ATTE HAARALA

Inflammation and Early Atherosclerosis

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ACADEMIC DISSERTATION
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To my family

Contents

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 Haarala A, Eklund C, Pessi T, Lehtimäki T, Huupponen R, Jula A, Viikari J, Raitakari O, Hurme M (2009): Use of combined oral contraceptives alters metabolic determinants and genetic regulation of C-reactive protein. The Cardiovascular Risk in Young Finns Study. Scandinavian Journal of Clinical & Laboratory Investigation 69(2):168-74.

II Jylhävä J*, Haarala A*, Eklund C, Pertovaara M, Kähönen M, Hutri-Kähönen N, Levula M, Lehtimäki T, Huupponen R, Jula A, Juonala M, Viikari J, Raitakari O, Hurme M (2009): Serum amyloid A is independently associated with metabolic risk factors but not with early atherosclerosis: the Cardiovascular Risk in Young Finns Study. Journal of Internal Medicine 266(3):286-95.

III Haarala A, Kähönen M, Eklund C, Jylhävä J, Koskinen T, Taittonen L, Huupponen R, Lehtimäki T, Viikari J, Raitakari OT and Hurme M (2011): Heart rate variability is independently associated with C-reactive protein but not with Serum amyloid A. The Cardiovascular Risk in Young Finns Study. European Journal of Clinical Investigation 41(9):951–7.

IV Jylhävä J*, Haarala A*, Kähönen M, Lehtimäki T, Jula A, Moilanen L, Kesäniemi YA, Nieminen MS and Hurme M (2011): Pentraxin 3 (PTX3) is associated with cardiovascular risk factors: the Health 2000 Survey. Clinical & Experimental Immunology 164(2):211-7.

V Haarala A, Kähönen M, Lehtimäki T, Aittoniemi J, Jylhävä J, Hutri-Kähönen N, Taittonen L, Laitinen T, Juonala M, Viikari J, Raitakari OT and Hurme M (2012): Relation of high cytomegalovirus antibody titers to blood pressure and brachial artery flow-mediated dilation in young men: the Cardiovascular Risk in Young Finns Study. Clinical & Experimental Immunology 167(2):309-16.

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Abbreviations

Apo	apolipoprotein
BMI	body mass index
CAC	carotid artery compliance
CD	cluster of differentiation
Cdist	carotid artery distensibility
CMV	cytomegalovirus
COCs	combined oral contraceptives
CRP	C-reactive protein
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
ERT	estrogen replacement therapy
FcγR	constant/crystal fragment γ receptors
FMD	flow-mediated dilation
GWAS	genome-wide association study
HDL	high-density lipoprotein
HF	high frequency
HRV	heart rate variability
IDO	indoleamine 2,3 dioxygenase
Ig	immunoglobulin
IL	interleukin
IMT	intima-media thickness
INF	interferon
IUD	intrauterine device
LDL	low-density lipoprotein

LF	low frequency
LPS	lipopolysaccharide
MCP-1	monocytes chemoattractant protein 1
mRNA	messenger ribonucleic acid
PPARγ	peroxisome proliferator-activated receptor γ
PTX3	pentraxin-3
RMSSD	square root of the mean squared difference of successive R-Rs
SAA	serum amyloid A
SDNN	standard deviation of R-R intervals
SNP	single nucleotide polymorphism
Th	T helper cell
TNF	tumor necrosis factor
TP	total power
T-reg	regulatory T cell
VCAM-1	vascular cell adhesion molecule-1
α7nAChR	alpha 7 nicotinic acetylcholine receptor

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

Abstract

Traditionally atherosclerosis has been considered merely as a lipid storage disease. It has been known that the excess lipid molecules accumulate in the artery walls, which eventually leads to narrowing of the lumen. Nowadays it is also known that atherosclerosis is a chronic inflammatory process that will develop over decades of human life. During that process the artery wall loses its normal function. The feared end-points of atherosclerosis are cardiovascular diseases, which are the main reasons for disability and mortality in the world. Typical atherosclerotic risk factors have been known for decades: age, male sex, smoking, diabetes, high blood pressure and high cholesterol values. In addition to these traditional risk factors many inflammatory parameters have been shown to be increased in cardiovascular disease patients as well in people who had atherosclerotic changes in the arteries. The C-reactive protein (CRP) especially has been shown to be a significant independent biomarker. It has also been shown that preventive treatment for patients with increased CRP values is beneficial. Many other inflammatory markers or immunity mediators have been related to atherosclerosis and cardiovascular disease risk. However, only limited information exists about the role of these factors in healthy people and whether they can be considered as independent risk factors for early atherosclerosis.

Data from two large Finnish cohorts were used in this dissertation. The Cardiovascular Risk in Young Finns Study, an ongoing follow-up study involving participants between 24 and 39 years of age in the 21-year follow-up conducted in 2001 (n=2,283). Inflammatory markers, CRP and serum amyloid A (SAA), as well as cytomegalovirus (CMV) antibodies were measured from the participants of the Cardiovascular Risk in Young Finns Study. In the data analysis we found that CRP levels were significantly higher in those women who used combined oral contraceptives. Triglyceride levels were also elevated in combined oral contraceptive users and triglyceride levels were associated

with elevated CRP levels. Additionally, the effect of oral contraceptive use on CRP levels was so decisive that it overwhelmed the effect of CRP genetics on CRP values. Increased SAA concentrations were also associated with use of combined oral contraceptives. Interestingly, the use of an intrauterine device was associated with decreased SAA values. SAA levels were also associated directly with body mass index, leptin (a hormone secreted by adipose tissue) and with HDL cholesterol or its surface apoliprotein-A1. SAA levels correlated with early vascular changes but these associations were not independent in multivariate models. Decrease in heart rate variability has been shown to be a marker of dysregulation of the autonomic nervous system. We demonstrated that heart rate variability is independently associated with CRP but not with SAA levels. High CMV antibody titers was shown to be related with blood pressure values and inversely with endothelial function in men. These relations were not seen in women.

The Health 2000 Study included 1,867 participants between 46 to 76 years of age. PTX3 levels were measured from the participants of the Health 2000 Study. PTX3 levels were associated with atherosclerotic risk factors, including LDL cholesterol levels, pulse pressure and indoleamine 2,3 dioxygenase levels. There was no relation between early vascular changes and PTX3 values.

In conclusion, measured inflammatory markers were related to several atherosclerotic risk factors such as metabolic and blood pressure values. However, only high CMV antibody levels were independently associated with unfavourable changes in vascular function.

