apoptotic cells, exposed phospholipids, and other ligands known to bind CRP.

Indeed, the production of CRP in normal and atherosclerotic aortas (Reynolds and Vance 1987, Venugopal et al. 2005) and in atherosclerotic lesions (Zhang et al. 1999) has been detected. Yasojima and colleagues observed that both CRP mRNA and protein are expressed in arterial plaque tissue in 10-fold concentrations compared to the normal artery. The mRNA was localized mainly to macrophages and smooth muscle-like cells (Yasojima et al. 2001).

4. CRP genetics

The basal concentration of CRP has been estimated to be about 40-52% heritable according to family and twin studies (Pankow et al. 2001, Vickers et al. 2002, Retterstol et al. 2003, MacGregor et al. 2004). Therefore variations in genes inducing *CRP* gene expression as well as variations in *CRP* gene itself may predispose to different basal CRP concentrations. The single copy of the human *CRP* gene is located on 1q23.2 (Figure 1). It is about 2.5 kbp in length and consists of two exons and one intron. The entire human region of 1p22 to 1q32 is among those mammalian chromosome segments whose organisation seems to have been conserved throughout evolution and contains several genes coding for proteins of immunological importance (Kingsmore et al. 1989) These include e.g. Fc receptors for IgE and IgG and the already mentioned pentraxin genes. Mammalian *CRP* and *SAP* are thought to be products for an ancestral gene duplication event and share 51% amino acid and 59% nucleotide sequence identity. Also, a single non-functional pseudogene with 50-80% region-specific homology is found close to the authentic *CRP* gene (Goldman et al. 1987).

The first exon of *CRP* gene encodes a putative signal peptide consisting of 18 amino acids and the first two amino acids of the mature protein. The rest of the polypeptide chain (204 amino acids) and a 1.2 kbp 3' untranslated region (3' UTR) are encoded by the second exon. The nucleotide sequence of the intron contains two characteristic repetitive elements; a run of 16 adenines and a polymorphic GT repetitive sequence. It has been pointed out that this region can adopt the Z-form of DNA and therefore could have a role in chromatin activation.

The promoter region of human *CRP* gene contains many transcription factor binding elements. In 1990, two acute phase response elements were described, each containing a binding site for the liver specific transcription factor (Toniatti et al. 1990). In 1996, two IL-6 responsive elements were discovered, namely two C/EBP beta (CCAAT/enhancer binding protein beta) binding sites (Li and Goldman 1996). At the beginning of 2000, an IL-1 responsive element was found, namely a κB site, which binds Rel P50 molecule. The κB site overlaps with the proximal C/EBP binding site, and the binding of Rel P50 to the κB site seems to enhance and stabilize the binding of C/EBP to the promoter and amplify CRP expression. Thus both IL-6 and IL-1 appear to affect CRP transcription (Cha-Molstad et al. 2000, Agrawal et al. 2001a). The promoter also contains four E-box elements, which were described in 2005 (Carlson et al. 2005, Szalai et al. 2005b). The core consensus sequence of these elements is CAnnTG and they are known to bind the transcription factors upstream stimulatory factor 1 (USF1), USF2, Myc and Max. The first (E-box 1) $^{-412}$CAC<u>G</u>TG^{-407} contains SNP -409 G/A which modulates the transcription factor binding. This SNP is polymorphic only in African Americans, but not in Caucasians. The second element (E-box 2) $^{-394}$CACT<u>T</u>G^{-389} also contains a SNP, a triallelic SNP -390 C/T/A, which is also called -286 C/T/A. This SNP supports transcription factor binding only when the T allele is present. At least two other SNPs in the *CRP* promoter lie within E-box elements, -198 C/T in E-box 3 and -861 T/C (also called -757 T/C) in E-box 4. The USF1 binding motif is of importance because USF1 is a potentially important regulator of glucose and lipid-metabolism (Pajukanta et al. 2004). USF1 binds to the promoter region of its target genes as a homodimer and recognizes the CAnnTG motif, resulting in transcriptional activation in response to various stimuli, such as glucose and dietary carbohydrates (Pajukanta et al. 2004).

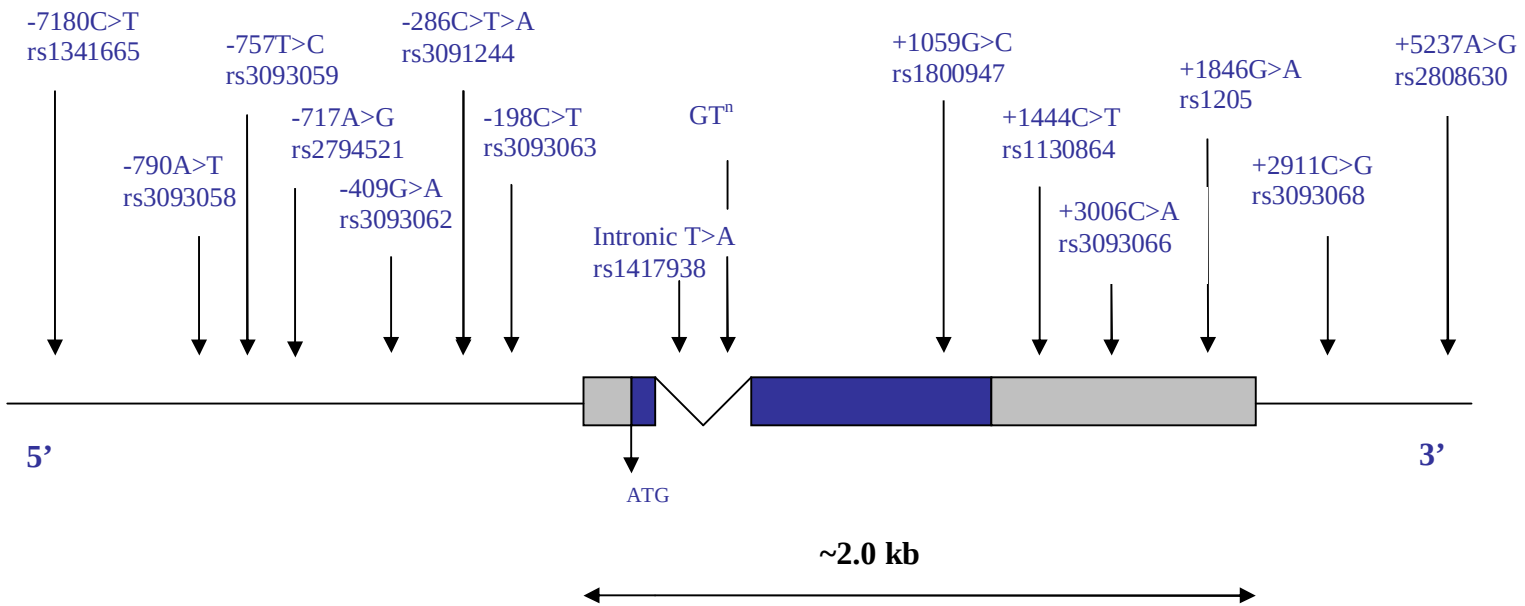

Figure 1. The *CRP* gene region. Structure of human *CRP*, located at chromosome band 1q23. The mRNA (grey) is 2015 bps in length. The coding regions of exons 1 and 2 (blue) correspond to a 224 amino acid peptide separated by a single ~280 bp intron. Information is based on common *CRP* transcript (ENST00000255030) in Ensemble database and on protein sequence (NM000567) in NCBI database. Diagram not to scale.

4.1. CRP gene polymorphisms

The *CRP* gene is polymorphic. In 2005, Carlson and colleagues resequenced the entire *CRP* gene plus 1700 kb upstream and 2800 kb downstream of the gene in 47 individuals and found only 31 SNPs overall, which of 13 were polymorphic in whites and 30 polymorphic in blacks. According to the Ensemble genome browser (http://www.ensembl.org), however, there are 63 SNP's in the *CRP* gene area when the gene area is defined as gene plus 1000 bp upstream and 2000 bp downstream regions. Without upstream and downstream regions there are 38 SNPs (accessed October 30, 2006). On closer examination, the ensemble database contains a lot of SNPs with only one genotype, being thus artefacts of the database, and SNPs with very low (below 0.02) minor allele frequency. Because of this, Carlson and colleagues' 31 SNPs is probably closer to the true value of SNPs in *CRP* gene area. Taken together, it seems that the genome browser contains a lot of SNPs which are, in fact, artefacts (only one genotype exists) or SNPs that have a very low minor allele frequency (below 0.05). The literature has generally used a nomenclature for *CRP* polymorphism positions relative to the ATG start codon, approximately, which will also be used in this dissertation. The association studies have focused either on the association of these SNPs with CRP concentration or on association with diseases. Interestingly, no common amino acid changing SNPs have been found in the whole gene area, but there are some very rare non-synonomous SNPs with a very low frequency (Crawford et al. 2006b).

4.2. CRP genetic association studies

4.2.1. CRP promoter region polymorphisms

In the promoter region of *CRP* gene there are four reported common polymorphisms; -717A>G, a tri-allelic variation site -286C>T>A, -757T>C and -7180C>T (also called -7000C>T in the literature). In addition, there are three polymorphisms which seem to be polymorphic only in African Americans; -198C>T, -409G>A and -790A>T.

In a study by Wolford and co-workers, the -717 A allele was first associated with increased risk of type 2 diabetes mellitus in Pima Indians (n=1300) (2003). Later an association between A allele and increased risk of CHD was found in a case-control study of male Han Chinese (cases n=619, controls n=615) (Chen et al. 2005). However, contradictory results were reported in a subsequent study of 610 cases and 610 controls, where an association between A allele and decreased

risk of atherothrombosis was found [odds ratio (OR) for any event 0.80 (95% CI 0.66-0.97), OR for MI 0.66 (95% CI 0.52-0.85, p=0.001)] (Miller et al. 2005). None of these studies found an association between this SNP and CRP concentration. However, recently an association between G-allele carriage and increased CRP concentration after an acute ischemic stroke/transient ischemic attack (n=219 patients) was found in a study by Ben-Assayag and colleagues, where CRP was measured within 24 hours of hospital admission (2007).

The tri-allelic SNP-286 CA and TA genotypes were associated with increased CRP concentration in 357 patients with first MI by Kovacs and associates (2005). Subsequently the same association was found by Wang and colleagues (2006) and Kathiresan and co-workers (2006). Similar associations were found by Miller and colleagues, where carriage of allele A was associated with increased CRP in three different study populations (n=717, n=1110 and n=509) (2005) and by Suk Danik and associates in a study of 1827 acute coronary syndrome patients (2006). Szalai and associates reported that TT genotype had a tendency for increased CRP in 287 ostensibly healthy patients, but this difference did not reach statistical significance (2005b). Other studies have investigated the association between this SNP and age-related macular degeneration (Schaumberg et al. 2006), ischemic stroke (IS) (Ladenvall et al. 2006) and SLE (Russell et al. 2004), but no association with these phenotypes was found.

SNP-757T>C allele C has been associated with increased CRP concentration in a study by Miller and co-workers (2005), where the association was found in three different study populations. Subsequently this finding was replicated by Suk Danik and colleagues in 1827 acute coronary syndrome patients, where the association was found to exist during and four months after acute coronary syndrome (2006), and by Wang and associates in 1296 Caucasians (Wang et al. 2006). However, no association of this SNP with MI or IS was found in the study by Miller and co-workers (2005).

Morita and the group studied the association of -7180C>T with CRP concentration and pulse wave velocity in 315 healthy elderly Japanese patients (2006a), as well as with IS in a case-control setting of elderly Japanese (cases n=152, controls n=304) (2006b), but no associations were found.

For the three polymorphisms found solely in African Americans, only -790A>T was significantly associated with CRP concentration (n=687) and MI (n=67) (Lange et al. 2006). Subjects with allele T had higher CRP concentrations, and subjects homozygous for allele T had a fourfold risk of MI compared with subjects homozygous for allele A. No association between -409G>A and CRP concentration was found in 287 ostensibly healthy individuals (Szalai et al. 2005b) and no results are at present available for -198C>T polymorphism.

4.2.2. CRP exonic polymorphism

The exonic polymorphism +1059G>C of *CRP* gene is a silent mutation (Leu184Leu) and was first reported in 2000 (Cao and Hegele 2000). Since then the minor C allele of this SNP has been associated with decreased CRP concentration in several subsequent reports (Zee and Ridker 2002, Russell et al. 2004, Davey Smith et al. 2005, Miller et al. 2005, Suk et al. 2005, Balistreri et al. 2006, Lange et al. 2006, Thalmaier et al. 2006), of which several were large-scale studies (n≥1000) (Davey Smith et al. 2005, Miller et al. 2005, Suk et al. 2005). However, there is one study showing no association (Araujo et al. 2004). In the literature there seems to be consensus that this SNP regulates CRP concentrations although it is a silent mutation, but the mechanisms remains unknown.

Despite the modulating effect of the CRP concentration, the evidence on an association between this SNP and disease phenotypes is sparse. Association with IS was found in a study by Lange and associates, where decreased CVD mortality was found in GC heterozygous individuals compared to GG homozygotes [hazard ratio (HR) 0.65 (CI 0.48-0.88) total n= 3889, CVD deaths n=490] (2006). In another smaller study on elderly Japanese patients (cases n=152, controls n=304) contradictory results were obtained. There allele C carriage was associated with increased risk for IS (Morita et al. 2006b). Interestingly, there is also one study showing no association between IS and this SNP (264 IS pairs) (Miller et al. 2005). Ladenvall and colleagues in turn found an association between C-allele carriage and cardioembolic stroke (OR 2.2 (CI 1.26-3.97, p=0.006) (2006), and Balistreri and associates found association of allele C and increased risk of acute MI in young Sicilian male patients (Balistreri et al. 2006). There has been quite a lot of interest in this SNP in the literature and there are reports showing lack of association between this SNP and various disease phenotypes, such as hypertension (Davey Smith et al. 2005), arterial thrombosis (Zee and Ridker 2002), venous thromboembolism (Zee et al. 2004a), Alzheimer's disease (Flex et al. 2004) and incidence of post angioplasty restenosis (Zee et al. 2004b). The frequency of the rare allele C of this SNP seems to be somewhat higher in white (6%) than black (1%) population (Carlson et al. 2005).

4.2.3. CRP intronic polymorphisms

There are two reported polymorphisms in the intron area of the *CRP* gene; a GT repeat and a T>A polymorphism (also called IVS1). The GT^n repeat was first discovered in the 1980s at the time of the characterization of the *CRP* gene (Lei et al. 1985, Woo et al. 1985). Roy and co-workers first reported an association of GT^{16} with increased risk for invasive pneumococcal disease (cases n=205, controls n=346) (2002) and in the same year Szalai and associates found an association of GT^{16} and GT^{20} alleles with decreased CRP concentration in 244

healthy controls and 312 SLE patients. However, there were no differences in allele distributions between SLE patients and controls (2002). Subsequently Russell and colleagues reported over-transmission of GT^{16} allele in 344 UK SLE families, but noted in haplotype analysis that this was due to linkage of this repeat with SNP +1846 minor allele (2004). The following year Szalai and associates reported an association of GT^{20} variant with increased vascular events in 25 multiethnic SLE patients (2005a).

In 2004, Zee and co-workers published two association studies of intronic T/A. A matched, prospective case-control sample (cases n=130, controls n=130) of the Physician's Health Study found no association between the intronic T/A polymorphism and venous thromboembolism (2004a). The other study found no association between intronic T>A and incidence of post-angioplasty restenosis in Spanish patients, 342 cases and 437 controls (2004b). In the same year Obisesan and colleagues studied the association between T/A and CRP concentration before and after exercise training in 63 sedentary adults, but no association was found (2004). Later Suk and associates conducted a community based large-scale association study of intronic T/A and CRP concentration in 2397 US participants (mean age 62 years), and found increased CRP concentrations in subjects with A-allele (2005). This association persisted after adjusting for age, sex, BMI, ethnicity, hypertension, smoking, diabetes, hyperlipidemia and aspirin use, and the effects were also present in subgroup analysis limited to those with (n=1063) and without (n=1334) prevalent coronary disease. The associations also persisted in analysis among Caucasians only (>80% of subjects). This association was subsequently verified by Lange and colleagues, who conducted a large scale study of the association of intronic T>A with CRP concentration, IS, MI and CVD mortality in 3941 US white (European American) and 700 black (African American) participants (2006). The participants were older than in the study by Suk and associates (≥ 65 years). It should be noted that the alleles of intronic T>A are coded incorrectly in this paper; T allele is said to be the rarer allele but in fact it should be allele A. Taking this into account, the rarer allele A was associated with increased CRP concentration in both whites and blacks, and this association persisted in covariance models after adjusting for age, sex, clinic site, BMI, smoking status, triglycerides and clinical or subclinical cardiovascular disease at baseline. In the whites the AA genotypes had 1.4 fold risk of stroke compared to TT genotypes (HR 1.40 (CI 1.06-1.87)), and also increased risk of CVD mortality (HR 1.40 (CI 1.10-1.90)). Yet another study was conducted in 2006 by Ladenvall and co-workers, who found no association between risk of IS and intronic T/A in a matched case-control study of 600 cases and controls participating in the Sahlgrenska Academy Study on Ischemic Stroke (2006).

4.2.4. CRP 3'UTR polymorphisms

In the *CRP* gene 3'UTR there are four common SNPs, +1444C>T, +1846G>A, +2911C>G and +5237A>G. In addition, there is one SNP which seems to be polymorphic only in African Americans; +3006C>A. The location of the

+3006C>A lies between +1444 and +1846, thus the nomenclature used in literature for this SNP is misleading.

In 2003 Brull and colleagues first described a new polymorphism of the *CRP* gene, a +1444C>T at the 3'UTR region of the *CRP* gene. They studied the association of this variant with CRP concentration in two different inflammatory response models; perioperatively in 193 coronary artery bypass graft patients (mean age 62 years, model of major inflammatory response) and in 250 British white male army recruits at baseline and after an intensive 48-hour military endurance exercise (mean age 19 years, model of mild inflammatory response). They found an association of +1444C>T allele C with decreased post-operative CRP concentration in the artery bypass graft patients in analysis with age, sex, BMI, smoking, diabetic status, therapy with drugs, length of bypass, operation duration and aortic cross-clamp time as covariates. The same allele was associated with decreased CRP in army recruits at baseline and after exercise (Brull et al. 2003). However, contrary results were reported by Obisesan and associates, who studied 63 sedentary white and non-white men and women aged 50-75 years participating in an exercise training intervention with low-fat diet (2004). Participants' CRP was measured at baseline and after training for 6 months. Training was conducted three times a week and consisted of jogging, stair stepping, cycle and rowing ergometry. Associations of +1444C>T allele T with decreased baseline and after training CRP concentrations were found after adjusting for age, gender, and ethnicity. In regression analysis further adjustment with body weight and body fat did not change the result. Subsequent studies concurring with Brull and colleagues soon appeared, D'Aiuto and associates reported an association of C-allele with decreased CRP concentration in 55 periondontitis patients after a mild inflammatory stimulus (2005), Marsik and co-workers found an association between C-allele carriers and decreased CRP in 91 healthy young male Caucasian volunteers after an LPS challenge (2006) and finally Miller and associates showed an association between C-allele and decreased CRP in a large-scale study of three different study populations (n=717, n=1110 and n=509). In this study the association between this variant and IS/MI risk was also analysed, but it was not there (Miller et al. 2005). Later Casas and colleagues reported a large-scale meta-analysis of an association between risk of coronary event and +1444C>T in 4659 European men from six different studies and found no sinificant association, though the confidence limits were still compatible with modest causal effect (2006).

The +1846G>A (also named +2147G>A in the literature) was first reported by Russell and associates in 2004. They made a family-based association test of this variant with CRP concentration and SLE susceptibility and also analysed its effect on antinuclear autoantibody formation in 586 UK SLE families. They found association of the G allele with increased CRP concentration, and an association of the A allele with SLE and antinuclear autoantibody formation and concluded that reduced *CRP* expression predisposes to the development SLE (Russell et al. 2004). Subsequnetly Grocott and colleagues studied the effect of this polymorphism on the risk of stroke after cardiac surgery in 1635 patients. Twenty-eight of the patients suffered a stroke. Alone this SNP did not associate

significantly with stroke, but together with *IL6*-174 G>C variation a significant association was found. Carriers of both +1846 allele T and IL-6 -174 allele C had over 3-fold risk of stroke compared to non-carriers [OR 3.3 (CI 1.4-8.1)] (Grocott et al. 2005). The possible association of this SNP with CRP concentration was not studied in this investigation. Later Miller and co-workers reported an association of +1846 allele A with decreased CRP concentration in three different study populations (n=717, n=1110 and n=509) but found no association with risk of IS or MI (2005). Nor was any association with IS found in a study of 152 elderly Japanese IS patients and 304 controls (Morita et al. 2006b). However, recently Lange and colleagues found an association between AA homozygous and decreased CVD mortality in whites in a large-scale study of 3941 US whites with 490 CVD deaths, HR being 0.65 (2006). In addition, in a population-based prospective association study (The Rotterdam Study) the relationships between this polymorphism and the risk of dementia and Alzheimer's disease were studied in 5972 Dutch patients. Six hundred and seven (607) of the patients developed dementia during the nine-year study period. The association was estimated using Cox's proportional hazard model and indicated an association between the allele A and decreased CRP concentration and also between lower risk of dementia and genotype GA (HR 0.73 (CI 0.71-1.00)) and genotype AA (HR 0.86 (CI 0.53-0.98)) compared to genotype GG. Likewise, reduced risk of Alzheimer's disease was found for genotype AA HR 0.73 (CI 0.53-0.98) (van Oijen et al. 2006). This variant has also been associated for risk of lung cancer in the Rotterdam Study with over 7000 participants (age ≥ 55). The authors studied the effect of this variant on the risk of colorectal, lung, breast and prostate cancer and found that homozygosity of the allele A increased lung cancer 2.6-fold (OR 2.6 (CI 1.6-4.4)). As the A allele is usually a marker of decreased CRP concentrations, the authors suggested that an impaired defence mechanism in the form of reduced CRP response might result in prolonged inflammation and increased tissue damage in lung cancer patients (Siemes et al. 2006).

Another 3'UTR polymorphism, +2911C>G was associated with lung cancer in the same study. The CG heterozygotes had 60% reduced risk of lung cancer compared to CC homozygotes (OR 0.38 (CI 0.15-0.93)) (Siemes et al. 2006).

The SNP +5237A>G has generally been used as a part of the CRP haplotype in association studies (Carlson et al. 2005, Crawford et al. 2006a, Lange et al. 2006), but in the study by Lange and colleagues the individual effect of this SNP on CRP concentration is also shown. It suggests that allele G may be associated with increased CRP, but only in black people (Lange et al. 2006).

4.3. Haplotype association studies

The association studies using *CRP* haplotypes are shown in Table 6. Only studies with more than 500 participants are included in the list. The study by Kathiresan and colleagues is not included in the table due to the large genomic region size studied (26 kbp), which extends far beyond the CRP gene (2006). The studies

included investigated *CRP* haplotype association with CRP concentration, disease phenotype or both. As different studies have not used the same SNPs in haplotype inference, the results are hard to compare. However, it seems that allele C of -286, allele C of +1444 and allele A of +1846 either with or without allele C of +1059 are often present in haplotypes associated with decreased CRP values. The other alleles of these SNPs are present, naturally, in the haplotypes associated with increased CRP values (Russell et al. 2004, Carlson et al. 2005, Miller et al. 2005, Timpson et al. 2005, Crawford et al. 2006a, Ladenvall et al. 2006, Suk Danik et al. 2006). It appears that the association studies with disease phenotypes are still too sparse in number to actually permit comparison.

Table 1. *Haplotype association studies of CRP gene.*

Study population	N	Haplotype forming SNPs	Haplotypes	Allele freq. (%)	CRP conc.	IS	MI	CHD	CVD mort.	SLE	DM	OR/RR/*p*-value (95% CI)	Refs.
The Rotterdam Study	6007 Dutch ≥55y	1444/1846/2911	H1 C A C	32.8	R	-	R	R	-	-	-	NS	Kardys et al. 2006
			H2 T G C	31.7	HIGH	-	N	N	-	-	-	NS	
			H3 C G C	29.5	HIGH	-	N	N	-	-	-	NS	
			H4 C G G	5.9	HIGH	-	N	N	-	-	-	NS	
The Cardio-vascular Health Study	3941 EA whites	IVS1/1059/1846/5237	E4 A G G A	30.2	HIGH**	R	R	-	R	-	-	1.00	Lange et al. 2006
			E1 T C A A	6.8	LOW	N	N	-	Y	-	-	0.65 (0.50-0.85)	
			E2 T G A A	26.9	LOW	Y	N	-	Y	-	-	IS 0.80 (0.68-0.94) CDV 0.81(0.70-0.95)	
			E3 T G G G	27.7	NS	N	N	-	Y	-	-	0.85 (0.73-0.99)	
			E5 T G G A	8.1	HIGH	Y	N	-	N	-	-	0.74 (0.58-0.96)	
PROVE IT-TIMI 22 Study	1847 EAs with ACS	-757/-717/-286/IVS1/ 1059/1444/1846	H1 T A T T G T G	31.7	HIGH	-	-	-	-	-	-	-	Suk Danik et al. 2006
			H5 C A A A G C G[%]	5.4	HIGH	-	-	-	-	-	-	-	
			H4 T A C A C C A	6.0	LOW	-	-	-	-	-	-	-	
			H3 TA C A G C A	26.6	LOW	-	-	-	-	-	-	-	
WHS+PRINCE+PHS cohorts	550+1071+ 446 CA	-757/-717/-286/IVS1/ 1059/1444/1846	H1 T G C A G C G	26.9	R*	N	Y[#]	-	-	-	-	0.64 (0.52-0.90)	Miller et al. 2005
			H3 T A C A G C A	26.0	LOW	N	N	-	-	-	-	NS	
			H4 T A C A C C A	6.8	LOW	N	N	-	-	-	-	NS	
			H5 C A A A G C G	5.9	HIGH	N	N	-	-	-	-	NS	
			H2 T A T T G T G	26.5	HIGH	N	N	-	-	-	-	NS	

The	1557 AAs	-790/-286/IVS1/	H2 A C A G C A A	28/18[¤]	*	-	-	-	-	-	-	-	Carlson et
Coronary	1820 EAs	1059/3006/1846/5237	H1 A C A C C A A	6/1	LOW	-	-	-	-	-	-	-	al. 2005
Artery Risk			H5 A T T G C G A	29/12	HIGH	-	-	-	-	-	-	-	
Development			H6 T T A G C G A	0/17	HIGH	-	-	-	-	-	-	-	
in Young			H7 A A A G C G A	6/3	HIGH	-	-	-	-	-	-	-	
Adults			H8 A A A G A G A	0/22	HIGH	-	-	-	-	-	-	-	
Collection of	536	-286/GT[n]/	H1 T 16 G T G	28	HIGH**	-	-	-	-	N	-	NS	Russell et
UK SLE		1059/1444/1846	H2 C 16 G C A	24	LOW	-	-	-	-	Y	-	p=0.017 (TDT)	al. 2004
families			H3 C 21 G C C	23	MID[&]	-	-	-	-	N	-	NS	
			H4 A 20 G C G	6	MID[&]	-	-	-	-	N	-	NS	
			H5 T 16 C C A	5	LOW	-	-	-	-	N	-	NS	
The	5972	1444/1846/2911	H1 C A C	32.8	-	-	-	-	-	-	R	1.00	van Oijen
Rotterdam	(607 with		H2 T G C	31.4	-	-	-	-	-	-	N	NS	et al. 2006
Study	dementia)		H3 C G C	29.9	-	-	-	-	-	-	Y	1.21 (1.05-1.41) †	
			H4 C G G	5.9	-	-	-	-	-	-	N	NS	
Sahlgrenska	600 cases	-717/-286/1059/1444	H1 T C G C	27.5	R	-	-	-	-	-	R	NS	Ladenvall
Academy	and 600		H2 T T G T	31.1	HIGH	-	-	-	-	-	N	NS	et al. 2006
Study on	controls,		H3 C C G C	29.3	NS	-	-	-	-	-	N	NS	
Ischemic	Swedish		H4 T C C C	6.5	LOW	-	-	-	-	-	N	NS	
Stroke			H5 T A G C	5.5	NS	-	-	-	-	-	N	NS	
NHANES III	2630	-790/-286/IVS1/	H1 A C A C C A A	<5/<5[+]	NS	-	-	-	-	-	-	-	Crawford
American	whites,	1059/3006/1846/5237	H2 A C A G C A A	31/20	R	-	-	-	-	-	-	-	et al. 2006a
population	2108		H3 A C A G C G A	<5/8	NS	-	-	-	-	-	-	-	
based sample	blacks and		H4 A C A G C G G	28/15	NS	-	-	-	-	-	-	-	

	2073 MA		H5 A T T G C G A	30/12	HIGH∈	-	-	-	-	-	-	-	
			H6 T T A G C G A	<5 /17	HIGH∈	-	-	-	-	-	-	-	
			H7 A A A G C G A	6/<5	HIGHf	-	-	-	-	-	-	-	
			H8 A A A G A G A	<5/23	HIGH∈	-	-	-	-	-	-	-	
British	3218	1059/1444/1846	G C Gp,b	37	HIGH	-	-	-	-	-	-	-	Timpson et
Women's	women		G C A	26	HIGH	-	-	-	-	-	-	-	al. 2005
Heart and			G T G	30	HIGH	-	-	-	-	-	-	-	
Health Study			C C A	7	LOW	-	-	-	-	-	-	-	
NHLBI	1296 CA	-7180/-757/-717/-	CTAAAC/TTAGGC	27.9 ‡	MID	-	-	-	-	-	-	-	Wang et al.
Family Heart		286/1444/7598	CTGGGT/TTAGGC	14.6	LOW	-	-	-	-	-	-	-	2006
Study cohort			TTAGGC/TTAGGC	13.4	LOW	-	-	-	-	-	-	-	
			CTAAAC/CTGGGT	12.1	MID	-	-	-	-	-	-	-	

ABBREVIATIONS: PROVE IT –TIMI 22, Pravastatin or Atorvastatin and Infection TIMI 22 clinical trial; WHS, Women's Health Study; PRINCE, Pravastatin Inflammation/CRP Evaluation; PHS, Physician's Health Study; NHLBI, National Heart, Lung, and Blood Institute; SLE, systemic lupus erythematosus; NHANES III, Third National Health and Nutrition Examination Survey; ACS, acute coronary syndrome, EA, European American; CA, Caucasians; AA, African American; MA, Mexican American; IVS1, intronic T>A polymorphism; R, reference; N, no; Y, yes; NS, non- significant; IS, ischemic stroke; MI, myocardial infarction; CHD, coronary heart disease; CVD mort., coronary vascular disease mortality; DM, dementia; OR, odds ratio; RR, risk ratio; TDT, transmission disequilibrium test.

* No association with CRP levels

** was used as a reference level in analysis

Association tested only in PHS cohort (n=346 MI pairs)

¤ Frequencies in EAs and AAs, respectively

% Haplotype which remained significantly associated with CRP adjusted analysis

& CRP level not significantly different from reference haplotype

†Age and sex adjusted odds ratio (Cox' proportional hazards model)

‡ CRP concentration reported only for diplotypes

+ Frequencies in non-hispanic whites and blacks respectively, frequencies in Mexican Americans not shown

∈ High producer in non-hispanic black people only

f High producer in non-hispanic white people only

p All alleles reported as opposite DNA strand alleles in the article

b +1059 alleles coded incorrectly in the article, corrected here

Aims of the study

The present study was undertaken in order to:

1. Study whether polymorphisms in CRP gene alone or in combination with IL1B gene polymorphism are associated with plasma CRP concentrations

2. Investigate whether genotypes of pro-inflammatory and anti-inflammatory genes *IL1A, IL1B, IL1RA* and *IL6* are associated with circulating plasma CRP concentrations

3. Elucidate the relationship between *IL1B, IL6* or *CRP* genotypes and CRP or IL-6 plasma concentration, fat mass or BMI in obese men before and after weight loss

4. Analyse whether *CRP* genotypes are associated with acute phase or recovery phase CRP concentrations of bacteraemia patients and if these polymorphisms are associated with bacteraemia mortality

5. Examine whether CRP genotypes are associated with carotid artery compliance as measured by ultrasonography

Subjects and methods

1. Subjects

1.1. Studies I and II

Blood samples (buffy coats) were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere, Finland. The donors were healthy middle-aged adults (19-64) and they had not had any sign of infection during a 2-week period before donating blood. All the donors were of the same ethic origin, Finnish Caucasians. The 336 subjects investigated in Study II were also included in Study I (n=338).

1.2. Study III

The subjects were participants in a trial on weight reduction and maintenance which consisted of three phases and lasted for 31 months. In this study, however, only the first phase, the weight reduction phase (WR), was analysed. WR lasted for two months and the participants followed a very low energy diet (2 MJ/day) for 8 weeks. Before the WR, the participants were on a low-energy diet (5 MJ/day) for one week. The inclusion criteria for the men were age 35-50 years, body mass index 30-40 kg/m^2 and waist circumference over 100 cm. All subjects fulfilled the following criteria in screening examinations: they were non-smokers, did not use any regular medication, they were not physically active (leisure time exercise<twice weekly), resting blood pressure was ≤160/105 mmHg, fasting serum cholesterol was ≤ 8 mmol/l, triglycerides were ≤ 4 mmol/l and blood glucose was ≤ 6.7 mmol/l. The participants were assessed before and after WR. The blood samples were drawn between 8.00 a.m. and 9.00 a.m. after 12-hour fast. Ninety obese, but otherwise clinically healthy men participated in the study and completed the WR. At the time of this study, DNA was available from 72 men.

1.3. Study IV

Blood samples and a verified positive blood culture were obtained from 149 Caucasian patients with symptoms and signs of systemic infection during the study period from June 1999 to February 2004. Patients with bacteraemia caused

by *Streptococcus pneumoniae*, *Staphylococcus aureus*, β-haemolytic streptococci or *Eschericia coli*, the four most common causative organisms of community acquired bacteraemia, were included in the study. The genotyping was successful for 147 patients, as DNA could not be extracted from one blood sample (risk of infection) and another patient's DNA failed in genotyping.

Symptoms and signs of systemic infection were fever or hypothermia, tachycardia or tachypnoea combined with leukocytosis or leukopenia and/or elevated CRP. Each patient was interviewed and examined by clinicians. Symptoms of infection before treatment had persisted 0 to 14 days (median 2 days). All patients were treated with empirical intravenous antibiotics, which were started immediately after the blood cultures were taken and the antibiotic regimen was modified according to the blood culture results. The empirical antibiotics were effective in all patients according to resistance testing.

1.4. Study V

The subjects in the study comprised participants of the ongoing Cardiovascular Risk in Young Finns Study, a five-centre follow-up study involving five university hospital cities in Finland. The study was initiated in 1980, when 3596 participants aged 3, 6, 9, 12, 15 and 18 were randomly selected for the study. The most recent follow-up was conducted in 2001, when the participants (n=2283) were 24-39 years of age. Cardiovascular risk factors, including serum lipids, BMI, blood pressure values, CRP, alcohol consumption, diabetes and smoking habits were recorded in 2001. In addition, carotid artery compliance and intima-media thickness were measured by ultrasonography in 2001.

2. Methods

2.1. CRP measurements

In Studies I, II and III, the plasma CRP concentrations were analysed by particle enhanced immunonephelometric method using the Dade Behring N High Sensitivity CRP on the Dade Behring Nephelometer II (Dade Behring, Marburg, Germany). The lower detection limit for CRP was 0.16 mg/l. In Study IV, plasma CRP concentrations were analysed by a particle enhanced immunoturbidimetric method using the Cobas Integra 700 automatic analyser (Hoffmann La Roche Ltd., Basel, Switzerland) with the COBAS® Integra C-Reactive Protein (Latex) reagent. The lower detection limit was 0.10 mg/l. The CRP was measured at several time points: on blood culture day and on at least three days thereafter. In addition, recovery phase CRP was measured 2-3 months

after the positive blood culture day. Top CRP was determined as the highest CRP measured from a patient in any detection day. In Study V, CRP concentrations were analysed by high-sensitive latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The lower detection limit was 0.06 mg/l.

2.2. Measurement of cytokine plasma concentrations

In Study III, the plasma IL-6 concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA; CLB, Pelikine Compact Human IL-6 ELISA kit, Amsterdam, The Netherlands). The lower detection limit of the assay was 0.60 pg/ml.