Tiivistelmä

Perinteisesti ateroskleroosin ajateltiin olevan ainoastaan rasva-aineenvaihdunnan häiriö, jossa rasvamolekyylit kertyvät ylimäärin valtimoseinämään aiheuttaen valtimon seinämän paksuuntumisen. Nykytiedon valossa ateroskleroosin tärkeänä osana on myös hidas vuosikymmeniä kestävä tulehduksellinen prosessi, jonka aikana normaali valtimoseinämä menettää vähitellen luonnollisen toimintakykynsä. Sen pelätyt seuraukset ovat sydän- ja verisuonitaudit, mitkä ovat suurin syy toimintakyvyttömyydelle ja kuolleisuudelle maailmassa. Ateroskleroosin tyypilliset riskitekijät ovat olleet tiedossa vuosikymmenien ajan: korkea ikä, mies sukupuoli, tupakointi, diabetes, korkeat verenpainearvot ja kohonneet kolesteroliarvot. Näiden perinteisten riskitekijöiden lisäksi monien tulehdusmerkkiaineiden kohonneita pitoisuuksia on mitattu sydän- ja verisuonitautipotilailta kuten henkilöiltä, joilla on ateroskleroottisia muutoksia valtimoissa. Erityisesti C-reaktiivisen proteiinin (CRP) pitoisuuksien on osoitettu olevan merkittävä itsenäinen riskitekijä. Lisäksi uusimmat tutkimukset ovat osoittaneet, että ennaltaehkäisevä hoito on hyödyllistä henkilöille, joilla CRP pitoisuudet ovat koholla. Monien muidenkin tulehdusmerkkiaineiden ja immuuniparametrien on osoitettu olevan yhteydessä ateroskleroosiin ja sydän- ja verisuonitauteihin. Kuitenkin tulehdusmerkkiaineista ja niiden itsenäisistä yhteyksistä varhaisiin valtimotaudin muutoksiin on vain vähän tietoa nuorilla aikuisilla.

Tutkimuksissamme käytimme kahta laajaa suomalaista aineistoa. Lasten ja nuorten sepelvaltimotaudin riskitekijät (LASERI) pitkittäistutkimuksen 21-vuotisseurantaan osallistui yhteensä 2 283 henkilöä vuonna 2001. Tutkittavat henkilöt olivat tällöin iältään 24-39 vuotiaita. LASERI aineistosta mittasimme CRP, seerumin amyloidi A (SAA) ja sytomegalovirus (CMV) vasta-aine pitoisuudet. Tutkimuksessamme huomasimme CRP pitoisuuksien olevan selkeästi korkeammat niillä naisilla, jotka käyttivät yhdistelmäehkäisyvalmisteita. Näillä naisilla oli lisäksi korkeammat

triglyseridipitoisuudet ja ne olivat yhteydessä kohonneisiin CRP tasoihin. Lisäksi yhdistelmäehkäisynvalmisteiden käyttöön yhteydessä ollut CRP tasojen nousun todettiin olevan niin voimakas, että se pystyi peittämään CRP:n geneettisen säätelyn vaikutukset. Seerumin SAA pitoisuuksien osoitettiin myös olevan korkeammat yhdistelmäehkäisyvalmisteita käyttävillä naisilla, kun taas hormonikierukan käyttö liittyi alentuneisiin SAA tasoihin. SAA tasojen osoitettiin olevan vahvasti yhteydessä painoindeksin, rasva-aineenvaihduntaan liittyvän hormonin leptiinin ja HDL kolesterolin tai sen pinnalla esiintyvän apoliproteiini-A1:n kanssa. SAA pitoisuudet korreloivat varhaisten valtimotaudin muutosten kanssa, mutta monimuuttajamallissa yhteys ei säilynyt itsenäisenä selittävänä tekijänä. Sydämen sykevaihtelun vähentymisen on osoitettu olevan merkki autonomisen hermoston epätoiminnasta. LASERI aineistossa pystyimme osoittamaan sydämen sykevaihtelun olevan itsenäisesti yhteydessä CRP tasoihin, mutta SAA tasoihin itsenäistä yhteyttä emme havainneet. Lisäksi pystyimme osoittamaan korkeiden CMV vasta-ainetasojen olevan yhteydessä kohonneisiin verenpainetasoihin ja alentuneeseen verisuonen toimintakykyyn miehillä. Näitä yhteyksiä emme kuitenkaan havainneet naisilla.

Toiseen käytössämme olleeseen aineistoon - Terveys 2000 tutkimukseen osallistui 1 867 henkilöä ja he olivat iältään 46-76 vuotiaita tutkimushetkellä. Terveys 2000 aineistosta mittasimme pentraksiini-3 (PTX3) pitoisuudet. PTX3 tasojen osoitettiin olevan yhteydessä valtimotaudin riskitekijöihin, erityisesti LDL kolesterolin tasoihin, pulssipaineeseen ja indoliamiini 2,3-dioksigenaasin pitoisuuksiin. Varhaisten valtimotaudin muutosten ja PTX3 tasojen välillä emme huomanneet yhteyttä.

Yhteenvetona voidaan todeta, että mittaamamme tulehdusmerkkiaineet olivat yhteyksissä moniin tunnettuihin valtimotaudin riskitekijöihin, kuten rasva-aineenvaihduntaan ja verenpainetasoihin. Kuitenkin vain CMV vasta-ainepitoisuuksilla pystyimme osoittamaan olevan itsenäinen yhteys epäedullisiin muutoksiin valtimonseinämän toiminnassa.

Introduction

Atherosclerosis is the main reason for mortality and disability in the industrialized countries (Walt, 2004). Atherosclerosis is a tedious process that is probably ongoing in some extent in the arteries of all people. The atherosclerotic process can start already in childhood (Oliveira et al., 2010). Atherosclerotic changes have been found one of six teenagers and the prevalence increases with increasing age, with the prevalence over 85% in people over 50 years old (Tuzcu et al., 2001). There are two important pathological mechanisms at the beginning of this process: lipid accumulation in the arteries and inflammation. Through these processes the artery loses its normal capacity to function and the lumen is constricted. This eventually leads to inadequate blood flow in the artery and the manifestation of cardiovascular diseases (CVD) including conditions like coronary artery disease and stroke (Pearson et al., 2003). Decisive preventive measures have been taken in order to decrease the incidence of these diseases. Recommendations for healthier lifestyles have been given by the World Health Organization in order to decrease CVD risk, including measures like decreasing smoking, increasing physical activity, decreasing use of saturated fat and increasing use of vegetables and fruits (2007). In addition, pharmacologic interventions with medications to lower elevated blood pressure, fasting glucose and cholesterol levels have been shown to decrease the CVD risk (2007). Therefore blood pressure, fasting glucose and cholesterol levels measurements have established their role in the preventive examinations of CVD risk patients. However, studies have shown that these so called traditional atherosclerotic risk factors underestimate the risk of CVD in young adults (Akosah et al., 2000; Akosah et al., 2003).