2.3. Measurement of carotid artery compliance by ultrasound

In Study V, the ultrasound studies were measured by Sequoia 512 ultrasound mainframes (Acuson, CA, USA) with 13.0 MHz linear array transducer. To assess the carotid artery elasticity indices, the best quality cardiac cycle was selected from the 5-second clip images. The common carotid diameter 10 mm from carotid bifurcation was measured from the B-mode images using ultrasonic calipers at least twice in end-diastole and end-systole respectively. The mean of the measurements was used as the end-diastolic and the end-systolic diameter (D). Ultrasound and concomitant brachial blood pressure measurements were used to calculate the carotid artery compliance= $([D_{systolic} - D_{diastolic}]/D_{diastolic})/$ (systolic blood pressure - diastolic blood pressure).

2.4. Calculation of physical activity index

In Study V, the physical activity index was constructed by combining the information on the frequency, intensity and duration of physical activity, including leisure time physical activity and commuting to the workplace. Participants were asked about the frequency of their participation in physical activity and its intensity outside school and working hours. Participants were offered multiple-choice answers. When estimating physical activity during the journey to work, we considered the length of the journey and whether it was made on foot or by bicycle. The coefficients for the variables were estimated from existing tables (Ainsworth et al. 1993).

2.5. Analysis of IL1A, IL1B and IL6 genotypes and IL1RA VNTR

Genomic DNA was extracted from buffy coats (Studies I and II) or whole blood (Studies III, IV and V) using either the QIAamp DNA blood Mini Kit (QIAGEN Inc., USA) (Studies I, II, III and IV) or DNA extraction robot (Biorobot M48, Qiagen GmbH, Hilden, Germany) with MagAttract DNA blood Mini M48 Kit (Qiagen GmbH, Hilden, Germany) (Study V).

Amplification of the *IL1A* gene promoter region polymorphic site at position -889 was done by PCR using primers and restriction enzyme *NcoI* as described earlier (McDowell et al. 1995). The PCR reaction was performed in a 50 µl reaction containing 40 pmol of each primer, 0.1 mM dNTPmix, 1x PCR buffer for *Taq* DNA polymerase (Fermentas, International Inc., Burlington, Canada), 2.5 Units of *Taq* DNA polymerase (Fermentas International Inc., Burlington, Canada) and 200 ng of template DNA. The PCR conditions were as follows: 96 °C for 1 min, the 39 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1min, and finally 72 °C for 4 min and 55 °C for 5 min. The *NcoI* digestion of the PCR-products was done in 50 µl reaction in +37 °C for 3 hours and contained 30 µl of the PCR product, 1x NEBuffer 4, 0.5 µl of BSA buffer, and 6U of *NcoI* enzyme (New England BioLabs inc., Boston, USA). Digested fragments were separated by electophoresis in 4% agarose gel and visualized with ethidium bromide staining under UV-light.

IL1B promoter region polymorphism at position -511 was amplified by PCR in 50 µl reaction containing 100 ng of template DNA, 20 pmol of each primer, 0.1 mM dNTPmix (Pharmacia Biotech), 1 mM $MgCl_2$, 1x PCR buffer for DyNAzyme (Finnzymes, Espoo, Finland) and 1U of DyNAzyme polymerase (Finnzymes, Espoo, Finland) using the primers earlier described (di Giovine et al. 1992). The PCR conditions used were as follows: 95 °C for 2 min, then 36 cycles of 95 °C for 1 min, 55 °C for 1 min and 74 °C for 1 min, and finally 74 °C for 4 min. After amplification the PCR products were digested for 3 hours at +37 °C with *AvaI* restriction enzyme (New England Biolabs inc., Boston, USA) in 50 µl reaction containing 25 µl of the PCR product, 6U of *AvaI* and 1x NEbuffer 4 (New England Biolabs inc., Boston, USA). The fragments were separated by electrophoresis in 2% agarose gel and visualized with ethidium bromide staining under UV light.

Amplification of the other *IL1B* polymorphism at position +3954 was amplified using PCR primers 5' GTTGTCATCAGACTTTGACC 3' and 5' TTCAGTTCATATGGACCAGA 3'. The PCR reaction was done in 50 µl reaction containing 40 pmol of each primer, 0.1 mM dNTPmix, 1x buffer for DyNAzyme (Finnzymes, Espoo, Finland), 1 mM $MgCl_2$, 100 ng of template DNA and 1U of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland). The PCR conditions were as follows: First 3 cycles at 97 °C for 2 min, 55 °C for 2 min and 74 °C for 1 min, then 2 cycles at 97 °C for 2 min, 55 °C for 2 min, and 74 °C for 2 min. After this 33 cycles at 97 °C for 1 min, 55 °C for 1 min and 74 °C for 1 min, and finally 73 °C for 10 min. The PCR products were digested for 3 hours in +65 °C in a 50 µl reaction containing 40 µl of PCR product, 1x Taq buffer, 1 mM BSA and 5U of *Taq*I restriction enzyme (Fermentas International

Inc., Burlington, Canada). The fragments were separated by electrophoresis in 2% agarose gel and visualized with ethidium bromide staining under UV light (Pociot et al. 1992).

The *IL6* promoter region polymorphism -174 was amplified either by PCR and fragment restriction analysis (Study I) or by PCR with TaqMan chemistry (Study III). The fragment restriction PCR was done using primers 5' TGACTTCAGCTTTACTCTTGT 3' and 5' CTGATTGGAAACCTTATTAAG 3'. The PCR reaction was done in 50 µl reaction containing 20 pmol of each primer, 0.2 mM dNTPmix, 0.1 mM $MgCl_2$, 1x buffer for DyNAzyme, 0.5U of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland) and 200 ng of template DNA. The PCR conditions were as follows: first 6 cycles at 94 °C for 9 min, 52 °C for 1 min and 72 °C for 3 min, then 31 cycles at 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min and finally 72 °C for 10 min. The PCR products were digested overnight at +37 °C in a 50 µl reaction containing 45 µl of the PCR product and 5 U of *NlaIII* restriction enzyme (New England BioLabs inc., Boston, USA). The fragments were visualized on 3% agarose gel in UV-light after ethidium bromide staining. Genotyping of IL6 -174 by TaqMan® chemistry was performed using the ABI (Applied Biosystems, CA, USA) PRISM 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA, USA) with designed unlabelled primers and TaqMan® MGB probes. The universal PCR conditions were: first 50 °C for 2 min and 95 °C for 10 min, and then 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The PCR reaction was done in 25 µl reaction with 1x TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI, CA, USA), 1x Assay Mix (primers and probes) and 10-100 ng of template DNA.

The *IL1RA* VNTR in intron two was amplified using primers 5'CTCAGCAACACTCCTAT3' and 5' TCCTGGTCTGCAGGT 3'. The PRC reaction was done in 50 µl reaction containing 40 pmol of each primer, 0.1 mM dNTPmix, 1x PCR buffer for DyNAzyme, 1 mM $MgCl_2$, 1U of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland) and 100 ng of template DNA. The PCR reaction conditions were as follows: 96 °C for 1 min 30 sec, then 35 cycles at 94 °C for 1 min, 60 °C for 1 min and 70 °C for 1 min and finally 72 °C for 5 min. Twenty µl of the PCR products were loaded on a 2% agarose gel and stained with ethidium bromide (Tarlow et al. 1993). Five different alleles were seen under UV light: 240 bp (allele 2), 325 bp (allele 4), 410 bp (allele 1), 500 bp (allele 3) and 595 bp (allele 5), representative of 2, 3, 4, 5, and 6 copies of the 86-bp sequence.

2.6. Analysis of CRP -717, -286, +1059, +1444 and +1846 genotypes

The *CRP* gene polymorphisms -717, +1444 and +1846 were genotyped with TaqMan® chemistry using the ABI PRISM 7000 or ABI PRISM 7900HT

Sequence Detection System for both PCR and allelic discrimination (ABI, CA, USA). Designed unlabelled PCR primers and fluorogenic TaqMan® MGB probes were used (Assay by Design, ABI, CA, USA). The universal PCR thermal cycling conditions from ABI were followed: first 50 °C for 2 min and 95 °C for 10 min, and then 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The PCR reaction was done in 5 µl (386 well plate) or 25 µl (96 well plate) reaction (for 7900HT or 7000 Sequence Detection Systems respectively) containing 1x TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI, CA, USA), 1x Assay Mix (primers and probes, ABI, CA, USA) and 10-100 ng of template DNA. The genotypes were called automatically by the SDS software (ABI PRIMS 7900HT) or selected manually from the allelic discrimination tab (ABI PRISM 7000).

The *CRP* gene polymorphism +1059 was genotyped by either ABI PRISM 7000 or 7900HT Sequence Detection System using a commercial kit from ABI (Assay on Demand, C_177490-10 CRP). Universal PCR thermal conditions were followed in either 5 µl or 25 µl reaction volume depending on the Sequence Detection System used. PCR reaction contained 1x TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI), 1x Assay Mix (ABI) and 10-100 ng of template DNA. The genotypes were called automatically by the SDS software or selected manually from the allelic discrimination tab depending on the Sequence Detection System used.

The tri-allelic *CRP* gene promoter region polymorphism -286 was genotyped with designed primers and probes as described previously using the ABI PRISM 7900HT Sequence Detection System (Carlson et al. 2005), except for the genotype calling, which was done manually from the PCR run component tab.

2.7. Statistical analyses

For skewed continuous variables non-parametric statistics were used (Kruskall-Wallis test, Mann-Whitney U-test). For normally distributed continuous variables (or log transformed skewed variables) one-way ANOVA or ANCOVA were used. For detecting differences in mean values, T-test for dependent samples was used in Study III. Spearman's rank correlation coefficients were used to quantify the relation between continuous variables in Study III. In Study I, the Arlequin program was used for both haplotype frequency calculation and Hardy-Weinberg equation testing (ver. 2.0., A software for population genetics data analysis. Schneider S, Roessli D, Excoffer L. Genetics and Biometry Laboratory, Geneva, Switzerland). Chi-square statistics were used to analyse the differences in haplotype frequencies. In Studies I and II, odds ratios and their 95% intervals were calculated using the CIA software (ver. 1.1., copyright by M. J. Gardner and *The British Medical Journal*, 1989). In Study IV, polymorphisms were tested for their association with mortality using Fischer's exact test, and Kaplan-Meier survival analysis was carried out to estimate the probability of survival. In Study V, multiple linear regression was used to study the association between study variables and PHASE v2.0.2 program was used for reconstructing

the haplotypes (Stephens et al. 2001, Stephens and Scheet 2005). Statistica software (ver. Win.5.1.D or ver. Win.6., StatSoft Inc., Tulsa, OK, USA) and SPSS (ver. 11.5, ver. 13.0 and ver. 14.0, SPSS inc., Chicago, IL, USA) were used for the statistical analyses. A two-tailed p-value <0.05 was considered statistically significant.

2.8. Ethics

In Studies I and II, the ethical committee of the Finnish Red Cross Blood Transfusion Centre approved the use of human blood. In Study III the ethical committee of Pirkanmaa Hospital District approved the study and written informed consent was obtained from the participants. In Study IV the Ethics Committee of Tampere University Hospital approved the study and written informed consent was obtained from the patients or a first degree relative. In Study V, the Cardiovascular Risk in Young Finns -Study plan was approved by the local ethics committees and all subjects gave written informed consent.

Results

1. The effect of *IL1A*, *IL1B*, *IL1RA* and *IL6* gene polymorphisms on CRP concentration (Study I)

Results from family studies implied strong heritability in basal CRP concentrations (Vickers et al. 2002). As IL1 complex molecules and IL-6 in plasma were known to affect liver CRP production (Weinhold et al. 1997), and *IL1B*+3954 gene polymorphism was already shown to be associated with CRP concentration in cardiac patients (Berger et al. 2002), and furthermore, *IL6*-174 polymorphism was shown to be associated with CRP concentration in hypertensive patients (Vickers et al. 2002), we hypothesised that the genetic polymorphisms of these genes might have an effect on the plasma baseline CRP concentration of healthy people. Therefore we examined the plasma CRP concentration and determined genotypes of healthy middle-aged blood donors and conducted an association study between CRP concentration and genotypes of these individuals.

The median CRP concentration was 0.72 mg/l, ranging from 0.16 to 30 mg/l, only one CRP value being above 10 mg/l. The genotype frequencies were in accordance with the Hardy-Weinberg equation. When the CRP concentrations of different genotypes were compared, *IL1B*+3954 genotypes showed a significant difference in CRP concentrations (CC 0.87 mg/l (n=189), CT 0.68 mg/l (n=122) and TT 0.52 mg/l (n=26), p=0.038, Kruskall-Wallis analysis of variance), but the genotypes of other polymorphisms did not differ significantly in CRP concentrations. However, a trend was observed in CRP concentrations of *IL1B*-511 genotypes (CC 0.56 mg/l (n=120), CT 0.82 mg/l (n=168) and TT 0.77 mg/l (n=50), p=0.101). The *IL1B*+3954 was further analysed by allele carrier status, which showed that the carriers of allele T had significantly lower CRP concentrations than non-carriers (0.57 and 0.87 mg/l, p=0.027, Mann-Whitney U-test).

IL1 complex gene polymorphisms were further analysed by haplotype analysis, so that the subjects were divided into two groups by median CRP value; ≥ 0.72 mg/l and <0.72 mg/l. A significant difference in haplotype frequencies between these groups was observed in *IL1B* bilocus haplotype analysis of -511 and +3954 (p=0.0088), but no differences were found in three or four gene haplotype analysis, probably because the groups became too small to reach statistical difference. The bilocus haplotype of -511/+3954 formed four haplotypes (h); h1 T-C, h2 C-C, h3 C-T and h4 T-T, with frequencies of 0.405, 0.385, 0.185 and 0.003 in above median group and with frequencies of 0.357,

0.342, 0.292 and 0.009 in below median group respectively. There was a significant difference in h3 frequency between the groups (p=0.049), the haplotype being more common in the below CRP median group. The composite genotype analysis confirmed this result; genotype -511 C-C/ +3954 T-T was almost 5 times less frequently observed in the above median group [OR 0.206 (95% CI 0.07-0.62)]; in other words, it was over 4 times more common in the below median CRP group [OR 4.86 (95% CI 1.61-14.7)].

1.2. The epistatic effect of CRP +1059 and IL1B +3954 polymorphisms on CRP concentration (Study II)

As *CRP* gene polymorphism +1059 was already shown to be associated with CRP concentration in a study by Zee and colleagues (2002), we wanted to investigate the epistatic effect of both *IL1B*+3954 and *CRP*+1059 on baseline CRP concentrations. To achieve this, we genotyped healthy blood donors for *CRP*+1059 polymorphism and computed association analyses of these two polymorphisms on CRP concentrations.

The median CRP concentration was 0.71 mg/l, ranging from <0.16 to 8.52 mg/l. In men (n=186), the median CRP was 0.64 mg/l, and in women 0.89 mg/l (n=150). The genotype frequencies followed the Hardy-Weinberg equation. The genotypes of *CRP*+1059 and *IL1B*+3954 polymorphisms were significantly associated with CRP concentration, but only in men: carriers of +1059 C-allele had significantly lower CRP concentrations than non-carriers (0.43 mg/l (n=21) and 0.66 mg/l (n=165) respectively, p=0.009, Mann-Whitney U-test), and carriers of +3954 T-allele had significantly lower CRP concentrations than non-carriers (0.51 mg/l (n=79) and 0.76 mg/l (n=107) respectively, p=0.032, Mann-Whitney U-test). In women we did not detect any difference between the genotypes of either SNP.

When allele C non-carriers of *CRP*+1059 (CRP2-) were further divided by *IL1B*+3954 allele T-carriage status, we found that IL1B T-allele carriers (IL1BT+) had significantly lower CRP concentrations than allele T-non carriers of *IL1B*+3954 (IL1BT-) (0.50 mg/l (n=84) vs. 0.93 mg/l (n=93) respectively, p=0.013). When the same stratification by IL1B allele T-carriage status was done for allele C carriers of *CRP*+1059 (CRPC+), there were no differences between the groups (IL1BT+ 0.51 mg/l (n=7) vs. IL1BT- 0.38 mg/l (n=13), p=0.360).

Accordingly, when the data was stratified by *IL1B* allele T carriage status and *CRP*+1059 C-allele carriage status, the IL1BT-/CRPC+ had significantly lower CRP concentrations than IL1BT-/CRPC- (0.35 mg/l (n=13) vs. 0.93 mg/l (n=93), p=0.004 respectively). However, no difference was found between IL1BT+/CRPC- and IL1BT+/CRPC+ (0.49 (n=72) vs. 0.62 mg/l (n=7), p=0.557 respectively).

To compare and evaluate the effect of composite genotypes on CRP concentrations in men, we divided the subjects according to CRP tertiles (1st tertile <40 mg/l, 3rd tertile >1.01 and <8.52 mg/l). The analysis showed that the carrier genotype combination CRPC-/IL1BT- was almost three times more common in the CRP 3rd tertile group than in the 1st tertile group (OR 2.84 [95% CI 1.33-6.07]).

2. The effect of *IL1B*, *IL6* and *CRP* gene polymorphisms on CRP concentration, plasma IL-6 concentration and fat mass in weight reducing men (Study III)

The results of several studies have suggested that obese women have increased CRP concentrations, which tend to decrease with weight loss (Heilbronn et al. 2001, Tchernof et al. 2002, Esposito et al. 2003). Therefore we wanted to investigate the effect of *IL1B, IL6* and *CRP* gene polymorphisms on CRP concentration in obese, weight reducing men.

Subjects with CRP concentrations ≥ 10 mg/l, indicating clinically relevant inflammatory conditions were excluded from the analysis. The genotype frequencies did not deviate from the Hardy-Weinberg equation. At baseline, median CRP concentration was 1.72 mg/l. A significant reduction in CRP concentration was observed after weight loss in the participants, being 1.22 mg/l at two months ($p<0.02$, t-test). Similar significant reductions in weight, BMI, fat mass, fat-free mass and waist circumference were detected (Table 2), as well as in glucose and insulin (data not shown). IL-6 concentrations, however, did not change. At baseline, CRP concentration correlated significantly with BMI and insulin concentration ($r=0.46$ and 0.43 respectively, $p<0.001$ for both), but not with fat mass or IL-6 concentration ($r=0.18$, $p=0.12$ and $r=0.23$, $p=0.06$ respectively). After weight reduction, CRP correlated significantly with BMI, insulin and fat mass ($r=0.34$, $r=0.35$ and $r=0.31$ respectively, $p<0.01$ for all), but not with IL-6 concentration ($r=0.20$, $p=0.08$).

Table 3 shows participants' fat mass, IL-6 and CRP concentrations according to *IL6*-174G>C genotypes at baseline and after weight loss. A significant difference in CRP concentrations between the genotypes of *IL6*-174G>C was found after weight reduction. Post-hoc analysis showed a significant difference between CC and GG homozygotes (1.93 vs. 1.01 respectively, $p=0.007$), which remained significant after adjusting for fat mass ($p=0.006$). After Bonferroni correction the result remained significant ($p=0.002$). It should be noted that in the abstract of the original article the alleles are incorrectly marked, GG should be CC in the ANOVA post-hoc test. At baseline no difference was observed among the genotypes. However, the change in CRP concentration from baseline to 2 months was significantly different among *IL6*-174G>C genotypes, being largest in GG and GC genotypes. No difference between the *IL6*-174G>C genotypes was observed in fat mass or IL-6 concentration.

CRP gene polymorphism +1059G>C was significantly associated with CRP concentration at baseline (GG 1.81 vs. GC 1.02 mg/l, p=0.01), but no association was found after weight loss. No other statistically significant associations were found between CRP+1059G>C polymorphism and fat mass, IL-6 or CRP concentrations (data not shown). Nor were significant associations found between *IL1B*+3954C>T polymorphism and CRP, fat mass or IL-6 concentration at any time point.

Table 2. *Participant characteristics at baseline (0 month) and after weight reduction (2 months).*

Variable	0 month (n=77-78)	2 months (n=78)	*P* (t-test)*
Weight (kg)	105.8 (103.7-108)	91.5 (89.4-93.6)	p<0.001
BMI (kg/m^2)	32.9 (32.3-33.4)	28.4 (27.8-29.0)	p<0.001
Fat mass (kg)	37.4 (35.7-39.0)	27.3 (25.6-29.1)	p<0.001
Fat-free mass (kg)	68.5 (67.2-69.8)	64.1 (62.9-65.4)	p<0.001
Waist (cm)	113 (111.0-114.0)	98.0 (96.0-100.0)	p<0.001
CRP (mg/l)	1.72 (1.02-2.94)	1.22 (0.58-2.48)	p<0.02
IL-6 (pg/ml)	2.66 (2.25-3.07)	2.65 (2.19-2.62)	p= n.s.

Values are expressed as means (95% CI) except for CRP, which is expressed as median (25-75%). * T-test for dependent samples.

Table 3. *Fat mass, IL-6 and CRP concentrations by IL6-174G>C genotype at baseline and after weight reduction and the effect of the genotypes on their changes.*

IL-6 genotypes	0 month	*p*	2 months	*p*	Δ 0-2	*p*
Fat mass (kg)						
GG	34.0	0.05	24.2	0.11	-29.6	0.70
GC	36.8		27.1		-26.9	
CC	39.7		29.5		-26.5	
CRP (mg/l)						
GG	1.34	0.19	1.01	0.03	-9.52	0.01
GC	1.91		1.12		-45.44	
CC	1.82		1.93		-0.24	
IL-6 (pg/ml)						
GG	2.40	0.76	2.61	0.97	+2.98	0.59
GC	2.91		2.79		+3.61	
CC	2.74		2.72		+4.58	

For normally distributed data (fat mass and IL-6) ANOVA statistics are used and mean values are presented, for skewed data (CRP) Kruskall-Wallis test is used and median values are presented. At 0 and 2 months n=72: 13 GG, 34 GC and 25 CC genotypes. Δ 0-2 values are calculated using following formula: (2 months- 0 month)/0 month*100.

3. The effect of *CRP* gene polymorphisms on bacteraemia mortality (Study IV)

C-reactive protein concentration is a surrogate marker of inflammation in various infectious diseases. CRP concentration rapidly increases in bacterial infections, participating in defence against microbes in several ways. As *CRP* polymorphisms were associated with CRP concentration in our earlier investigation and also in other earlier investigations, we wanted to examine if *CRP* polymorphisms were associated with top CRP (patient's highest CRP concentration) or recovery phase CRP (60 to 90 days from positive blood culture) concentrations, or with mortality from bacteraemia. Three *CRP* SNPs -717A>G, +1059G>C and +1444C>T were selected for analysis.

The mean age of the patients was 59 years (range 16-93 years); 78 of them were male and 69 female. The predisposing factors and underlying diseases of the patients are shown in Table 4. The genotype frequencies of the SNP's studied

did not deviate from the Hardy-Weinberg equation (p>0.05). Nineteen (13%) of the 147 patients died within 30 days of the positive blood culture. The non-survivors (n=19) had significantly higher CRP concentrations than the survivors (n=128) on the blood culture day (248 vs. 186 mg/l respectively, p=0.02), but there was no significant difference in their top CRP concentration (310 vs. 268 mg/l respectively, p=0.206). When the CRP concentrations were analysed by *CRP* genotypes, a modest but significant association of +1059 with recovery phase CRP was found in the survivors (GG 3.00 vs. GC+CC 1.40 mg/l, p=0.04). Top or blood culture day CRP concentrations did not differ between the genotypes when survivors and non-survivors were analysed together (data not shown). The other polymorphisms, -717 and +1444, were not associated with CRP concentration at any time point (data not shown). Due to the limited number of patients, the associations between CRP and genotypes were not tested separately according to different causative organisms.

When the genotype distributions of the survivors were compared with those of the non-survivors, a significant difference was found in the -717A/G polymorphism (Table 5). Three out of 19 deceased (16%) had GG genotype, whereas only three out of 128 survivors (2%) had the same genotype (p=0.03). Moreover, when the genotype frequencies of the *Streptococcus pneumoniae* infected survivors were compared against those of the deceased, the result was even more striking; 38% (n=3) of the deceased had GG genotype compared to 6% (n=2) of the survivors (p=0.05). The odds ratio for mortality in *S. pneumoniae* infected patiens with GG genotype was 9.6 (95% CI 1.3-72.5) compared to AA+AG genotype individuals. Kaplan-Meier survival analysis showed that the effect of GG genotype was significant both in all patients (p=0.004) and in *S. pneumoniae* infected patients (p=0.01). In the logistic regression model the effect of *CRP*-717 GG on mortality remained significant after adjusting for all those variables which were significant in univariate analysis (smoking, alcohol, BMI; data not shown). The genotype distribution did not differ in patients with diabetes, haematological/solid malignancies, patients formerly on corticosteroid treatment, in current or ex-smokers, or by alcohol consumption (data not shown). The other two SNP's genotype distributions were not associated with mortality in all patients or in patients analysed by differential aetiology (data not shown).

Table 4. *Predisposing factors and underlying diseases of bacteraemia.*

Predisposing factor or underlying disease	All patients n=147 (%)	*S. aureus* n=40 (%)	*S. pneumoniae* n=42 (%)	ß-haemolytic streptococci n=23 (%)	*E. coli* n=42 (%)	*p*-value*
Current smoker or ex-smoker[1]	65 (49)	17 (47)	26 (65)	11 (58)	11 (28)	0.009
Alcohol abuse	24 (16)	5 (13)	9 (21)	6 (26)	4 (10)	0.235
Diabetes type 1 or 2	34 (23)	10 (25)	5 (12)	4 (17)	15 (36)	0.065
Haematological malignancy	10 (7)	2 (5)	3 (7)	1 (4)	4 (10)	0.820
Solid malignancy	15 (10)	6 (15)	3 (7)	1 (4)	5 (12)	0.489
Male sex	78 (53)	28 (70)	24 (57)	14 (61)	12 (29)	0.001
Earlier corticosteroid treatment[2]	17 (12)	6 (15)	4 (10)	2 (9)	5 (12)	0.844
Healthy[3]	32 (22)	8 (20)	15 (36)	6 (26)	3 (7)	0.015

*Difference between groups of patients with bacteraemia caused by different causative organisms

[1]Data available from 134 patients

[2]Corticosteroids used in a dose of over 5mg per day for one month prior to the episode of bacteraemia

[3]Patient had no chronic illnesses.

Table 5. *Survival stratified by CRP-717 A>G genotype in all patients (panel A) and in Streptococcus pneumoniae infected patients (panel B).*

Genotype	Survivors n (%)	Deceased n (%)	*p**
A.			
AA	95 (74)	11 (58)	**0.03**
AG	30 (24)	5 (26)	
GG	3 (2)	3 (16)	
B.			
AA	23 (68)	3 (38)	**0.05**
AG	9 (27)	2 (25)	
GG	2 (6)	3 (38)	

*p-value is based on Fisher's exact test

4. The effect of *CRP* gene polymorphisms on CRP and early atherosclerosis changes in young Finns (Study V)

At least three studies described an association between cardiovascular disease and *CRP* gene polymorphisms (Chen et al. 2005, Miller et al. 2005, Lange et al. 2006), but little information was available about association of *CRP* gene variation with preclinical markers of vascular changes, e.g. IMT and CAC. We wanted to investigate if CRP gene SNPs -717, -286, +1059, +1444 or +1846 are associated with these markers in a population based study cohort of young Finns.

All SNPs studied followed the Hardy-Weinberg equation and were strongly linked, D' values ranging from 0.98 to 0.99 (Kivimaki et al. 2007). Haplotype analysis of the SNPs revealed five common haplotypes (h) (frequency 5% of greater); h1 ATGTG (35%), h2 ACGCA (30%), h3 GCGCG (21%), h4 ACCCA (6.3%) and h5 AAGCG (6.0%). These haplotypes formed five common haplotype pairs (Hps); Hp1 ACGCA/ATGTG (20%), Hp2 ATGTG/GCGCG (15%), Hp3 ACGCA/GCGCGA (13%), Hp4 ATGTG/ATGTG (13%), and Hp5 ACGCA/ACGCA (9%).

The association between CRP concentration and *CRP* gene SNPs was strong; all five SNPs were significantly associated with CRP concentration in females, and all but -717 in males. Figure 2 shows the distribution of CRP values by different *CRP*-286 genotypes and association between -286C>T>A and CRP is shown in Table 6. In order to study which SNP affected CRP concentration the

most, the subjects were divided into three groups by CRP tertiles, males and females separately and the number of genotypes in the first tertile was compared to those in the third tertile for each polymorphism (Table 7). In men the best odds ratio was found for SNP +1059 [2.11 (1.30-3.41)], and in women best odds ratio was found for -286 [2.31 (1.37-3.91)].

Haplotype analysis showed that CRP concentrations were significantly different according to haplotype pairs, Hp4 having the highest median [interquartile range; IQR] values and Hp5 the lowest (Hp1 0.72 [1.1] mg/l, Hp2 0.58 [1.2] mg/l, Hp3 0.51 [1.0] mg/l, Hp4 0.76[1.4] mg/l and Hp5 0.48 [1.0] mg/l, p=0.001). A linear regression model was constructed to analyse the possible independent association of SNP -286 with CRP. Logarithmically transformed CRP was the dependent variable and other risk factors were independent variables. In females, the variables which remained significantly associated with logCRP were age, body mass index, (log)triglycerides, use of hormonal contraceptives, (log)leptin and -286 C-allele carriage ($p<0.0001$). Similarly, the associations between other SNPs and CRP remained significant after adjustment for risk factors (-717 G-carriers p=0.007, +1059 C-carriers p=0.004, +1444 T-carriers p=0.001 and +1846 A-carriers p=0.002). A corresponding model was constructed for males, and the variables which remained significantly associated with logCRP were HDL cholesterol, body mass index, diastolic blood pressure, daily smoking, (log)leptin, (log)insulin and SNP -286 C-allele carriage (p=0.001). The association between other SNPs and CRP also remained significant, except for +1444, which attenuated to the null after adjusting for the above-mentioned factors (+1059 C-carriers p=0.002, +1444 T-carriers p=0.070 and +1846 A-carriers p= 0.001).

The SNPs -286C>T>A, +1444C>T and +1846G>A were associated with CAC in males, but no association was found in females (see Table 6. for -286 and CAC association). The levels of circulating CRP were not independently associated with CAC, although univariate analysis showed a weak association (males $r=-0.18$, $p<0.001$, females $r=-0.06$, p= n.s.). There was strong interaction between SNP-286 and sex in relation to CAC values (p=0.006). In linear regression, the association between -286 allele C-carriers and CAC in men remained significant after adjustment for age, body mass index, systolic blood pressure, daily smoking and physical activity (p=0.005). However, the other SNPs +1444C>T and +1846G>A were not independently associated with CAC. At the haplotype level, carrying of haplotype 1 was significantly associated with decreased CAC (carriers 1.96 (n=502) vs. non-carriers 2.06 %/10 mmHg (n=361), p=0.029) and carriying of haplotype 2 with higher CAC (carriers 2.06 (n= 446) vs. non-carriers 1.93 %/10 mmHg (n=417), p=0.005).

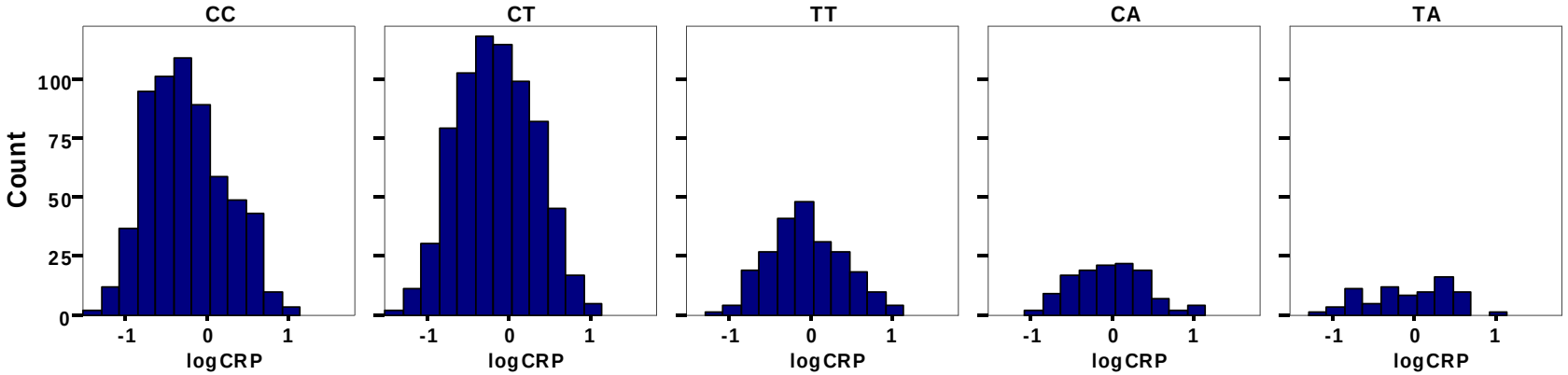

Figure 2. Distribution of CRP values in different CRP-286 genotypes (men and women combined).

Table 6. *Median CRP and mean CAC in females and males by CRP -286 genotypes.*

	FEMALES				MALES			
	CRP*		CAC		CRP*		CAC	
SNP-286	mg/L (N)	*p*	%/10 mmHg (N)	*p*	mg/L (N)	*p*	%/10 mmHg (N)	*p*
CC	0.51 (312)	<.0001	2.29 (410)	.380	0.45 (299)	.001	2.06 (325)	.005
CT	0.75 (367)		2.31 (466)		0.57 (340)		2.01 (391)	
TT	0.88 (127)		2.43 (151)		0.71 (103)		1.88 (114)	
CA	1.07 (63)		2.28 (82)		0.81 (59)		2.13 (68)	
TA	1.24 (39)		2.32 (53)		0.74 (38)		1.75 (43)	

*Subjects with CRP values >10 mg/L, triglycerides above 4 mmol/L, history of recent infection, chronic rheumatic disease, diabetes, lactating women and pregnant women were excluded from the analysis.

Table 7. *Comparison of subjects in the first and third CRP tertile by CRP gene single nucleotide polymorphism allele carriage.*

Sex	*CRP* loci	Alleles	3rd CRP tertile (N)	1st CRP tertile (N)	Odds ratio* (95% CI)
Males	-286	CA+AA	44	27	1.81 (1.09-2.83)
		CC+CT+TT	237	263	
	+1059	GG	270	243	2.11 (1.30-3.41)
		GC+CC	29	55	
	+1444	TT	35	21	1.76 (1.00-3.09)
		CC+CT	262	276	
	+1846	GG	126	98	1.48 (1.06-2.07)
		GA+AA	167	192	
Females	-717	AA	197	173	1.51 (1.09-2.09)
		AG+GG	102	135	
	-286	CA+AA	47	23	2.31 (1.37-3.91)
		CC+CT+TT	250	283	
	+1059	GG	285	270	1.60 (1.01-2.53)
		GC+CC	35	53	
	+1444	TT	49	34	1.53 (0.96-2.45)
		CC+CT	269	286	
	+1846	GG	145	122	1.38 (1.00-1.89)
		GA+AA	171	198	

*Chi-square test

Discussion and conclusions

1. Association between CRP concentration and cytokine gene polymorphisms

1.1. Association between CRP concentration and IL1B SNP +3954

Among healthy Finnish blood donors, the *IL1B* gene SNP +3954C>T was associated with CRP concentration, so that carriers of +3954 allele T had lower CRP values than non-carriers (Study I). Further analysis showed men carrying the high producer allele G of *CRP* gene SNP +1059 had still low CRP values if they simultaneously carried the *IL1B*+3954 allele T (Study II). However, we did not find an association between *IL1B*+3954 and CRP concentration in obese men (Study III), nor in healthy Finns participating in the Cardiovascular Risk in Young Finns Study (n=2282) (unpublished data).

There are two other published reports about an association between *IL1B* polymorphisms and CRP concentration, both of which contradict our results. In 2002 Berger and colleagues studied 454 US individuals undergoing coronary angiography and found higher CRP concentrations in subjects with *IL1B*+3954 allele T (CC 2.02, CT 2.89, TT 4.33, p= 0.001) (2002). Two years later Latkovskis and colleagues found a similar association in Latvian patients with CHD (n=160); carriers of T-allele had higher CRP concentration than non-carriers in univariate analysis ($p<0.01$) and in adjusted analysis (TT+CT 2.77 vs. CC 1.74 mg/l, p=0.002). The CRP concentration was adjusted for smoking status, BMI, triglycerides and diabetes (Latkovskis et al. 2004).