In recent decades, the new pathogenenic mechanism of atherosclerosis has emerged. Inflammation has been shown to play a significant role in the pathogenesis of atherosclerosis from the beginning to end (Libby et al., 2009). However, many details are

still unknown. Although inflammation has an obvious role in the pathogenesis of atherosclerosis, there is no single outstanding inflammatory marker that could reflect this ongoing inflammation in the body. The C-reactive protein (CRP) is the most studied inflammatory marker and increased CRP levels have been shown to be associated and predict future CVD events (Kaptoge et al., 2010). However, there is still controversy about the causal role of CRP and whether it will yield any additional benefit beyond the traditional risk factor assessment (Zacho et al., 2008; Zacho et al., 2010). American recommendations favour the CRP measurement in order to achieve more exact CVD risk assessments but the European recommendation still favours only the measurement of conservative risk factors (Graham et al., 2007; Pearson et al., 2003).

This dissertation focuses on the role of inflammation in the pathogenesis of the atherosclerosis and the role of inflammatory markers in early atherosclerosis. The subjects in the first, second, third and fifth studies were young adults participating in the ongoing Cardiovascular Risk in Young Finns Study. The subjects in Study IV were the subjects of the Finnish Health 2000 Study, including middle-aged and older individuals. Inflammatory markers CRP, Serum amyloid A and pentraxin-3; and cytomegalovirus antibodies, as a marker of past of present infection, were measured. All the markers were determined from the young adults, except the pentraxin-3 levels which were measured from the participants of the Health 2000 Study. In addition, other traditional atherosclerotic risk factors were measured from all these subjects and early atherosclerotic changes of arteries were measured with ultrasound examinations. We aimed to resolve whether these inflammatory markers were related to the early vascular changes and which traditional atherosclerotic risk factors are related to increased inflammation parameters in healthy young adults and older middle-aged adults.

Review of the literature

1. Inflammation and atherosclerosis

Atherosclerosis is a chronic process where the normal arterial wall is thickened and loses its normal capability to function. Atherosclerosis manifests typically in bifurcations of arteries and other places where blood flow is not laminar. The traditional risk factors for atherosclerosis have been known for decades: smoking, overweight, hypertension, diabetes, physical inactivity, saturated fat and cholesterol in the diet (2007). Atherosclerosis pathogenesis includes a highly complex series of inflammatory, metabolic, thrombotic and other known, possibly also some as yet unidentified mechanisms. The feared endpoints of atherosclerosis are cardiovascular diseases (CVD) which are the main causes of death and disability in developed countries (Walt, 2004). Traditionally atherosclerosis has been thought to be solely a lipid accumulation disease in the arteries (Ross and Glomset, 1976). The current view is that inflammation also plays a substantial role in the development and aggravation of atherosclerosis (Libby et al., 2009). There is no single inflammatory cascade behind atherosclerosis; instead all the branches of the immune system are known to participate in the pathogenesis of atherosclerosis.

1.1. Innate immunity in atherosclerosis

Innate immunity is the rapid and blunt arm of human immune defence against pathogens. The elements involved in innate immunity, such as monocytes, dendritic cells, mast cells and platelets have been shown to have a role in the pathogenesis of atherosclerosis (Libby

et al., 2009). The first step of the inflammatory process in atherosclerosis is the recruitment of non-activated monocytes from the arterial lumen to intima. The recruitment involves the attachment of monocytes to activated endothelial cells by leukocyte adhesion molecules (Rocha and Libby, 2009). There are several subtypes of these adhesion molecules; vascular cell adhesion molecule-1 (VCAM-1) especially is known to be important. It is still partly unclear which mediators stimulate the expression of these adhesion molecules. For example, endothelial cells have been shown to express VCAM-1 in response to cholesterol, modified lipoproteins, interleukins and shear stress of haemodynamic flow (Nakashima et al., 1998). Another important mediator for monocytes recruitment to the intima includes specialized cytokines – chemokines, which can chemoattract attached monocytes to migrate into the intima. The most studied pair is the monocyte chemoattractant protein 1 (MCP-1) and its receptor CC-receptor-2. In experimental animal models deficiency in MCP-1 and CC-receptor-2 leads to reduced burden of atheroclerosis (Boring et al., 1998). There are also other known chemokines such as CC-chemokine ligand 5, its receptor CC-receptor 5 and CX3-chemokine receptor 1.

In the intima, monocytes are activated and become macrophages which lead to up-regulation of pattern-recognition receptors, including scavenger receptors and toll-like receptors (Janeway and Medzhitov, 2002; Peiser et al., 2002). These receptors recognize a wide broad range of molecules, including bacterial endotoxins, apoptotic cell fragments and oxidized low density lipoprotein (LDL) particles. Recognization of these particles is followed by the phagocytosis of these particles, especially LDL particles, in atherosclerosis. Eventually, if the macrophages internalize enough lipid particles they transform into lipid rich foam cells. These activated macrophages release growth factors and cytokines which further aggravates inflammation (Hansson, 2005). This migration of monocytes and activation continues even in established atherosclerotic lesions and not just in the initial stages of lesion formation (Swirski et al., 2006).

Monocytes in atherosclerosis have heterogeneity (Libby et al., 2008). In mice, the monocytes that are more proinflammatory are distinguished by the presence of high

levels of Ly6C marker. The less inflammatory monocytes are known for the low levels of Ly6C, but have major surveillance function in homeostasis. Hyperlipidemia stimulates the enrichment of high Ly6C monocytes in mice and the number of high Ly6C monocytes is increased in atherosclerosis (Swirski et al., 2007). These high Ly6C monocytes express high levels of proinflammatory cytokines and other macrophage mediators, including matrix metalloproteinases (Libby et al., 2009). In humans, there is also equivalent distribution of macrophages to proinflammatory and homeostatic. These proinflammatory macrophages are classically activated (M1) and rest alternatively activated (M2). M1 are associated with higher levels of atherosclerosis but this bisection is only a simplification from a larger heterogeneity (Johnson and Newby, 2009).