The allele frequencies of *IL1B*+3954 are similar in these three studies, which was expected, as almost all participants were Caucasians. Among healthy Finnish blood donors the frequency of allele C was 0.74, in the study by Berger and colleagues it was 0.76 and in that by Latkovskis and colleagues it was 0.72. There are, however, differences in CRP concentrations between the studies. In our study the median CRP concentration was 0.72 mg/l, but was over 2 mg/l in US participants and 1.7 mg/l in the Latvian study population, probably due to unhealthy and somewhat older individuals. This difference is perhaps not an explanation for the discrepancies in the results, however. We could not replicate our primary result in our other study populations, and there are no other studies in the literature confirming it. It may simply be a by chance finding.

1.2. Association between CRP concentration and IL6 SNP-174

The polymorphic site -174G>C in the regulatory region of the *IL6* gene has been associated with altered rate of expression of the *IL6* gene (Fishman et al. 1998). We found an association of this SNP with CRP concentration in men participating in a weight reduction and maintainance programme where *IL6*-174 was associated with CRP concentration after weight reduction (Study III). The CC homozygotes had the highest CRP concentration after weight loss. These individuals did not reduce their CRP concentration with weight loss, as opposed to those with the other genotypes. However, we could not find an association between *IL6*-174 and CRP concentration in healthy blood donors (Study I). The lack of association in this study could be due to the very low CRP concentrations in these individuals. It could be that there are no 'induced' CRP concentrations in these individuals (median CRP concentration 0.71 mg/l), i.e. IL-6 simply does not induce CRP levels of these individuals. In contrast, in obese people the CRP concentrations are slightly elevated (median 1.72 mg/l), and differences in IL-6 induced production can be therefore found between genotypes.

Previously Vickers and colleagues found an association between CRP concentration and *IL6*-174 genotype in 128 British Caucasians with essential hypertension and in their family members (total n=588) (2002). As in our results, subjects with CC genotype had higher CRP concentration than GG or GC genotypes. In a study by Humphries and associates a similar trend was found in 494 healthy UK males, but did not reach statistical significance (2001). Contradictory results were found in 467 postmenopausal US women, where lower CRP concentrations were associated with the simultaneous presence of *IL6*-174 allele C and *IL6*-572 allele C, but the association of *IL6*-174 alone with CRP did not reach statistical significance (Ferrari et al. 2003). The frequency of the C allele was 0.55 in Finnish healthy blood donors and 0.56 in Finnish obese men, but was lower in the UK and USA populations (0.43 and 0.39 respectively).

The role of *IL6*-174 in CRP induction awaits future research. The effect of this SNP should be investigated in larger populations before any firm conclusions can be drawn; at this moment there is some evidence that this SNP could be important in CRP determination. It may be that individuals with CC genotype are somewhat more resistant to reductions in CRP concentrations when weight is lost, and there seem to be more of these individuals in Finland than in UK or USA.

2. Association between and *CRP* genotypes and CRP concentration

2.1. CRP gene promoter region polymorphisms

An association between *CRP* promoter region polymorphism -717A>G and CRP concentration was found in young Finnish females participating in the Cardiovascular Risk in Young Finns Study (Study V), but not in bacteraemia patients (Study IV). The latter study had fewer participants, which may affect the results. In the former study, allele G was associated with low CRP concentration, and remained significant after adjustment for confounding variables. As can be seen from the review of the literature, there are no earlier reports showing an association of this SNP with CRP concentration. Future studies will show the importance of this finding both in Finnish and other populations.

We found an association of -286 with CRP concentration in both males and females in Study V. The highest CRP values were associated with the rare A-allele, and low values with C-allele. This association persisted after adjustment for confounding variables. From the five SNPs studied in Study V, the allele A of -286 was the strongest risk marker when the numbers of genotypes in highest and lowest tertiles of CRP were compared. The A-allele has also been associated with increased CRP concentration in other studies; in patients with first MI (Kovacs et al. 2005), in patients with acute coronary syndrome (Suk Danik et al. 2006) and in a study by Miller and colleagues, where this allele was associated with increased CRP in three different study cohorts (2005). However, contradictory results were reported by Szalai and associates, who found an association of high CRP concentration with allele T, but this result was based on quite a small number of individuals (n=287)(2005b). Thus it seems that allele A predisposes people to increased CRP values. The frequency of this allele was 0.03 in the Finnish study cohort and ranged between 0.05 and 0.07 in the other studies mentioned.

2.2. CRP gene exonic polymorphism +1059

We found an association of *CRP*+1059G>C allele C with decreased CRP concentration in healthy male blood donors (Study II), in obese men before weight loss (Study III), in bacteraemia patients during the recovery phase (Study IV) and in the Cardiovascular Risk in Young Finns participants (Study V). In study V, the association persisted after adjustment for confounding factors. The homozygosity of allele G was the main risk marker for increased CRP values in females. In light of these results, carrying +1059 allele C seems to protect Finnish people from high hereditary CRP concentrations. The C allele frequency ranged from 0.047 to 0.065 in these cohorts, showing sadly that this allele is not frequent in our population. There is an abundance of reports showing a similar association of this allele with low CRP concentration in different study cohorts

(Zee and Ridker 2002, Russell et al. 2004, Davey Smith et al. 2005, Miller et al. 2005, Suk et al. 2005, Lange et al. 2006, Thalmaier et al. 2006). Thus it can be categorically stated that this SNP is associated with CRP concentration, although it is a silent mutation and the mechanism through which it occurs is not known.

2.3. CRP gene 3'UTR polymorphisms

An association of *CRP*+1444C>T allele T with increased CRP concentration was found in the Cardiovascular Risk in Young Finns Study participants (Study V) but not in the bacteraemia patients for either top or recovery phase CRP concentrations (Study IV). However, there was a trend for higher CRP in T-allele carriers in bacteraemia patients both in top and recovery phase CRP concentrations which did not reach statistical difference probably due to the small patient numbers in each group (top CRP CC 247, CT 300 and TT 268 mg/l, p= 0.09; recovery phase CRP CC 2.79, CT 2.89 and TT 4.61 mg/l, p=0.49; unpublished results). There are numerous studies concurring with our results showing an association of C allele with decreased CRP concentration (Brull et al. 2003, D'Aiuto et al. 2005, Miller et al. 2005, Casas et al. 2006, Marsik et al. 2006). Interestingly, a study showing an association of the opposite allele T with decreased CRP has also been published, but in that study the patient number was too small (n=63) to attach much importance to this association (Obisesan et al. 2004). In light of these reports it seems obvious that this SNP has a role in CRP concentration determination.

An association of *CRP*+1846G>A allele A with decreased CRP concentration was found in the Cardiovascular Risk in Young Finns Study participants (Study V). Our finding is in line with earlier reports showing a similar association (Russell et al. 2004, Miller et al. 2005, Lange et al. 2006), thus this association would appear to be generalizable to different populations.

3. Association between bacteraemia mortality and *CRP* gene polymorphisms

Pneumococcal infection is a major global cause of mortality and morbidity (Laupland et al. 2004). CRP is an important first line defence molecule of the host in the early stages of infection. Transgenic mice with human CRP infected with *S. pneumoniae* have reduced bacteraemia and longer survival time compared to wild-type controls, suggesting functional importance of CRP (Szalai et al. 1995).

We found an association for the first time, to our knowledge, between -717A>G polymorphisms and bacteraemia mortality caused by *Streptococcus pneumoniae*. Patients with GG genotype were 9.6 times more likely to die than other genotypes (CI 1.3-72.5). This association remained significant in the logistic regression model after adjusting for smoking, alcohol and BMI.

Interestingly, we could detect no association between this SNP and CRP concentration in our study population. However, the population studied was quite small and an association could possibly be found in a larger number of individuals. Nevertheless, we cannot rule out the possibility that this finding is a result of linkage of the -717 SNP with some other *CRP* gene region SNP that we did not investigate in this study. As the number of patients in our study was limited, larger studies are needed to confirm our finding.

4. Association between carotid artery compliance and *CRP* gene polymorphisms

Although elevated CRP is an independent risk marker for cardiovascular disease and has predicted the risk of coronary events along with the traditional risk factors such as cholesterol levels, BMI, smoking and diabetes (Danesh et al. 2000, Danesh et al. 2004), the evidence on the association between CRP genetic variants and CHD is less consistent. An association has been reported between *CRP*+1059 and risk of cardiovascular disease mortality (Lange et al. 2006), but no association has been found between *CRP*+1059 and risk of arterial thrombosis (Zee and Ridker 2002), *CRP* +1444 and non-fatal MI (Casas et al. 2006), *CRP*+1059 and blood pressure (Davey Smith et al. 2005), *CRP*-286 and MI/IS (Miller et al. 2005), CRP haplotype and IMT (Kivimaki et al. 2007) or CRP haplotype and the occurrence of CHD (Kardys et al. 2006).

However, we found an association of CAC, a vascular marker of atherosclerosis with three *CRP* SNPs, -286C>T>A, +1444C>T and +1846G>A in unadjusted analysis of men participating in the Cardiovascular Risk in Young Finns Study. After adjustment for traditional risk factors only the association between *CRP* -286 allele C non-carriage (TT and TA genotypes) and decreased CAC remained significant in linear regression analysis. The analysis of the *CRP* haplotype association with CAC supported the individual SNP finding. The carriers of the commonest haplotype ATGTG, which includes -286 allele T, had decreased CAC compared to the non-carriers. Also, the non-carriers of the second commonest haplotype ACGCA, had significantly decreased CAC compared to the carriers. In women, variants in the *CRP* gene were not associated with CAC.

Interestingly, levels of CRP were not independently associated with CAC, although univariate analysis showed a weak association in males ($r=-0.18$, $p<0.001$). This is in agreement with earlier studies on this cohort and other study populations showing a non-significant association between circulating CRP and IMT and coronary artery calcification after adjustment for risk factors (Reilly et al. 2003, Kivimaki et al. 2007) and suggests that the association of the polymorphism -286 with CAC may be driven by pathological mechamisms other than circulating CRP levels. Taken together, in Finnish young men, the carriage of allele C of CRP-286 seems to protect from decreased carotid artery elasticity,

but futher research is needed to determine whether this association is generalizable to other populations.

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Original publications

EPISTATIC EFFECT OF C-REACTIVE PROTEIN (CRP) SINGLE NUCLEOTIDE POLYMORPHISM (SNP) +1059 AND INTERLEUKIN-1B SNP +3954 ON CRP CONCENTRATION IN HEALTHY MALE BLOOD DONORS

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Summary

Baseline C-reactive protein (CRP) concentrations are indicative of persons prone to cardiovascular diseases and are about 40-50% heritable. We have previously shown that IL1B +3954 allele T is associated with lower CRP concentration. In this study, we aimed to examine the effect of this polymorphism together with CRP+1059 gene polymorphism on baseline CRP concentrations, and genotyped 336 healthy blood donors for CRP+1059 (G>C) and IL1B+3954 (C>T) polymorphisms. In men, the carriers of the CRP+1059 C-allele had significantly lower CRP values than GG homozygotes (0.66 versus 0.43 mg/l, up to -35%, p=0.009). No significant difference was found in women. When the data were stratified for both of these polymorphisms in men, CRP+1059 GG homozygotes had low CRP concentrations only if they were allele-T carriers of IL1B +3954 simultaneously (0.93 versus 0.50 mg/l, p=0.013). Genotype CRP+1059 GG/IL1B+3954 CC was associated to almost 3-fold risk of higher baseline CRP value (OR 2.84 (1.03-6.07)). Thus, both IL1B+3954 (C>T) and CRP +1059 (G>C) polymorphisms influence baseline CRP values and act independently of each other in male subjects. These polymorphisms might be predictive markers of persons prone to cardiovascular diseases.

Introduction

C-reactive protein (CRP) is an acute-phase protein produced in response to inflammation, infection and trauma. It is a sensitive marker of inflammation as well as an independent risk factor for coronary heart disease (CHD), as many studies have shown (Danesh et al., 2000). Recently the metabolic syndrome, which is a known risk factor for coronary artery diseases, was also associated with elevated CRP levels and low grade inflammatory state in healthy reference range (Tamakoshi et al., 2003). The baseline level of CRP is less than 1 mg/l but during inflammation it may rise 1000-fold. The major organ synthesizing CRP is the liver, which produces CRP during the acute phase in response to pro-inflammatory cytokines, e.g. interleukin (IL)-6 and IL-1β. IL-6 is assumed to be the major regulator of blood CRP (Weinhold and Ruther, 1997). However, *in vivo* as well as *in vitro* studies have shown that IL-1β affects the acute-phase reaction and CRP transcription, possibly by enhancing the CRP transcription. (Agrawal et al., 2001;Cha-Molstad et al., 2000;Szalai et al., 2000;Zheng et al., 1995)

Because the basal values of CRP appear to be significantly heritable (~40-50%) (MacGregor et al., 2004;Pankow et al., 2001;Vickers et al., 2002), it is very likely that polymorphisms in genes controlling CRP expression influence CRP levels. The IL6–174 (G>C) promoter region single nucleotide polymorphism (SNP) was shown to influence CRP levels in studies by Vickers et al. (2002), and Ferrari et al. (Ferrari et al., 2003). However, we did not find this association in our previous study of healthy blood donors (Eklund et al., 2003), nor did Margaglione et al. (Margaglione et al., 2001) in a study of asymptomatic hospital employees. An association of IL1B+3954

(C>T) gene SNP with CRP levels has been shown in two studies. We previously showed an association of IL1B+3954 SNP with CRP levels (Eklundet al., 2003), and Berger et al (2002) showed association of this SNP with CRP levels in a study of cardiac symptoms patients. The most logical gene polymorphism associated with CRP level is polymorphism in the CRP gene itself. There are three published polymorphisms which have an influence on CRP levels: a dinucleotide repeat in CRP gene intron (Szalai et al., 2002), a CRP(G>C)+1059 SNP in exon two (Zee and Ridker, 2002) and a CRP(C>T)+1444 polymorphism in the 3' UTR region (Brull et al., 2003) .

We have previously shown that IL1B +3954 SNP is associated with CRP concentrations in healthy blood donors. In view of the possibility that CRP +1059 SNP together with IL1B +3954 SNP could regulate basal CRP values, and therefore increase the risk for future cardiovascular diseases, we performed an association study of these polymorphisms on CRP levels of healthy subjects. This was performed in healthy, middle-aged blood donors.

Methods

Blood donors

Blood samples were collected from 336, healthy, volunteer adult blood donors at the Finnish Red Cross Blood Transfusion Centre, Tampere, Finland. All the blood donors were of the same ethnic origin, Finnish Caucasians. The persons fulfilled the general requirements for blood donation, e.g. they had no chronic diseases and used no regular medication. Also they did not have any signs of infectious diseases during

the two weeks period prior to blood donation. The age range was 21-64 years in men (mean 46.6, n= 186), and 19-62 years in women (mean 41.3, n=150). The ethical committee of Finnish Red Cross Blood Transfusion Centre approved the use of human blood.

Genotyping

The IL1B gene polymorphic site at nucleotide (nt) position +3954 of exon 5 was amplified by PCR using nucleotides 5' GTTGTCATCAGACTTTGACC 3' and 5' TTCAGTTCATATGGACCAGA 3' as primers. PCR products were digested with *TaqI* restriction enzyme and analyzed on 2% agarose gel (Pociot et al., 1992). The reliability of the IL1B genotyping was verified by ABI PRISM 7000 Sequence Detection System (Assay By Design, Applied Biosystems, CA, USA).

Genotyping of CRP gene polymorphism at nt position +1059 of exon 2 was done using the ABI PRISM 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA, USA). A commercial kit from Applied Biosystems was used (Assay On Demand, C_177490-10 CRP), which corresponds to GenBank SNP database rs number 1800947.

CRP assay

The plasma CRP concentrations were analyzed by particle-enhanced immunonephelometric method using the Dade Behring N High Sensitivity CRP on

the Dade Behring Nephelometer II (Dade Behring, Marburg, Germany). The lower detection limit for CRP was 0.16 mg/l (0.016 mg/dl)(Erlandsen and Randers, 2000).

Statistical analysis

The results for CRP are expressed as median and interquartile ranges (25-75%), as the variable was not normally distributed. Non-parametric statistics were used for testing the significance of an association between CRP and genotypes (Kruskal-Wallis test and Mann-Whitney U-test). Statistical analyses were calculated using Statistica software (ver. Win.5.1D StatSoft Inc, Tulsa, OK). Odds ratios (ODs) and their 95% confidence intervals (CIs) were calculated using CIA software (version 1.1, copyrighted by M J Gardner and British Medical Journal, 1989).

Results

The median CRP value in healthy blood donors was 0.71 mg/l, ranging from <0.16 to 8.52 mg/l. In men, the median CRP value was 0.64 mg/l (range <0.16 to 8.52), and in women 0.89 mg/l (range <0.16 to 8.34). The genotype distributions of CRP SNP+1059 and IL1B SNP +3954 were in accordance with those expected under the Hardy-Weinberg equation and allele frequencies did not differ from previous work (Berger et al., 2002;Cao and Hegele, 2000;Zee and Ridker, 2002) (Table 1). The genotypic data shown in Table 2 show the genotype frequencies of allele 2 carriers (=2+) and non-carriers (=2-) of CRP SNP +1059 and IL1B SNP +3954. In men, the allele carriers and non-carriers of allele 2 of CRP SNP +1059 and IL1B SNP +3954 showed significantly different CRP levels (Mann-Whitney test, p= 0.009 and 0.032, respectively). The allele 2 carrier genotypes (1.2 and 2.2) of both of these SNPs in men were associated with lower CRP levels. We could not detect any significant

associations between these genotypes and CRP levels in women. Stratification of the data by both CRP +1059 and IL1B +3954 SNPs (in men) showed that among the CRP allele 2 non-carriers (CRP2-) the CRP values differed significantly according to IL1B carrier status; allele 2 non-carriers had the highest values (0.93 vs. 0.50 mg/l, p=0.013, Kruskal-Wallis test, Table 3). We stratified the data also by IL1B +3954 allele 2 carriers (IL1B2+) and non-carriers (IL1B2-). The CRP values of IL1B2- genotypes differed significantly according to CRP +1059 carrier genotypes (p=0.004), so that the CRP2- genotypes had the highest values.