Mast cells are only a minor fraction of the leukocyte population in the arteries. However, mast cells release many mediators e.g. histamine, leukotrienes, certain serine proteinases and heparin. These factors have many potentially harmful actions. These substances have vasoactive effects, they may act as growth factors and stimulate angiogenesis. In mice models, mast cells have been shown to participate in atherogenesis (Sun et al., 2007). However, the role of mast cells in human atherosclerosis is still uncertain, although there is evidence that mast cells may participate also in human atherosclerosis and CVD especially abdominal aorta aneurysm formation (Kovanen, 2009; Swedenborg et al., 2011).

1.2. Adaptive immunity in atherosclerosis

Active T-cell infiltration is present in atherosclerotic lesions. CD4+ T cells or helper T-cells are a prominent subclass in the inflammation of atherosclerosis. Known antigens for T-cells include oxidized LDL particles, heat-shock proteins and microbial surface proteins (Rocha and Libby, 2009). Four subtypes of helper T-cells are currently known: Th1, Th2, Th17 and Treg cells. Th1 response is aggravated in atherosclerosis and leads to increased secretion of proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These cytokines augment the production of many

inflammatory and cytotoxic molecules in macrophages and vascular cells, further promoting atherosclerosis (Hansson, 2005). The role of Th2 cells is still controversial (Libby et al., 2009). Th2 cells release interleukin-4 (IL-4) and IL-13 cytokines which have been shown to have anti-inflammatory effects by attenuating the deleterious effects induced by IFN-γ. However, some studies suggest that Th2 responses may promote aneurysm formation by inducing elastolytic enzymes (Shimizu et al., 2004). More recently discovered T-cell subtypes, Th17 and Treg cells are probably also involved in the pathogenesis of atherosclerosis. Th17 cells probably drive proinflammatory effects in atherosclerotic lesions, although opposite reports have also been presented (Chen et al., 2010). Treg cells possess anti-inflammatory nature in atherosclerosis. Treg cells can suppress the functions of proinflammatory helper T-cells and produce anti-inflammatory cytokines – IL-10 and transforming growth factor-β (Rocha and Libby, 2009). Transfusion of Treg cells to atherosclerosis susceptible mice has been shown to reduce lesion burden (Ait-Oufella et al., 2006).

One third of T-cells found in human atherosclerotic lesions are CD8+ T-cells (Libby et al., 2009). These cells have the capability to recognize viral antigens and destroy cells with cell-cell contact. Activation of CD8+ T-cells in a mouse model have been shown to cause destruction of smooth muscle cells and macrophages in arteries, further accelerating atherosclerosis (Ludewig et al., 2000). A minor proportion of T-cells in early atherosclerotic lesions are natural killer T-cells. In contrast to other T-cells which recognize protein antigens, the natural killer T-cells' activation is dependent on lipid antigens (Tupin et al., 2004). The activation of natural killer T-cells leads to the production of proinflammatory cytokines and promotion of atherosclerosis.

1.3. Humoral immunity in atherosclerosis

B lymphocytes can secrete antibodies that recognize numerous antigens *e.g.* pathogens' membrane structures. B cells are responsible for humoral immunity. The current evidence indicates that humoral immunity has a protective role in atherosclerosis. B-cells produced

in spleen have anti-atherogenetic effects and splenectomy has been shown to increase the risk of vascular disease (Caligiuri et al., 2002; Witztum, 2002). This phenomenon is probably due to the result of the similarities in epitopes of oxidized LDL and pneumococcal antigen (Binder et al., 2003). These epitopes are phospholipids such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (Shaw et al., 2000). Immunization of rabbits with oxidized LDL has been shown to attenuate atherosclerosis. Also autoantibodies against LDL surface molecule, apolipoprotein B (apoB), epitopes are associated with less atherosclerosis and CVD events in humans (Sjogren et al., 2008). Currently vaccines are developed for humans. It has been shown that Treg stimulation might be one possible mechanism behind these favourable effects of humoral immunization (Wigren et al., 2011).

2. Inflammatory markers

2.1 C-reactive protein

2.1.1. Function of C-reactive protein

C-reactive protein (CRP) is a classic short pentraxin protein (Bottazzi et al., 2006). CRP molecule consists of a five noncovalently associated identical subunits that are arranged symmetrically in a cyclic configuration around a central pore, with the total molecular weight of a 23 kDa (Oliveira et al., 1979). CRP can also be categorized as an acute-phase reactant based on its biological properties. It is produced basically in response to any kind of inflammatory stimulus e.g. during infections, acute injuries, autoimmune inflammatory diseases and malignancies (Wilson et al., 2006). CRP is produced mainly by liver in response to inflammatory signals, most prominently IL-6 and IL-1β. The most important cells that secrete IL-6 and IL-1β are stimulated monocytes and macrophages, endothelial cells, fibroblast cells and plasma cells (Kato et al., 1990; Shearer, 1995; Tocci, 1997). CRP can also be directly synthesized by the cells at the site of inflammation e.g. monocytes, endothelial cells, fibroblasts and adipocytes (Morley and Kushner, 1982).

The function of CRP is to recognize non-familiar particles *i.e.* antigens. Due to its properties, CRP is categorized as a part of innate immunity (Janeway and Medzhitov, 2002). CRP recognizes particles that express phospocholine (Volanakis and Kaplan, 1971). This molecule can be found in numerous pathogens, including *Streptococcus pneumoniae, Clostridium spp., Lactococcus spp., Bacillus spp., Haemofilus influenzae, Neisseria meningitidis* and *N. gonorrhoeae.* In humans phospocholine is located between phospholipids in cell membranes and in normal conditions it is coated so that CRP cannot interact with it. However, in situations when the cell membrane is damaged, CRP is able to interact with the phospocholine and initiate inflammatory response. This could be an

important mechanism in the apoptotic and necrotic cells elimination. In addition, CRP can be bound to nuclear material *i.e.* histones, chromatins and ribonucleic acid particles. In light of all these interactions it has been suggested that the function of CRP could be the removal of disposable materials and thereby possibly prevent the development of autoimmune diseases (Szalai, 2004).

CRP is capable of activating innate immune response via activating complement cascade. C1q is one of the binding sites of CRP (Siegel et al., 1974). CRP stimulates early phases of complement cascade (C1, C2 and C4) without leading to the formation of membrane attack complex C5b-9 (Berman et al., 1986). This is enabled by the presence of a factor H, a molecule that controls complement activation. Factor H is located on the surface of the cell membrane and is able to bind with CRP thereby preventing the further activation of complement. This mechanism enables CRP to conserve late factors of complement and reduce inflammation (Mold et al., 1999).

CRP and phagocytic cells interact via Fcγ receptors. There are four subclasses of Fcγ receptors but only two of them are able to bind with CRP – FcγRI and FcγII (subtypes a and b). These receptors are found on the surface of neutrophils, monocytes and macrophages (Nimmerjahn and Ravetch, 2006). It has been demonstrated that there is allele-specific variation between individuals in these receptors' genomes and their functional differences have been illustrated (Stein et al., 2000). The activation of monocytes and neutrophils via Fcγ receptors leads to the phagocytosis of CRP bound particles and the expression of proinflammatory cytokines (Ballou and Lozanski, 1992). The strength of this activation depends on the number of receptors activated (Mold et al., 1999).

2.1.2. CRP genetics

In twin studies, CRP concentrations were shown to be up to 40 percent hereditary (MacGregor et al., 2004). Human CRP gene is located on 1q23.2 and consists of 2 exons and 1 intron. The gene is polymorphic and contains at least 31 SNPs, of which five have

been a subject of greater interest (Carlson et al., 2005). These five SNPs, -717 A/G, -286 C/T/A, +1059 G/C, +1444 C/T and +1846 G/A; have been shown to associate with CRP concentrations (Ben-Assayag et al., 2007; Eklund et al., 2008; Kovacs et al., 2005). In modern genome-wide association studies (GWAS) considerably more SNPs have been identified as possible genetic regulators of CRP levels. The following loci have been associated with CPR levels: genes encoding CRP, leptin receptor, IL-6 receptor, glucokinase regulator, hepatic nuclear factor 1-α, apolipoprotein E, achaete-scute complex homolog 1, pyrin domain containing 3, IL-1 family member 10, protein phosphatase 1, regulatory subunit 3B, hepatocyte nuclear factor 4-α, RAR-related orphan receptor A, Sal-like 1, poly(A) binding protein, cytoplasmic 4, B-cell chronic lymphocytic leukemia/lymphoma 7B, proteasome assembly chaperone 1, protein tyrosine phosphatase, nonreceptor type 2, G protein-coupled receptor family C group 6 member A and interferon regulatory factor 1 (Dehghan et al., 2011). All in all, these results indicate a wide interplay between genes involving the immune system, metabolism and chronic inflammation on CRP levels regulation. Additionally, genetic studies have shown that CRP gene polymorphisms associated with increased CRP levels are not associated with increased CVD risk (Wensley et al., 2011; Zacho et al., 2008).

2.1.3. Role of CRP in diseases

Under physiological conditions, normal human CRP concentration is approximately 0.8 mg/l (Shine et al., 1981). CRP levels can increase up to 1,000-fold in 24-72 hours in infectious and non-infectious conditions, *e.g.* tissue damage, sepsis and gout. It has been used widely by physicians as a marker of acute inflammation and values <10 mg/l have been considered “negative” and values over 10 mg/l have been considered as an implication of ongoing inflammation in the body. However, *e.g.* modern high-sensitive latex turbidometric immunoassay methods can also precisely measure these “negative” CRP levels. Measurements have revealed that only a minority of people have CRP concentrations in physiological levels. In fact, one third of Americans have CRP concentrations over 3 mg/l (Kushner et al., 2006).

These slightly increased CRP concentrations have been associated with various clinical conditions, including: diabetes mellitus, metabolic syndrome, obesity, cancers, Alzheimer's disease, acute myocardial infarction and stroke (Lavie et al., 2009). The use of CRP as a predictor of CVD has especially been the subject of a wide range of research and it has been shown in numerous publications that increased levels of CRP are a strong biomarker for CVD indices (Kaptoge et al., 2010; Pearson et al., 2003), although criticism has been voiced about the quality of these publications (Hemingway et al., 2010). It is hypothesized that increased levels of CRP indicate an ongoing low grade inflammation in the body and that this could aggravate pathologies such as atherosclerosis. CRP levels can be stratified into values <1 mg/l (low risk), 1-3 mg/l (intermediate risk) and >3 mg/l (high risk) for cardiovascular diseases. It has been suggested that CRP could offer advantage for physicians to categorize intermediate risk patients according to traditional risk factors to either high risk or low risk individuals for CVD (Lavie et al., 2009). Adding CRP value as an additional risk marker into the traditional CVD risk model, the Framingham algorithm, with other risk factors such as age, sex, cholesterol levels and blood pressure values yields more exact predictions (Ridker et al., 2007; Ridker et al., 2008b). Despite the usefulness of CRP in CVD risk prediction, the current view is that CRP is unlikely to be causally related to atherosclerosis and increased CVD risk (Nordestgaard, 2009).

The usefulness of CRP values is not limited to risk prediction. Therapeutic intervention studies with statins have shown that patients whose cholesterol and CRP levels were reduced by therapy were at lower risk of having CVD outcomes when compared to those whose cholesterol levels, but not CRP levels, were reduced (Ridker et al., 2005a; Ridker et al., 2005b). These findings and other evidence have suggested that statins have a dualistic effect; lipid lowering and anti-inflammatory (Libby et al., 2009). The obvious next step was to test the possibility of using statins to lower the CVD risk for those individuals with normal cholesterol levels but increased CRP levels. This indicates that there is likely to be ongoing low-grade inflammation and development of atherosclerosis in these individuals. In a large prospective epidemiological setup, subjects with increased CRP levels (≥2 mg/l) but normal LDL cholesterol levels (<130 mg/dl) were randomized

to take either rosuvastatin 20 mg daily or placebo (Ridker et al., 2008a). During follow-up, the intervention group achieved 44% reduction in CVD end-points compared to those taking placebo. Although it is fair to point out that there was also a significant reduction on LDL cholesterol values in the intervention group. In later analysis it was estimated the number needed to treat value for statin therapy to CVD prevention in 5 years would be 25 (Ridker et al., 2009).

2.2. Pentraxin-3

2.2.1. Function of Pentraxin-3

Pentraxin-3 (PTX3) is a protein belonging to a long pentraxin family. It was first discovered in the 1990s in guinea pig apexin in spermatozoa (Noland et al., 1994). PTX3 is a 45 kDa molecule that assembles to form high molecular weight multimers linked by interchain disulphide bounds (Bottazzi et al., 1997). The C-terminal domain of PTX3 contains homology with classic short pentraxins (*e.g.* CRP), whereas the N-terminal domain does not show any significant homology (Mantovani et al., 2008). PTX3 has remained highly conserved during evolution, unlike CRP. In humans, the serum levels of PTX3 vary from undetectable levels to 800 ng/ml during infections and inflammatory conditions (Muller et al., 2001). While CRP is mainly produced in the liver, PTX3 is produced and released *in situ* by many different cell types, including dendritic cells, macrophages, fibroblasts, endothelial cells, renal cells and neutrophils (Bassi et al., 2009). PTX3 production is stimulated by lipopolysaccharides (LPS), IL-1, IL-10 and TNF-α. IFN-γ has been shown to have an inhibitory effect or no effect on PTX3 expression and interestingly IL-6 seems to have no effect on PTX3 production (Bassi et al., 2009). Genetic polymorphism of PTX3 genome has also been identified and SNP rs6788044 especially is associated with higher PTX3 levels as well as with increased fertility (May et al., 2010). No GWAS on PTX3 has been performed.