To compare and evaluate the effect of composite genotypes on men's CRP levels, we divided the subjects according to CRP tertiles (1^{st} tertile <40 mg/l, 3^{rd} tertile >1.01 <8.52 mg/l, Table 4). The analysis revealed that the carrier genotype combination CRP +1059 2-/ IL1B +3954 2- markedly increased the CRP levels (OR 2.84 (95% CI 1.33-6.07)) The opposite effect could not be seen, unfortunately, due to limited number of subjects carrying this composite genotype.

Discussion

In this study, we showed that CRP SNP +1059 and IL1B +3954 have an epistatic effect on baseline CRP levels in healthy men. This report extends our previous finding concerning the association of IL1B +3954 with CRP levels in healthy individuals (Eklundet al., 2003). The present data shows that allele 2 of both CRP SNP +1059 and IL1B SNP +3954 is associated with low baseline CRP levels in healthy men. It seems that carriage of either allele 2 of IL1B +3954 or allele 2 of CRP +1059 is enough to keep the CRP concentration down. These alleles seem to compensate each other, so that only in case when neither one of them is present is the CRP value raised.

Interestingly, the carriage of allele 2 of both of these genes simultaneously does not further decrease the CRP value, indicating that the baseline is already reached by one of them. However, in induced states it might be that both of these alleles are needed to assert the down-regulating effect on CRP values. It is also of interest that this association is found only in males. The reason might be female hormones, e.g. oestradiol concentrations have been found to modulate CRP levels in women (Tchernof et al., 2002).

The CRP +1059 polymorphism is a silent mutation. Zee & Ridker (2002) have shown that carriage of allele 2 (=allele C) decreases plasma CRP levels in apparently healthy men. Our results verify this finding. The molecular mechanism of this effect remains to be resolved.

There is conflicting data about IL1B SNP +3954 and its effect on IL1B gene expression. *In vitro*, Pociot et al.(1992) showed allele 2 homozygotes to produce 4-fold more protein than other genotypes, Dominici et al. (Dominici et al., 2002) found secretion not to be altered by IL1B+3954 genotype and (Santtila, Savinainen and Hurme, 1998) showed that allele 2 carriers secrete less protein than others. *In vivo* association studies have not found significant associations between IL1B +3954 polymorphisms and IL-1B levels, neither in induced states (in people with disease) (Hefler et al., 2001;Wieser et al., 2003) nor in healthy blood donors (Eklund et al, unpublished data 2003), but this probably reflects merely the autocrine and paracrine nature of IL-1β molecule. The molecular mechanism of this exonic polymorphism is as yet undetermined. However, at present the possibility that the observed effect for

both IL1B +3954 and CRP +1059 is the result of an unknown gene locus, located near these gene polymorphisms, cannot be excluded.

In conclusion, this study shows that, in men, the composite genotype CRP +1059 GG/IL1B +3954 CC increases the risk for higher CRP values by almost 3-fold (OR 2.84). This genotype combination could be a predictive marker for persons prone to cardiovascular diseases.

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Table 1. The genotype distributions and allele frequencies of C-reactive protein +1059 and interleukin 1B+3954 in blood donors.

Genotype	All	Men	Women
CRP +1059			
1.1	300 (89.3%)	165 (92%)	135 (90.0%)
1.2	35 (10.4%)	21 (8%)	14 (9.3%)
2.2	1 (0.3%)	0	1 (0.7%)
Allele 1	0.945	0.944	0.947
Allele 2	0.055	0.056	0.053
IL1B +3954			
1.1	188 (56 %)	107 (58%)	81 (54%)
1.2	122 (36 %)	66 (35%)	56 (37%)
2.2	26 (8 %)	13 (7%)	13 (9%)
Allele 1	0.741	0.753	0.727
Allele 2	0.259	0.247	0.273

Table 2. C-reactive protein levels by CRP +1059 and IL1B +3954 carrier genotypes.

Genotype		**N (%)**	**CRP value (mg/l) (25-75%)**	***P*- value**
Men				
CRP +1059	2-	165 (89%)	0.66 (0.35-1.34)	0.009
	2+	21 (11%)	0.43 (0.25-0.70)	
IL-1B +3954	2-	107 (58%)	0.76 (0.35-1.51)	0.032
	2+	79 (42%)	0.51 (0.28-1.07)	
Women				
CRP +1059	2-	135 (90%)	0.87 (0.43-2.11)	0.890
	2+	15 (10%)	0.90 (0.38-2.22)	
IL-1B +3954	2-	81 (54%)	0.87 (0.50-2.36)	0.373
	2+	69 (46%)	0.90 (0.38-1.81)	

Values for CRP are expressed as medians and interquartile range, i.e. values between 25th and 75th percentiles. *P*-values are based on Mann-Whitney *U*-test.
CRP2+ = CRP +1059 carriers of allele C (=GC and CC genotypes)
CRP2- = CRP+1059 non-carriers of allele C (=genotype GG)
IL1B2+ = IL1B+3954 carriers of allele T (=CT and TT genotypes)
IL1B2- = IL1B+3954 non-carriers of allele T (=genotype CC)

Table 3. C-reactive protein levels arranged by CRP+1059 allele 2 carriage (CRP2+) or non-carriage (CRP2-) and interleukin (IL)1B+3954 genotypes in male blood donors.

Carrier genotype	**N**	**Median CRP levels arranged by genotype (25%-75%)**	**Median**	***P* -value**
CRP genotype		**IL1B +3954 genotype**		
CRP2-	93	2- 0.93 mg/l (0.39-1.55)	0.66 mg/l	0.013
	84	2+ 0.50 mg/l (0.29-1.08)		
CRP2+	13	2- 0.38 mg/l (0.00-0.48)	0.40 mg/l	0.360
	7	2+ 0.51 mg/l (0.26-0.71)		
IL1B genotype		**CRP +1059 genotype**		
IL1B2-	93	2- 0.93 mg/l (0.39-1.55)	0.76 mg/l	0.004
	13	2+ 0.35 mg/l (0.00-0.48)		
IL1B2+	72	2- 0.49 mg/l (0.29-1.08)	0.51 mg/l	0.557
	7	2+ 0.62 mg/l (0.26-0.71)		

P-values are based on Kruskal-Wallis analysis of variance.

Table 4. Composite carrier genotypes of CRP + 1059 and IL1B +3954 in male blood donors by CRP tertiles and their odds ratios (Ors) and confidence intervals (CIs).

Geno-type	IL1B +3954	CRP +1059	CRP 3rd tertile (1.01-8.52 mg/l)	CRP 1st tertile (0-0.40 mg/l)	OR (95% CI)
1.	2-	2-	36	24	2.84 (1.33-6.07)
2.	2-	2+	2	8	0.25 (0.05-1.21)
3.	2+	2-	17	26	0.71 (0.41-1.22)
4.	2+	2+	0	2	—
All N			**55**	**60**	

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ORIGINAL ARTICLE

Polymorphism of the C-reactive protein gene is associated with mortality in bacteraemia

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Abstract
C-reactive protein (CRP) is an important molecule in the defence against bacterial infections. To discover if variation in the *CRP* gene is associated with clinical outcome of bacteraemia, we investigated 147 microbiologically verified bacteraemia patients (mean age 59 y, range 19–93 y) and determined whether *CRP* −717A>G, +1059G>C or +1444C>T single nucleotide polymorphisms (SNPs) were associated with clinical outcome of bacteraemia and/or CRP concentration caused by Staphylococcus aureus, Streptococcus pneumoniae, β-haemolytic streptococci or Escherichia coli. The patients were genotyped for *CRP* gene polymorphisms, CRP was measured and clinical outcomes were recorded. The *CRP* −717A>G, a promoter region polymorphism was strongly associated with mortality from Streptococcus pneumoniae but did not correlate with plasma CRP concentration. These results suggest that mortality from Streptococcus pneumoniae may be associated with polymorphism of the promoter region of the *CRP* gene.

Introduction

C-reactive protein (CRP) is an acute phase protein originally characterized by its ability to bind C polysaccharide from the cell wall of pneumococci. CRP concentration is rapidly increased in blood in various infectious diseases and in inflammatory states, thus being a good marker for inflammation. It participates in defence against microbes in several ways, e.g. by activating the complement system and by enhancing complement-mediated opsonization [1–3]. However, CRP has a regulatory role also in the activation of the inflammatory response of the host. It is able to bind to Fcγ receptors of phagocytic cells and lymphocytes and can in this way regulate their function, e.g. by inhibiting the production of proinflammatory cytokines directly or by production of the anti-inflammatory cytokine IL-10 [4].

Identification of genes implicated in susceptibility to polygenic diseases is a rapidly growing field of research. The association studies report polymorphic sites associated with susceptibility to, mortality from, or severity of, common diseases. The *CRP* gene is located on chromosome 1q32 and consists of 2 exons and 1 intron. The gene is polymorphic; at least 31 SNPs have been reported (see reference [5]). Association studies on CRP have focused either on CRP concentration variability or on disease association.

SNPs +1059G>C, +1444C>T, and a GT dinucleotide repeat in first intron have been associated with blood CRP concentration either in healthy individuals or in various disease states [6]. The GT repeat has also been associated with increased susceptibility to invasive pneumococcal disease [7]. In addition, some polymorphisms have been associated to a disease without demonstrable effect on blood CRP concentration, e.g. −717 A/G allele A carriers had a 6.8-fold higher risk for developing coronary heart disease compared to the non-carriers [8] and the same promoter polymorphism is associated to type 2 diabetes [9].

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To examine the significance of CRP in bacteraemic infection, we analysed the association of CRP polymorphisms −717A>G, +1059G>C and +1444C>T with the clinical outcome of microbiologically verified bacteraemia in 147 patients.

Methods and patients

The study was approved by the Ethics Committee of Tampere University Hospital. Written informed consent was obtained from the patients or a first degree relative. Blood samples and a verified positive blood culture were obtained from 149 Caucasian patients with symptoms and signs of systemic infection during the study period from June 1999 to February 2004. Symptoms and signs of systemic infection were fever or hypothermia, tachycardia or tachypnoea combined with leucocytosis or leucopenia and/or elevated CRP. The BACTEC 9240 blood culture system (BD Diagnostic Systems, Sparks, MD, USA) was used. Each patient was interviewed and examined by clinicians (J.S. or J.L.). 149 out of 152 patients agreed to participate. Only patients with bacteraemia caused by Staphylococcus aureus, Streptococcus pneumoniae, β-haemolytic streptococci or Escherichia coli, the 4 most common causative organisms of community acquired bacteraemia, were included.

The CRP was measured at several time points: on blood culture day and at least on the 3 consecutively following day. In addition, in 115 out of 128 living patients CRP was measured in the recovery phase (2–3 months after the positive blood culture). The plasma CRP concentrations were analysed by a particle-enhanced immunoturbidimetric method using the Cobas Integra 700 automatic analyser (Hoffmann La Roche Ltd., Basel, Switzerland) with the COBAS® Integra C-Reactive Protein (Latex) reagent. The sensitivity determined by the smallest analysis concentration of CRP which can be reproducibly distinguished from a zero sample leads to a typical detection limit of 0.10 mg/l [10].

Table I. shows the most important underlying diseases and predisposing factors to bacteraemia in all these 147 patients and in relation to causative organisms. Symptoms of infection before treatment had lasted from 0 to 14 d (median 2 d, data available from 142 patients). All patients were treated with empirical intravenous antibiotics, which were started immediately after the blood cultures were taken and the antibiotic regimen was modified according to the culture results. In all patients the empirical antibiotics were effective according to resistance testing. Severely ill patients were transferred to the ICU.

Table I. Predisposing factors and underlying diseases in bacteraemia.

Predisposing factor or underlying disease	All patients $n=147$ (%)	S. aureus $n=40$ (%)	S. pneumoniae $n=42$ (%)	β-haemolytic streptococci $n=23$ (%)	E. coli $n=42$ (%)	p-value*
Current smoker or ex-smoker[a]	65 (49)	17 (47)	26 (65)	11 (58)	11 (28)	0.009
Alcohol abuse	24 (16)	5 (13)	9 (21)	6 (26)	4 (10)	0.235
Diabetes type 1 or 2	34 (23)	10 (25)	5 (12)	4 (17)	15 (36)	0.065
Haematological malignancy	10 (7)	2 (5)	3 (7)	1 (4)	4 (10)	0.820
Solid malignancy	15 (10)	6 (15)	3 (7)	1 (4)	5 (12)	0.489
Male gender	78 (53)	28 (70)	24 (57)	14 (61)	12 (29)	0.001
Previous corticosteroid treatment[b]	17 (12)	6 (15)	4 (10)	2 (9)	5 (12)	0.844
Healthy[c]	32 (22)	8 (20)	15 (36)	6 (26)	3 (7)	0.015

*Difference between groups of patients with bacteraemia caused by different causative organisms.
[a]Data available from 134 patients.
[b]Corticosteroids used in a dose of over 5 mg per d during 1 month prior to the episode of bacteraemia.
[c]Patient did not have any chronic illnesses.

Genotyping of *CRP*+1059G>C (rs1800947), −717A>G (rs 2794521) and +1444C>T (rs 1130864) gene polymorphisms was performed using the ABI PRISM 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA, USA). For *CRP*+1059 a commercial kit was used (Assay On Demand, C_177490_10 CRP). For *CRP*−717 and *CRP*+1444 the nucleotide sequences of the primers and fluorogenic allele-specific oligonucleotide probes were deduced from published sequences deposited in the GeneBank database and were chosen and synthesized in conjunction with Applied Biosystems.

Statistical analyses were performed using SPSS (version 11.5; SPSS Inc., Chicago, IL, USA). Descriptive results of continuous variables were expressed as mean or median (minimum-maximum). Variables were tested for their association with mortality by using Fisher's exact test for categorized data (polymorphisms) and Mann-Whitney *U*-test for numerical data (CRP concentration). Kaplan-Meier survival analysis was carried out to estimate the probability of survival.

Results

DNA could be extracted from blood samples of 147 out of 149 patients. The study population consisted of 40 patients with Staphylococcus aureus, 42 patients with Streptococcus pneumoniae, 23 patients with β-haemolytic streptococci and 42 patients with Escherichia coli bacteraemia. 78 of the patients (53%) were male and 69 (47%) female; their mean age was 59 y (16–93 y). Predisposing factors and underlying diseases of the patients are shown in Table I.

The genotype frequencies were as follows: −717 A>G AA 0.72, AG 0.24 GG 0.04, +1059G>C GG 0.90, GC 0.09, CC<0.01, and +1444C>T CC 0.43, CT 0.45, TT 0.12. According to previous studies, these polymorphisms are in strong linkage disequilibrium, i.e. the pairwise D' between −717 and +1444 was −0.98 [9] and 0.91 between +1059 and +1444 [9,11]. 19 of the 147 patients died within 30 d of the positive blood culture. Top median CRP concentration was similar with respect to mortality (min-max) (survivors 268 mg/l (60–633) vs non-survivors 310 (120–618) mg/l, $p = 0.206$, Mann-Whitney *U*-test); however, on blood culture d the non-survivors had significantly higher plasma CRP concentrations than the survivors (248 mg/l (56–618) vs 186 (2–633) mg/l, respectively, $p = 0.02$, Mann-Whitney *U*-test).

When the survivors/non-survivors were stratified according to genotypes, only the −717A>G genotype distribution was associated with 30-d mortality. The GG homozygotes were significantly more likely to die ($p = 0.03$, Fisher's exact test; Table II, panel A). When this effect was analysed separately in cases with differential bacterial aetiology, it could be observed only in pneumococcal bacteraemia ($p = 0.05$, Fisher's exact test; Table II, panel B). The odds ratio for mortality in patients with −717 GG genotype was 9.6 (95% CI 1.3–72.5) compared to the AA+AG individuals. Kaplan-Meier survival analysis showed that the effect of −717 genotype GG was highly significant both in all patients ($p = 0.004$) and in those with pneumococcal bacteraemia ($p = 0.01$, Figure 1). In the logistic regression model the effect of CRP −717 GG on mortality remained significant after adjusting for all those variables that were significant in univariate analysis (smoking, alcohol, BMI) (data not shown). The genotype distribution did not differ in patients with diabetes, haematological/solid malignancies, patients on previous corticosteroid treatment, in current or ex-smokers, or by alcohol consumption (data not shown). There were no association of the 2 other SNPs (+1444 or +1059) genotype distribution with 30-d mortality in patients analysed by differential aetiology (data not shown).

We found no effect of CRP −717A>G or +1444C>T on CRP concentration. However, a modest effect of +1059G>C on recovery CRP concentrations (60 to 90 d from positive blood culture) was found in all patients: C-allele carriers had significantly lower recovery CRP (min-max) concentration than non-carriers (GC+CC 1.40 mg/l (0.3–5.2) vs GG 3.00 mg/l (0.1–67.6), $p = 0.04$, Mann-Whitney *U*-test).

Discussion

This study found an association for the first time, to our knowledge, between the rare −717A>G GG genotype and mortality from Streptococcus pneumoniae bacteraemia. The GG genotype was found

Table II. Survival stratified by *CRP* −717 A>G genotype in all patients (panel A) and in Streptococcus pneumoniae infected patients (panel B).

	Survivors *n* (%)	Deceased *n* (%)	p^a
A. Genotype			
AA	95 (74)	11 (58)	0.03
AG	30 (24)	5 (26)	
GG	3 (2)	3 (16)	
B. Genotype			
AA	23 (68)	3 (38)	0.05
AG	9 (27)	2 (25)	
GG	2 (6)	3 (38)	

[a] *p*-value is based on Fisher's exact test.