PTX3 has been shown to have a role in immunity and inflammation, extracellular matrix construction and female fertility (Mantovani et al., 2008). As a member of innate immunity, the major task of PTX3 is to recognize microbial pathogens, activate complement cascade and stimulate the uptake of pathogens by phagocytes. *In vitro,* after preincubation of apoptotic cells, PTX3 has been shown to interact with complement C1q and to activate complement cascade (Nauta et al., 2003). On the other hand, fluid-phase binding of PTX3 has been shown to inhibit complement activation, giving rise to the idea that PTX3 has a dual role in complement activation. PTX3 can also interact with factor H (Deban et al., 2008). PTX3 binds to apoptotic cells and inhibits their removal via dendritic cells (van Rossum et al., 2004). PTX3 may affect plaque stability by binding to fibroblast growth factor, which plays a major role in the induction, proliferation, migration and survival of vascular smooth muscle cells (Mantovani et al., 2008). PTX3 has also been shown to increase tissue factor expression in mononuclear cells and endothelial cells, and this way PTX3 may promote coagulation (Napoleone et al., 2002; Napoleone et al., 2004).

2.2.2. Role of PTX3 in diseases

PTX3 behaves as an acute phase protein. Under normal conditions PTX3 plasma levels are <2 ng/ml in humans but may rise up to 200-800 ng/ml within 6-8 hours during inflammatory and infectious conditions (Mantovani et al., 2008). PTX3 values correlate with disease severity and have prognostic value in various infectious diseases, such as sepsis, septic shock, tuberculosis and dengue (Huttunen et al., 2011; Muller et al., 2001).

PTX3 is also associated with CVD. PTX3 molecules have been identified in human atherosclerotic plaques (Rolph et al., 2002). Plasma levels of PTX3 rise rapidly during acute myocardial infarction and PTX3 levels have been shown to predict mortality in ST elevation myocardial infarction (Latini et al., 2004; Peri et al., 2000). Also, PTX3 levels have been shown to have elevated in non-acute conditions. For example, in patients with unstable angina pectoris, PTX3 levels were three times higher compared to normal range (Inoue et al., 2007). After hospitalization for unstable angina or non ST elevation

myocardial infarction, PTX3 levels have also been shown to predict future CVD events (Matsui et al., 2010). In a cohort of elderly CVD-free subjects, increased PTX3 levels were shown to predict future CVD and all cause deaths independent of CRP and other traditional risk factors (Jenny et al., 2009). PTX3 has been shown to correlate with the presence of heart failure and aortic valve stenosis (Matsubara et al., 2011; Naito et al., 2010). In addition, PTX3 genetics has been linked with the tissue ischaemia-reperfusion injury and systemic inflammatory response after cardiac bypass surgery (Liangos et al., 2010).

Elevated PTX3 levels have also been identified in some autoimmune diseases, e.g. small vessel vasculitis and rheumatoid arthritis (Fazzini et al., 2001; Luchetti et al., 2000). Chronic kidney disease patients have been shown to have increased PTX3 concentrations and this was associated with increased mortality in these patients (Tong et al., 2007). Thus PTX3 has plausible prognostic values in chronic kidney disease patients. Levels of PTX3 have been shown also to be associated with pregnancy related diseases such as pre-eclampsia (Cetin et al., 2006), excessive maternal inflammatory response and placental vasculopathy (Assi et al., 2007).

2.3. Serum amyloid A

2.3.1. Function of serum amyloid A

Serum amyloid A (SAA) is a group of acute phase proteins that were originally described for their role in AA amyloidosis. Human SAA consists of 104 amino acids (Parmelee et al., 1982). Four SAA genes have been discovered in humans. SAA1 and SAA2 are acute phase SAA and these levels may increase over 1,000 times during inflammation e.g. infections, trauma, tumours and tissue damage. SAA3 is probably a pseudogene. SAA4 is constitutively expressed and is constant but minor component of high density lipoprotein (HDL) (Kisilevsky and Tam, 2002). Previously liver was thought to be the most

important secretion site of SAA. However, it is now known that in nonacute-phase conditions adipose tissue is the major site of SAA production (Poitou et al., 2006; Sjoholm et al., 2005; Yang et al., 2006). It has been also shown that macrophages, vascular smooth muscle cells and endothelial cells can express SAA locally (Meek et al., 1994).

Proinflammatory cytokines (IL-1, IL-6, TNF-α) induce SAA gene expression (Thorn et al., 2004). The role of IL-6 is the most critical because of its synergistic induction of SAA gene when stimulated with other proinflammatory cytokines (Hagihara et al., 2004). The precise mechanism is not known, but it involves steroids as co-stimulators. Oxidized LDL also induces SAA expression (Ray et al., 1999). Conversely, SAA treatment of vascular endothelial cells and monocytes markedly increased the production of IL-6, IL-8, TNF-α and MCP-1 (Yang et al., 2006), indicating that there may well be a positive feedback mechanism between these inflammatory markers. A recent GWAS has identified two genetic loci for SAA: SAA1 and leptin receptor (Marzi et al., 2010), suggesting that there is a close interplay between the genetic regulations of leptin and SAA. A specific SAA receptor has not been identified, but it has been shown that SAA can interact with following receptors: Toll-like receptors 2 and 4, CD36, class B scavenger receptor and G-protein-coupled receptor formyl peptide receptor like 1/lipoxin A4 receptor (King et al., 2011).

SAA has the features of an apolipoprotein. It is produced and it forms a heterogeneous fraction of HDL at normal levels, containing SAA and predominantly apolipoprotein A1 (apoA1). At elevated levels of SAA, it displaces apoA1 from HDL to become the dominant apolipoprotein of HDL (Kisilevsky and Tam, 2002). ApoA1 has a key role in reverse cholesterol transport. This change leads to impaired cholesterol transport from periphery to liver. HDL antioxidative properties are also impaired and this leads to the suggestion that HDL-SAA is a proinflammatory molecule (O'Brien and Chait, 2006). Furthermore, in obese mice, SAA molecules has been detected on apoB-containing lipoproteins VLDL and LDL (King et al., 2011). LDL/SAA complex has also been shown to be associated with obesity, metabolic syndrome and CVD events in Japanese

populations (Kotani et al., 2009; Ogasawara et al., 2004). SAA molecules also exist as a lipid free form in circulation (Malle and De Beer, 1996).