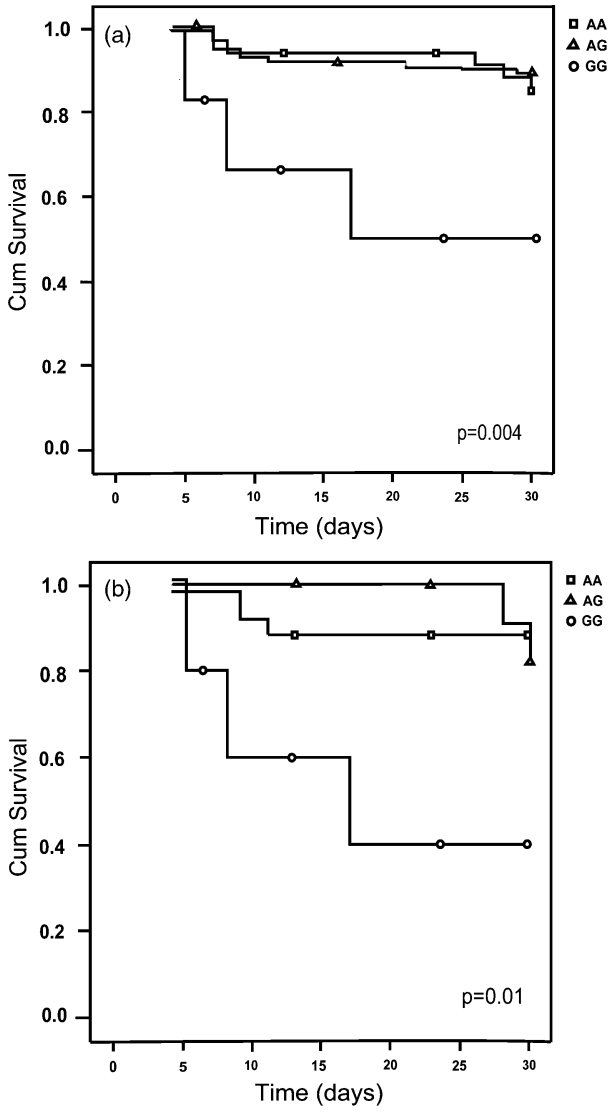

Figure 1. Survival stratified by *CRP* −717 A>G genotype in all patients (panel a) and in Streptococcus pneumoniae infected patients (panel b); Kaplan-Meier analysis.

significantly more often in non-surviviors than survivors of bacteraemia and appeared to be a genetic risk factor for death attributable to pneumococcal bacteraemia (odds ratio 9.6, 95% CI 1.3–72.5).

Pneumococcal infection is a major global cause of mortality and morbidity. CRP is an important acute phase reactant of the host that may be important in the early stages of infection. Compared to wild-type controls, transgenic mice with human C-reactive protein infected with S. pneumoniae have reduced bacteraemia and longer survival time, suggesting that CRP is functionally important [12].

We found that the non-survivors had significantly higher CRP concentration on blood culture d than the survivors. Similar results were recently reported in a study of critically ill patients where the incidence of infection was directly related to CRP concentration and plasma CRP correlated with mortality [13]. Opposite results were reported in a review article where survivors/non-survivors did not differ in their CRP concentration during sepsis [14]. However, we failed to demonstrate a significant correlation between plasma CRP concentration and *CRP* −717A>G polymorphism. At present, the mechanism of action of the *CRP*-717 polymorphism can only be speculated, as the data presented here do not permit assessment of the functional significance of the polymorphism. As the SNPs of the *CRP* gene seem to be quite strongly linked, linkage of −717A>G to some other seems likely. Interestingly, very recently a triallelic functional SNP, −390C>T>A (rs3091244) on the CRP promoter region was reported to be possibly in linkage with −717A>G due to their close proximity. This SNP was shown to affect transcription factor binding and also to alter the transcriptional activity of the *CRP* gene [15].

In an earlier study a similar lack of correlation between −717A>G and CRP concentration was noted. Chen et al. observed that the presence of the −717A allele increased the risk of coronary heart disease while no association with CRP concentration was found [8]. They suggested that the A to G transition would create a binding site for the transcription factor glucocorticoid receptor, thus changing glucocorticoid dependent regulation of the *CRP* gene. Another study found an association between −717A>G allele A and type 2 diabetes in Pima Indians [9]. It is interesting to note that to date there are no common amino acid changing SNPs in the *CRP* gene detected. Thus, linkage to a non-synonymous (=amino acid changing SNP) seems to be unlikely.

Carlson et al. recently haplotyped the entire *CRP* gene and identified 7 haplotype determinative SNPs (tagging SNPs) on the *CRP* gene forming 8 common haplotypes. They found that SNP +1059 was a determinant SNP for the lowest CRP haplotype [5]. We also noticed a weak association of this polymorphism with recovery phase CRP, allele C carriers having the lowest values.

The data presented in this report imply that CRP genetics has an important role in the defence mechanisms in bacteraemic infection and/or in the regulation of the infection-associated inflammatory responses of the host. The genes associated with sepsis mortality have been of inflammatory nature and, thus, CRP can be included in the list of those host genetic factors which are known to have an effect on sepsis mortality, e.g. the proinflammatory cytokine genes *TNFA, TNFB* and *IL1* (reviewed in [16]).

In conclusion, in this study mortality from bacteraemia caused by Streptococcus pneumoniae was increased in patients homozygous for CRP −717 GG genotype. The GG genotype appeared to be a genetic risk factor for death attributable to bacteraemia caused by pneumococci. Determining a patient's CRP genotype at the early stages of infection may enable the selection of a homogenous group of high-risk patients and may also have an influence on the selection of therapies. However, the number of patients in our study was limited and larger studies are needed to confirm our finding in a second and newly recruited population of individuals.

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C-reactive protein genetics is associated with carotid artery compliance in men in The Cardiovascular Risk in Young Finns Study

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Abstract

Although C-reactive protein (CRP) is known to predict cardiovascular events, its status as a causal risk factor is still controversial. CRP gene single nucleotide polymorphisms (SNPs) have been shown to associate with CRP concentration, but no direct independent effect on early atherosclerotic changes has been demonstrated. We aimed to determine if CRP gene polymorphisms or haplotypes are associated with CRP concentration or carotid artery compliance (CAC), an indicator of subclinical atherosclerosis. We genotyped CRP gene polymorphisms −717A > G, −286C > T > A, +1059G > C, +1444C > T and +1846G > A and measured CRP concentration and CAC in 2283 young adults participating in The Cardiovascular Risk in Young Finns Study. A strong association was found between CRP genotypes and CRP concentration, which was also seen at the haplotype level. Linear regression analysis showed an independent effect of each SNP on CRP concentration after adjustment for risk factors, except for +1444 in males. Moreover, −286C > T > A, +1444C > T and +1846G > A were associated with CAC in males, but not in females. Men carrying the SNP −286 allele C had increased CAC after adjusting for risk factors. These data suggest that the presence of high producer CRP genotype is deleterious to carotid elasticity in men.

Keywords: Atherosclerosis; CRP genetics; Elasticity

1. Introduction

C-reactive protein (CRP), an acute phase protein produced by the liver, is a sensitive marker of systemic inflammation and tissue damage. Being part of the calcium-dependent ligand-binding family of pentraxin proteins, CRP binds phosphocholine residues with high affinity as well as a variety of other molecules like native and modified plasma lipoproteins, damaged cell membranes, a number of different phospholipids and related compounds, small nuclear ribonucleoprotein particles, histones, chromatin and apoptotic cells [1]. CRP reacts with these components and aids in their removal via interaction with the phagocytic cells and the complement system. Thus, it is not surprising that CRP has been found on atherosclerotic plaques [2] as well as in acute

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myocardial infarction lesions. Elevated CRP is also an independent risk marker for cardiovascular disease [3] and has predicted the risk of coronary events along with the traditional risk factors such as cholesterol levels, BMI, smoking and diabetes [4,5]. In spite of the fact that there is extensive *in vitro* evidence showing a direct, proatherogenic effect of CRP in atherogenesis [6], evidence on the association between CRP and atherosclerosis is less consistent [7,8].

Twin studies suggest that the level of CRP is over 40% heritable [9] and several studies have shown the associations of CRP gene polymorphisms, such as −286, +1059, +1444 and +1846, with CRP concentration [10]. At least three studies have also reported an association between CRP gene polymorphisms and cardiovascular disease [11–13], but little is known about their association with preclinical markers of vascular changes. However, controversy persists as to whether the genetic polymorphisms of CRP are associated with cardiovascular traits [14]. CHD develops over a long time span and early atherosclerotic changes can be detected non-invasively by ultrasound methods. Valid markers for vascular changes are, e.g. measurements of carotid artery compliance (CAC) and carotid artery wall intima-media thickness (IMT). CAC is a measure of the elasticity of large arteries and IMT serves as a structural marker of atherosclerosis [15].

Genetic markers may provide information about the relationship between CRP and cardiovascular disease pathogenesis. In this study, we genotyped CRP polymorphisms which have been associated with CRP levels [10] or disease risk [12] in previous studies or have been classified as haplotype tagging SNPs by other investigations [16]. In addition, we collected data on adulthood CRP, CAC and IMT to explore if genetic variants at the human CRP gene influence early atherosclerotic changes or plasma CRP.

2. Methods

2.1. Subjects

The subjects in the study comprised participants of the ongoing Cardiovascular Risk in Young Finns Study, a five-centre follow-up study involving five university hospital cities in Finland. The first cross-sectional survey was conducted in 1980. Total sample size was 4320 boys and girls in six age cohorts (aged 3, 6, 9, 12, 15 and 18). These subjects were randomly chosen from the national register to include participants from the study centres (five university cities and rural communities in their vicinity). In practice, girls and boys of each age cohort in each community were separately placed in random order on the basis of the unique social security number. Every *k*th girl and every *k*th boy in each community was selected so that the sample consisted of the required number of boys and girls. The latest follow-up was conducted in 2001, when the subjects ($n = 2283$) were 24–39 years of age. Details of the study design have been presented elsewhere [17,18]. Cardiovascular risk factors, including serum lipids, BMI, blood pressure values, CRP, alcohol consumption, diabetes and smoking habits were recorded in 2001. In addition, CAC and IMT were measured by ultrasonography in 2001 [15]. This study was conducted on those subjects who participated in the latest follow-up in 2001, $n = 2283$.

2.2. Clinical characteristics and biochemical analyses

Height and weight were measured and body mass index (BMI) was calculated. A random zero sphygmomanometer (Hawksley & Sons Ltd., Lancin, UK) was used to measure blood pressure and a mean of three measurements was used in the analysis. Blood samples for the analysis of fasting plasma CRP, insulin, leptin, total cholesterol, HDL cholesterol and triglyceride concentrations were drawn. Smoking habits, alcohol consumption, hormone treatment, physical activity, history of recent infection, diabetes and chronic rheumatic disease were elicited by questionnaire. Smokers were classified as smokers if they reported smoking daily, otherwise they were classified as non-smokers.

Fasting plasma CRP concentration were analyzed by a high-sensitive latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The detection limit was 0.06 mg/L, and the coefficient of variation of repeated measurements was 3.3%. Details of the physical examination and other biochemical measurements have been presented elsewhere [19]. Subjects with chronic rheumatic disease ($n = 36$), history of recent infection ($n = 129$), diabetes ($n = 26$), pregnant women ($n = 62$), lactating women ($n = 2$) and subjects with CRP ≥ 10 mg/L ($n = 74$) were excluded from the main CRP analyses. Subjects with triglycerides above 4 mmol/L were also excluded ($n = 30$), as the Friedewald formula could not be applied to calculate LDL cholesterol concentrations.

2.3. Ultrasound measurements

Ultrasound studies were measured by Sequoia 512 ultrasound mainframes (Acuson, CA) with a 13.0 MHz linear array transducer, as previously described [19]. In short, to assess the carotid artery compliance indices the best quality cardiac cycle was selected from the 5-s clip images and manually analyzed to measure systolic and diastolic common carotid diameters, as previously described [20]. To measure the carotid IMT the image was focused on the posterior wall of the left carotid artery. A minimum of four measurements of the common carotid far wall were taken ∼10 mm proximal to the bifurcation to derive mean carotid IMT values [19].

2.4. Genotyping

DNA was extracted from whole blood using a commercially available kit (Qiagen Inc., Hilden, Germany) in 2001. Genotyping of CRP gene polymorphisms −717A > G (rs 2794521), −286C > T > A (rs3091244), +1059G > C (rs1800947), +1444C > T (rs1130864) and +1846G > A

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(rs1205) was performed using the ABI Prism 7900HT Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, Foster City, CA). For SNP +1059 a commercial kit from Applied Biosystems was used (Assay On Demand, C_177490_10 CRP). The other SNPs were genotyped using Assays By Design from Applied Biosystems under standard conditions, with the exception of the triallelic tagSNP, which was genotyped as previously described [16], except for the genotype calling, which was done manually from the PCR run component tab.

2.5. Statistical analysis

Statistical analysis was performed using SPSS versions 13.0 and 14.0 (SPSS Inc., Chicago, IL, USA). Genotypic frequencies were tested at each SNP locus against those expected by Hardy–Weinberg proportions. One-way ANOVA was used to test the heterogeneity of different genotype groups in normally distributed variables (CAC) and Kruskal–Wallis test was used for skewed variables (CRP). Sex by genotype interaction on carotid artery compliance was analyzed by two-way analysis of variance. The CAC–genotype association was additionally tested by two separate trimmings; after a random split of the data to two cohorts and in a subcohort excluding subjects with CAC values beyond ± 2S.D. from the mean. Linear regression was used to investigate the association between CRP levels and established risk factors as well as between CAC and IMT and risk factors. All those variables showing association ($p < 0.15$) with dependent variable in univariate testing were included in the multivariate model. However, co-linear variables (BMI and waist–hip ratio, blood pressures, insulin and glucose) were not included together into the model but rather the one showing higher association was selected for the model. After this all non-significant ($p > 0.05$) variables were dropped from the model one by one beginning from the least significant variable. Haplotypes were estimated from the five SNPs using the PHASE v2.0.2 program, which uses a Bayesian statistical method for reconstructing haplotypes from population genotype data, and lists the most probable haplotype pairs (Hp) for each individual. Individuals with complete data on all five SNP genotyping results were included in the haplotype estimation procedure ($n = 1992$). Of these, the program did not yield reliable results for eight individuals, and they were eliminated from the analysis, leaving us with 1984 individuals. Haplotype alleles were coded as haplotype numbers from 1 to 14 according to frequency in all, and the results are shown in the order −717, −286, +1059, +1444 and +1846, e.g. haplotype 1 is A-T-G-T-G.

3. Results

Of the five CRP gene SNPs selected for analysis, two are promoter region polymorphisms (−717 and −286), one is exonic (+1059) and two are in the 3′UTR region (+1444 and +1846). CRP −286 genotyping was successful in 2135 subjects, CRP −717 in 2153 subjects, CRP +1059 in 2281

Table 1
Median CRP and mean CAC in females and males by CRP genotypes

SNP	Females				Males			
	CRP[a] (mg/L) (*N*)	*p*	CAC (%/10 mmHg) (*N*)	*p*	CRP[a] (mg/L) (*N*)	*p*	CAC (%/10 mmHg) (*N*)	*p*
−286								
CC	0.51 (312)	<0.0001	2.29 (410)	0.380	0.45 (299)	0.001	2.06 (325)	0.005
CT	0.75 (367)		2.31 (466)		0.57 (340)		2.01 (391)	
TT	0.88 (127)		2.43 (151)		0.71 (103)		1.88 (114)	
CA	1.07 (63)		2.28 (82)		0.81 (59)		2.13 (68)	
TA	1.24 (39)		2.32 (53)		0.74 (38)		1.75 (43)	
−717								
AA	0.83 (566)	0.004	2.30 (746)	0.682	0.58 (507)	0.797	1.98 (575)	0.396
AG	0.57 (313)		2.33 (389)		0.53 (297)		2.03 (330)	
GG	0.64 (35)		2.40 (45)		0.78 (33)		1.90 (33)	
+1059								
GG	0.75 (840)	0.041	2.33 (1076)	0.072	0.59 (787)	<0.0001	1.99 (887)	0.102
GC + CC	0.62 (122)		2.21 (161)		0.36 (111)		2.10 (121)	
+1444								
CC	0.61 (395)	0.004	2.29 (519)	0.285	0.70 (344)	0.007	2.05 (407)	0.030
CT	0.79 (386)		2.30 (548)		0.82 (386)		1.99 (476)	
TT	0.90 (123)		2.40 (165)		1.05 (123)		1.88 (122)	
+1846								
GG	0.84 (399)	0.019	2.33 (495)	0.053	0.89 (358)	0.024	1.91 (396)	0.003
GA	0.74 (429)		2.33 (560)		0.76 (384)		2.06 (468)	
AA	0.51 (120)		2.17 (166)		0.61 (103)		2.04 (123)	

[a] Subjects with CRP values >10 mg/L, triglycerides above 4 mmol/L, history of recent infection, chronic rheumatic disease, diabetes, lactating women and pregnant women were excluded from the analysis.

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Table 2
Univariates and adjusted multiple linear regression model of (log)CRP in females and males

Variable	Females				Males			
	Univarite (n = 760–964)		Multivariate (n = 876)		Univarite (n = 801-898)		Multivariate (n = 791)	
	$B \pm$ S.E.	p	$B \pm$ S.E.	p	$B \pm$ S.E.	p	$B \pm$ S.E.	p
BMI (kg/m^2)	0.046 ± 0.003	<0.0001	0.020 ± 0.004	<0.0001	0.050 ± 0.004	<0.0001	0.017 ± 0.005	0.001
Waist	0.002 ± 0.000	<0.0001			0.002 ± 0.000	<0.0001		
Age (years)	−0.010 ± 0.003	0.001	−0.008 ± 0.003	0.003	0.010 ± 0.003	0.001		
Daily smoking (no/yes)	−0.003 ± 0.039	0.931			0.094 ± 0.033	0.005	0.154 ± 0.031	<0.0001
HDL cholesterol (mmol/L)	−0.008 ± 0.053	0.888			−0.290 ± 0.054	<0.0001	−0.158 ± 0.054	0.003
LDL cholesterol (mmol/L)	0.047 ± 0.021	0.025			0.071 ± 0.017	<0.0001		
(log)Triglycerides (mmol/L)	0.324 ± 0.027	<0.0001	0.135 ± 0.027	<0.0001	0.549 ± 0.070	<0.0001		
Diastolic BP (mmHg)	0.010 ± 0.002	<0.0001			0.011 ± 0.002	<0.0001	0.003 ± 0.002	0.040
Systolic BP (mmHg)	0.007 ± 0.001	<0.0001			0.006 ± 0.001	<0.0001		
(log)Insulin (mU/L)	0.717 ± 0.065	<0.0001			0.474 ± 0.061	<0.0001	−0.181 ± 0.076	0.018
Hormonal contraceptives[a] (yes/no)	0.284 ± 0.031	<0.0001	0.275 ± 0.028	<0.0001	–	–		
Glucose (mmol/L)	0.102 ± 0.036	0.005			0.086 ± 0.036	0.017		
(log)Leptin (mU/L)	0.837 ± 0.050	<0.0001	0.513 ± 0.066	<0.0001	0.682 ± 0.045	<0.0001	0.610 ± 0.072	<0.0001
Alcohol (no. drinks per week)	−0.001 ± 0.003	0.807			0.002 ± 0.001	0.199		
Physical Activity Index	−0.003 ± 0.001	0.027			−0.002 ± 0.001	0.073		
Carriage of −286 C-allele	−0.150 ± 0.042	<0.0001	−0.123 ± 0.034	<0.0001	−0.110 ± 0.042	0.009	−0.123 ± 0.037	0.001

Adjusted model R^2 = 0.36 in females and model R^2 = 0.28 in males.
[a] Hormone pills, intrauterine device or subcutaneous capsule.

subjects, CRP +1444 in 2281 and CRP +1846 in 2243 subjects. The genotype distributions of the SNPs did not deviate from the Hardy–Weinberg equation.

After exclusion of subjects with CRP values > 10 mg/L (n = 74), triglycerides above 4 mmol/L (n = 30), history of recent infection (n = 129), chronic rheumatic disease (n = 36), diabetes (n = 26), lactating women (n = 2) and pregnant women (n = 62), a strong association was found between CRP genotypes and CRP concentration (Table 1). SNPs −286C > T > A, +1444C > T, +1846G > A and +1059G > C were associated with CRP in both sexes, whereas −717A > G showed an association with CRP only in females. To assess the independent effect of −286 C-allele carriage on (log)CRP in females, a multiple linear regression model was constructed with (log)CRP as the dependent variable and age, LDL-cholesterol, (log)triglycerides, (log)leptin, (log)insulin, body mass index, physical activity index, use of hormonal contraceptives, diastolic blood pressure and −286 C-allele carriage as independent variables (Table 2). The variables which remained significantly associated with (log)CRP in female subjects were age, body mass index, (log)triglycerides, use of hormonal contraceptives, (log)leptin and −286 C-allele carriage ($p < 0.0001$; Table 2). Except for the genotype, the associations of these confounding factors with CRP levels have been published previously [21]. Similarly, the associations between other SNPs and CRP remained significant after adjustment for risk factors (−717 G-carriers p = 0.007, +1059 C-carriers p = 0.004, +1444 T-carriers p = 0.001 and +1846 A-carriers p = 0.002).

A corresponding multiple linear regression model was constructed for males with (log)CRP as the dependent variable and age, daily smoking, HDL-cholesterol, (log)leptin, physical activity index, diastolic blood pressure, (log)triglycerides, (log)insulin, body mass index and CRP −286 C-allele carriage as independent variables. The variables which remained significantly associated with (log)CRP in males were HDL cholesterol, body mass index, diastolic blood pressure, daily smoking, (log)leptin, (log)insulin and SNP −286 C-allele carriage (p = 0.001; Table 2). The association between other SNPs and CRP also remained significant, except for +1444, which attenuated to the null after adjusting for the above-mentioned factors (+1059 C-carriers p = 0.002, +1444 T-carriers p = 0.070 and +1846 A-carriers p = 0.001).

Importantly, −286C > T > A, +1444C > T and +1846G > A were associated with CAC in males, but no association was found in females (Table 1). There was strong interaction between SNP −286 and sex in relation to CAC values (p = 0.006). The association between CAC and SNP −286 in men was additionally analyzed in two randomly split halves of the male cohort and in a subcohort of men whose CAC was within 2S.D. from the overall mean. This replicated the results (p = 0.007, 0.04 and 0.005, respectively) suggesting that type II error (false positive) due to outliers is an unlikely explanation to our findings. SNP −286 was also associated with blood pressures in males (systolic CC 127.5 mmHg, CT 129.9 mmHg, TT 129.8 mmHg, CA 129.7 mmHg and TA 132.8 mmHg, p = 0.045; diastolic CC 73.9 mmHg, CT 75.6 mmHg, TT 74.9 mmHg, CA 74.8 mmHg and TA 77.8 mmHg, p = 0.031), but not in females. CAC correlated inversely with diastolic and systolic blood pressure in both sexes (diastolic males $r = -0.34$, females $r = -0.32$, $p < 0.0001$; systolic males and females $r = -0.41$, $p < 0.0001$). Associations of CAC with other variables in males are shown in Table 3.

In linear regression analysis in males with CAC as the dependent variable and body mass index, age, daily smoking, systolic blood pressure, physical activity index, LDL-cholesterol, (log)triglycerides, alcohol consumption

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Table 3
Univariates and adjusted multiple linear regression model of carotid artery compliance (CAC) in males

Variable	Univariate model		Adjusted model (model $R^2 = 0.29$)	
	$B \pm$ S.E.	p	$B \pm$ S.E.	p
BMI (kg/m^2)	−0.045 ± 0.005	<0.0001	−0.020 ± 0.006	0.001
Age (years)	−0.041 ± 0.004	<0.0001	−0.038 ± 0.004	<0.0001
Daily smoking (no/yes)	0.146 ± 0.045	0.001	0.090 ± 0.045	0.047
Systolic BP (mmHg)	−0.020 ± 0.001	<0.0001	−0.018 ± 0.002	<0.0001
Physical Activity Index	0.004 ± 0.001	0.005	0.0003 ± 0.001	0.006
Carriage of −286 C-allele	0.192 ± 0.058	0.001	0.151 ± 0.05	0.005
HDL cholesterol (mmol/L)	0.135 ± 0.074	0.069	–	–
LDL cholesterol (mmol/L)	−0.101 ± 0.022	<0.0001	–	–
(log)Triglycerides (mmol/L)	−0.460 ± 0.090	<0.0001	–	–
Diastolic BP (mmHg)	−0.025 ± 0.002	<0.0001	–	–
(log)CRP (mg/L)	−0.207 ± 0.041	<0.0001	–	–
Waist–hip ratio	−2.650 ± 0.318	<0.0001	–	–
Drinks per week	0.003 ± 0.002	0.144	–	–

and −286 C-allele carriage as independent variables, the association between −286 allele C-carriers and increased CAC remained significant after adjusting for age, body mass index, systolic blood pressure, daily smoking and physical activity (Table 3; $p = 0.005$). Alternative adjustment for waist-to-hip ratio instead for BMI and HDL-cholesterol instead of LDL-cholesterol did not change the result. Interestingly, CRP concentration had no independent effect on CAC, but carriage of CRP gene SNP 286C > T > A C-allele was an independent risk factor for CAC. There were no independent associations between other CRP gene polymorphisms and CAC in men.

Haplotype analysis revealed 14 haplotypes (h), 5 of which were common (frequency 5% or greater), with frequencies of 35.0, 30.1, 20.7, 6.3 and 6.0 (Table 4). The haplotypes formed five common (frequency 5% or greater) haplotype pairs (Hp), with the frequencies of Hp1 20.1, Hp2 14.9, Hp3 13.1, Hp4 12.7 and Hp5 9.0 (Table 4). C-reactive protein levels were significantly different according to haplotype pairs, Hp4 having the highest median [IQR] values and Hp5 the lowest (Hp1 0.72 [1.1] mg/L, Hp2 0.58 [1.2] mg/L, Hp3 0.51 [1.0] mg/L, Hp4 0.76 [1.4] mg/L and Hp5 0.48 [1.0] mg/L, $p = 0.001$). Separate analyses for males and females showed that the CRP levels did not significantly differ between the male haplotype pairs ($p = 0.106$, Kruskal–Wallis test), but that a significant difference was found for females ($p = 0.006$, Kruskal–Wallis test), where Hp4 had the highest CRP levels and Hp5 the lowest (Hp4 0.88 and Hp5 0.52 mg/L, $p = 0.01$, Mann–Whitney test). No differences in CAC were found between haplotype pairs in males or females.

Table 4
Haplotype and haplotype pair frequencies of CRP −717, −286, +1059, +1444 and +1846 SNPs in young Finns ($n = 1984$)

Haplotype	Frequency	Haplotype pairs	Frequency
1. A-T-G-T-G	0.350	1. ACGCA/ATGTG	0.201
2. A-C-G-C-A	0.301	2. ATGTG/GCGCG	0.149
3. G-C-G-C-G	0.207	3. ACGCA/GCGCG	0.131
4. A-C-C-C-A	0.063	4. ATGTG/ATGTG	0.127
5. A-A-G-C-G	0.060	5. ACGCA/ACGCA	0.090
6. A-C-G-C-G	0.009	6. ATGTG/AAGCG	0.047
7. A-C-G-T-G	0.003	7. ACCCA/ATGTG	0.044
8. A-T-G-C-G	0.001	8. ACGCA/AAGCG	0.042
Other	0.006	9. ACGCA/ACCCA	0.039
		10. GCGCG/GCGCG	0.038
		11. ACCCA/GCGCG	0.029
		12. AAGCG/GCGCG	0.023
		Other	0.042

Haplotypes can be scrutinized in several different ways. We used an additional approach to summarize the haplotype data, a haplotype carriage approach. Table 5 shows the CRP and CAC values according to haplotype carriage in males and females. In males, both CRP and CAC differed among carriers and non-carriers of haplotype 1 ($p = 0.01$ and 0.029, respectively), CAC differed among haplotype 2 carriers and non-carriers ($p = 0.005$) and CRP differed among haplotype 4 carriers and non-carriers ($p < 0.0001$). In females CRP values differed between haplotypes 1, 3, 5 carriers and non-carriers ($p = 0.007$, 0.001 and 0.001, respectively) but no significant differences were found in the CAC values.

4. Discussion

In this population-based study, both CRP genotypes and haplotypes were associated with CRP levels. In men, three of the five analyzed polymorphisms in the CRP gene were also associated with carotid artery compliance. Of these, a CRP gene promoter region polymorphism, −286C > T > A, remained an independent predictor of carotid artery compliance after adjustment for risk factors and this effect was also seen at the haplotype level. In women, variants in the CRP gene were not associated with carotid artery compliance.

Previously Carlson et al. [16] studied the association between CRP haplotypes and circulating CRP levels. They sequenced the whole CRP gene and found eight haplotypes (frequency > 1%) in the CRP gene region composed of seven selected SNPs. Our study shared three of the SNPs, namely −286, +1059 and +1846. Although it is not possible to exactly compare the haplotype structure in these populations, it seems that allele frequencies of these three SNPs are very

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Table 5
Comparison of median CRP and mean CAC values according to common CRP gene haplotype carriage in males and females

Carriage of haplotype	Males						Females					
	N	CRP (IRQ) (mg/L)	*p*	*N*	CAC (S.E.) (%/10 mmHg)	*p*	*N*	CRP (IRQ) (mg/L)	*p*	*N*	CAC (S.E.) (%/10 mmHg)	*p*
h1												
ATGTG+	441	0.63 (1.06)	0.01	502	1.96 (0.03)	0.029	489	0.79 (1.56)	0.007	622	2.35 (0.03)	0.17
ATGTG−	327	0.49 (0.95)		361	2.06 (0.03)		357	0.60 (1.36)		468	2.29 (0.03)	
h2												
ACGCA+	394	0.52 (0.91)	0.12	446	2.06 (0.03)	0.005	420	0.66 (1.47)	0.14	557	2.32 (0.03)	0.97
ACGCA−	374	0.61 (1.13)		417	1.93 (0.03)		426	0.79 (1.54)		533	2.32 (0.03)	
h3												
GCGCG+	303	0.54 (1.04)	0.94	336	2.03 (0.04)	0.34	323	0.58 (1.26)	0.001	399	2.35 (0.04)	0.43
GCGCG−	465	0.59 (0.97)		527	1.98 (0.03)		523	0.81 (1.55)		691	2.31 (0.03)	
h4												
ACCCA+	82	0.32 (0.76)	<0.0001	90	2.07 (0.07)	0.25	111	0.77 (1.25)	0.21	147	2.23 (0.06)	0.10
ACCCA−	686	0.59 (1.03)		773	1.99 (0.02)		735	0.70 (1.53)		943	2.34 (0.03)	
h5												
AAGCG+	89	0.82 (1.55)	0.06	103	1.96 (0.07)	0.57	98	1.07 (2.05)	0.001	130	2.29 (0.06)	0.56
AAGCG−	679	0.55 (0.97)		760	2.00 (0.02)		748	0.67 (1.44)		960	2.33 (0.03)	

Haplotype is composed of SNPs −717A > G/−286C > T > A/+1059G > C/+1444C > T/+1846G > A.

similar, and the frequencies of the haplotypes where alleles of −286, +1059 and +1846 are included are almost identical. Indeed, comparison of the effects of these haplotypes on CRP levels indicated that the associations were comparable. In the present study, the male carriers of haplotype 2 had higher CAC values than the non-carriers, and the male carriers of haplotype 1 had lower CAC than the non-carriers, showing that −286C allele carriage has an impact also at the haplotype level.

The Mendelian randomization approach uses genetic variants associated with CRP level as indicators of differences in life-long CRP exposure. The inheritance of genes is determined by the random assortment of maternal and paternal alleles at the time of gamete formation and is therefore independent of behavioural risk factors and environmental influences. Considering that the genetic variants are strongly associated with circulating CRP concentration, they may be considered as a confounding-free marker of CRP exposure. Mendelian randomization studies have provided no consistent support for a causal association of CRP with CHD, blood pressure, components of metabolic syndrome or carotid intima-media thickness [13,14,22–25], but due to methodological limitations or insufficient sample size [14], these studies have not been able to convincingly exclude the causality either. In this study, we demonstrate a direct effect of CRP genotype on one of the early markers of atherosclerotic changes, carotid artery compliance (CAC). Carotid artery compliance measures the ability of the arteries to expand in response to pulse pressure caused by cardiac contraction and relaxation. Decreased elasticity of large arteries is thought to represent an early risk factor or risk marker for cardiovascular disease. Elasticity of proximal large arteries is a consequence of a high elastin to collagen ratio in the arterial wall. Decreased arterial elasticity has been shown to be an independent predictor for cardiovascular events and mortality in high-risk individuals [26].

According to the principles of Mendelian randomization [27], associations of the CRP polymorphisms with circulating CRP levels and CAC and an association of the CRP level with CAC supports causal link between CRP and CAC. In this study, we found expected significant association between the genetic variant and CAC in men, but levels of circulating CRP were not independently associated with CAC, although univariate analysis showed a weak association (males $r = -0.18$, $p < 0.001$; females $r = -0.06$, $p =$ ns). This is in agreement with previous studies of this cohort and other study populations showing a non-significant association between circulating CRP and IMT and coronary artery calcification (a correlate of atherosclerosis) after adjustment for risk factors [24,28] and suggests that the association of the polymorphism −286C > T > A with CAC may be driven by pathological mechanisms other than circulating CRP levels.

There are at least two potential explanations for the lack of an association between circulating CRP and markers of atherosclerosis, such as CAC. First, although circulating CRP is a good correlate of overall inflammation, it is not necessarily informative about the local CRP concentration in the arterial wall. In terms of atherosclerosis pathology, the local CRP concentration is likely to be more important than circulating CRP. Compared to normal artery, Yasojima et al. detected 10-fold concentration of CRP and CRP mRNA in arterial plaque tissue [2]. One possible explanation is that there is a microenvironment in the intima, where the local production of CRP results in higher levels than in circulation, creating the potential for autocrine/paracrine loops among cells in the atherosclerotic lesion (macrophages, endothelial

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cells and smooth muscle cells) as suggested by Venugopal et al. [29]. This could be one reason for lack of association between CAC and circulating CRP, i.e. measurement of the centrally produced CRP by the liver may be an imprecise reflection of a locally produced CRP that is probably more important in atherosclerosis.

Second, the measured circulating CRP may not detect all the CRP molecules in the vasculature. The existence of modified/monomeric CRP (mCRP) adds complexity to this picture. CRP has been shown to dissociate to its subunits to form monomeric CRP molecules (mCRP) within few hours after binding to plasma membrane [30], and these monomeric subunits undergo a conformational change that significantly modifies CRP structure, solubility and antigenity. Both CRP isoforms (pentameric and subunits) are proposed to play a role in inflammation and may participate in the pathogenesis of cardiovascular disease. However, mCRP is a naturally occurring stable protein which is found in fibrous tissues of normal human blood vessel intima rather than in plasma [31]. In addition, recently Ji et al. reported that there is also a third form of CRP; a biologically active structural intermediate called mCRPm. According to this study, binding of pentameric CRP to membranes, including liposomes, lead to a rapid but partial structural change, producing molecules that express CRP subunit antigenicity but with retained native pentameric conformation [32]. The formation of mCRPm is associated with significantly enhanced complement fixation. However, it is not known if these structural variants of CRP are measured by turbidometric means. Although correlation between circulating CRP and mCRP concentrations probably exists, we do not know how good the correlation is.

In this study, men and women were analyzed separately. This was due to the strong interaction between sex and −286 in relation to CAC ($p = 0.006$); the alleles which associated with low CAC values in men were associated with high CAC values in women and vice versa. In addition, the females are approximately 10 years "younger" than men in relation to CAC values, when the CAC values are stratified by age [20] and in a previous study of this cohort, CRP was more strongly associated with IMT in men than in women [28]. Although the association between SNP −286 and CAC in men was robust in the present study, we cannot totally exclude the possibility that it was a false positive finding. To scrutinize this possibility, we tested our data against outliers, which is a common reason for false positive findings and observed that the associations remained unchanged. A further point against false negative finding involves unadjusted associations. Chance is a less likely explanation for the fact that actually three of five polymorphisms were associated with CAC among men (only one remained significant after adjustment for risk factors).

CAC is inversely associated with blood pressure. This was also observed in the present study: diastolic and systolic blood pressure correlated inversely with CAC in males and females and such converging evidence may reduce the likelihood of type II (false positive) error. However, the effect of SNP −286 genotype on blood pressures in males was much weaker than that on CAC. At the haplotype level, the carriage of haplotype 1 (ATGTG) was associated with higher diastolic and systolic blood pressure in males (systolic, carriers 130.3 mmHg versus non-carriers 128.0 mmHg, $p = 0.01$; diastolic, carriers 75.6 mmHg versus 74.3 mmHg, $p = 0.04$). However, no difference was found between subjects with different haplotype pairs. Interestingly, another marker of early atherosclerosis, intima-media thickness (IMT), was measured in our study and a US study but showed no association with CRP genetic variance [13,25]. It therefore seems that the CRP genetic variants are outcome specific being associated with CAC but not IMT.

In conclusion, this is apparently the first study to show an independent association between a CRP gene promoter region polymorphism, −286C > T > A, and carotid artery compliance in men. Further research is needed to determine whether this association is generalizable to other populations.

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