2.3.2. Role of SAA in diseases

Elevated serum SAA levels have been found to be associated with body mass index (BMI), obesity and body fat percentage in adults and also in children and adolescents (Gomez-Ambrosi et al., 2008; Yang et al., 2006; Zhao et al., 2010). SAA is said to be a possible direct link between obesity and inflammatory diseases like atherosclerosis and metabolic syndrome (Yang et al., 2006). Weight loss was found to significantly decrease SAA levels in obese individuals. The insulin sensitivity after weight loss correlated significantly with the decrease in SAA levels (Yang et al., 2006).

SAA is an independent predictor of CVD comparable to CRP or even better (Johnson et al., 2004; Kosuge et al., 2007; Ridker et al., 2000). SAA levels are also elevated in patients who have had myocardial infarctions (Bausserman et al., 1989). SAA is thought to be a prothrombotic and proinflammatory mediator in CVD. SAA has been shown to alter vascular proteoglycans and stimulate the production of proinflammatory mediators in endothelial cells and monocytes (Song et al., 2009; Wilson et al., 2008). Atherosclerotic plaques have been shown to contain SAA deposits (Maier et al., 2005).

SAA is also associated with insulin resistance and diabetes. Serum SAA concentrations are elevated in type 2 diabetics and high SAA concentrations have been shown to predict the development of diabetes (Herder et al., 2006). Tanis is a novel protein that is a possible link between inflammation, diabetes and SAA. Tanis is increasingly expressed in the liver of diabetic rats and diabetes has been shown to lead to dysregulation of Tanis (Walder et al., 2002). Further it was demonstrated that SAA is one ligand of the Tanis receptor. In type 2 diabetic humans Tanis messenger ribonucleic acid (mRNA) expression in skeletal muscle and adipose tissue is positively associated with circulating SAA levels (Karlsson et al., 2004). SAA has also been shown to down-regulate genes

associated with insulin sensitivity (Scheja et al., 2008) and treatment of adipocytes with SAA has been shown to decrease cellular insulin sensitivity (Ye et al., 2009).

Table 1.

Summary of different inflammatory markers features.

	C-reactive protein	Pentraxin-3	Serum amyloid A
Structure	Pentraxin	Pentraxin	Group of proteins with features of an apoliprotein
Main expression site	liver	*in situ*	adipose tissue
Expression stimulators	IL-1β, IL-6	lipopolysacharide, IL-1, IL-10, TNF-α	IL-1, IL-6, TNF-α, oxidized-LDL
Main function	pathogen recognization and opzonization, activation of complement, binding to apoptotic and necrotic cells	pathogen recognization and activation of complement, stimulation of extracellular matrix construction and promote fertility	mainly unknown, has features of an apolipoprotein and is a constituent of HDL cholesterol
Related diseases	Diabetes, metabolic syndrome, obesity, cancers, Alzheimer's disease and cardiovascular diseases	Heart failure, vasculitis, rheumatoid arthritis, chronic kidney disease, pre-eclampsia, placental vasculopathy and cardiovascular diseases	Amyloidosis, obesity, metabolic syndrome, diabetes, insulin resistance and cardiovascular diseases

2.4. Determinants of inflammatory markers

Measuring low concentrations of inflammatory markers and interpretation these results is challenging because many known internal and external atherosclerotic risk factors are associated with these inflammatory markers. The main determinants of CRP, PTX3 and SAA are summarized in Table 2.

Table 2.

Reported effects on inflammatory marker levels of presence or increase of various determinants. Reference numbers in parentheses.

	CRP		PTX3		SAA	
Age	↑	(1)	↑	(2,3,4)	↑ / -	(5,6,7,8,9,10,11)
Male sex	-	(1,12,13,14)	↑ / ↓ / -	(3,4,15)	↓ / -	(9,16,17)
BMI / waist circumference	↑	(1,18)	↑ / ↓ / -	(3,19,20,21)	↑	(16,22,23)
Leptin	↑	(18)	?		↑	(16)
Lipids	↑	(1)	↑ / ↓	(3,20,24)	↑	(9,10)
Blood pressure	↑	(1,25)	(-)	(15,24,26)	-	(9,11,27)
Glucose	↑	(1)	↑ / -	(15,26)	(-)	(9)
Insulin	↑	(1)	↑ / -	(15,26)	(-)	(9)
Metabolic syndrome	↑	(1)	(↑)	(24)	↑	(16,28,29)
Diabetes	↑	(1)	?		↑	(28)
COCs or ERT usage	↑	(13,14,30)	?		↑	(31,32)
Physical activity	↓	(1)	?		↓	(33)
Smoking	↑	(1)	↑	(34)	↑	(35)
Alcohol consumption	↑	(1)	?		-	(36)
CRP			↑	(4,15)	↑	(23)
SAA	↑	(23)	?			

References:

1. Kushner et al., 2006; 2. Matsui et al., 2010; 3. Yamasaki et al., 2009; 4. Jenny et al., 2009; 5. Lannergard et al., 2005; 6. Wu et al., 2007b; 7. Pertovaara et al., 2009; 8. Sondergaard et al., 2004; 9. Gomez-Ambrosi et al., 2008; 10. Johnson et al., 2004; 11. Delanghe et al., 2002; 12. Imhof et al., 2003; 13. Raitakari et al., 2005; 14. Williams et al., 2004; 15. Suliman et al., 2008a+b; 16. Lappalainen et al., 2008; 17. Sjoholm et al., 2005; 18. Viikari et al., 2007; 19. Miyaki et al., 2010; 20. Ogawa et al., 2010; 21. Shim et al., 2010; 22. Meyers et al., 2008; 23. van Dielen et al., 2001; 24. Zanetti et al., 2009; 25. Virdis et al., 2007; 26. Alberti et al., 2009; 27. Danesh et al., 1999; 28. Walder et al., 2002; 29. Yang et al., 2006; 30. Kluft et al., 2002; 31. Abbas et al., 2004; 32. van Rooijen et al., 2006; 33. Bergmann and Siekmeier 2009; 34. Dubin et al., 2010; 35. Rothenbacher et al., 2003; 36. De Bacquer et al., 2006

Deleterious effects on immune system are seen during aging. A direct correlation between age and CRP concentrations has been shown in various populations and in different age cohorts (Kushner et al., 2006). A direct correlation between age and PTX3 has been found in various adult cohorts. High PTX3 levels were associated with increased age in Japanese cohorts consisting of myocardial infarct patients (Matsui et al., 2010) and healthy adults (Yamasaki et al., 2009). In a CVD-free elder Caucasian cohort, there was a significant correlation between age and PTX3 levels (Jenny et al., 2009). Contradictory findings have been published about the correlation between age and SAA. In Swedish and Chinese cohorts with a wide range of subjects of all ages, SAA levels were shown to increase with increasing age (Lannergard et al., 2005; Wu et al., 2007b). In Sjögren's syndrome patients, SAA has also been shown to correlate with age (Pertovaara et al., 2009). On the other hand, in a young Iraqi refugee cohort, SAA levels did not correlate with age (Sondergaard et al., 2004) or in a cohort consisting Spanish children and adolescents (Gomez-Ambrosi et al., 2008). In a large middle-aged Caucasian female cohort with an increased risk of CVD, SAA levels did not correlate with age (Johnson et al., 2004), nor was any correlation seen in another CVD risk cohort (Delanghe et al., 2002). In a different approach, the SAA mRNA expression in adipocytes was not associated with age in obese women (Poitou et al., 2005). One possible explanation for these contradictory results is that age differences in SAA levels can only be detected between persons with a decisive age difference.

Originally it was thought that woman had higher CRP levels than men (Kushner et al., 2006). However, this idea has been questioned after the discovery that women who do not use oral contraceptives or hormone replacement therapy have similar CRP levels to those of men (Imhof et al., 2003; Raitakari et al., 2005; Williams et al., 2004). Contradictory results have been reported for PTX3 and sex based difference. PTX3 levels were higher in women than men in a healthy Japanese cohort (Yamasaki et al., 2009), whereas in CVD-free older American population, PTX3 levels were higher in men (Jenny et al., 2009). In a Swedish haemodialysis patient cohort no significant difference between sexes was observed (Suliman et al., 2008a). Women have been shown to have higher serum SAA levels than men (Lappalainen et al., 2008) and SAA mRNA levels have been

shown to be higher in women (Lappalainen et al., 2008; Sjoholm et al., 2005), whereas in Spanish children and adolescent there were no sex difference (Gomez-Ambrosi et al., 2008). All in all it is still controversial whether there are any sex-based differences in these inflammatory marker levels.

BMI, waist circumference, leptin and other markers to evaluate the composition and function of the body's adipose tissue have been shown to correlate closely with CRP and SAA. Direct associations between CRP and BMI have been seen in all ages and leptin levels especially seem to correlate closely with CRP levels (Kushner et al., 2006; Viikari et al., 2007). SAA levels have also been shown to be directly associated with BMI and leptin (Lappalainen et al., 2008; Meyers et al., 2008; van Dielen et al., 2001), while in the case of PTX3, the results are polarized. Obesity has been associated with both increased (Miyaki et al., 2010) and decreased PTX3 levels (Ogawa et al., 2010; Yamasaki et al., 2009). Variation in these results could be explained by the finding that PTX3 was positively associated with visceral fat but not with BMI (Shim et al., 2010). In any case it has been shown that human adipose tissue releases PTX3 (Alberti et al. 2009). Similar results have been reported between lipids and inflammatory markers. CRP and SAA have been shown to correlate directly with atherogenic lipid profile, with the exception that SAA levels correlate positively with HDL cholesterol (Johnson et al. 2004; Kushner et al. 2006; Gomez-Ambrosi et al. 2008), while in the case of PTX3, inverse correlations between PTX3 and triglyceride levels have been reported (Ogawa et al.; Yamasaki et al. 2009) and contradictory reports have again been presented (Zanetti et al., 2009).

Increased CRP concentrations are related to hypertension, arterial stiffness and increased blood pressure (Kushner et al., 2006; Virdis et al., 2007). However, after adjustment for life course confounding factors in a Mendelian randomization approach, elevated CRP levels are not associated with increased blood pressure values (Davey Smith et al., 2005), while in the case of SAA, there is a relation with hypertension but not with arterial stiffness (Pietri et al., 2006). At population level no correlation was found between SAA and blood pressure (Danesh et al., 1999; Delanghe et al., 2002; Gomez-Ambrosi et al., 2008). The current knowledge on a relation between PTX3 and blood pressure is scarce.

However, some studies have reported non-significant correlations (Suliman et al. 2008a; Alberti et al. 2009; Zanetti et al. 2009).

Glucose and insulin metabolism are also closely related to inflammation and atherosclerosis. CRP levels are associated with increased fasting glucose and insulin levels, insulin resistance, metabolic syndrome and diabetes (Devaraj et al. 2004; Kushner et al. 2006). SAA levels have been shown to be associated with insulin sensitivity and type 2 diabetes (Walder et al. 2002; Yang et al. 2006). However, negative correlations have been reported between SAA and fasting glucose and insulin levels in obese children and adolescents (Gomez-Ambrosi et al., 2008). Weight loss achieved by surgery has been shown to decrease abdominal tissue SAA mRNA expression but this decrease was not associated with glucose and insulin levels (Poitou et al. 2006), whereas in another study, SAA levels correlated with insulin sensitivity in obese women but not in men (Lappalainen et al. 2008). All in all, the relation between SAA, glucose and insulin metabolism is still under debate. PTX3 expression in visceral adipose tissue was shown not to correlate with fasting glucose and insulin levels (Alberti et al. 2009). PTX3 concentrations in plasma had no correlation between fasting glucose and insulin in chronic kidney disease patients (Suliman et al. 2008). However, it seems that PTX3 levels are higher in patients suffering from metabolic syndrome (Zanetti et al. 2009). However, this association is probably due the visceral fat component of metabolic syndrome (Shim et al., 2010).

Use of combined oral contraceptives (COCs) has been shown to markedly increase CRP levels in healthy premenopausal women (Raitakari et al., 2005; Williams et al., 2004). However, this increase occurs without the increase of IL-6 levels, indicating that the COCs stimulate CRP synthesis directly in the liver (van Rooijen et al. 2006). The mechanism is probably due to the estrogen component of COCs because pills containing only progestagen do not elevate CRP levels (Williams et al. 2004) and in postmenopausal women estrogen replacement therapy (ERT) containing only estrogen elevates CRP levels (Kluft et al., 2002). However, there is evidence that this progestagen component in COCs may also affect CRP levels (Buchbinder et al., 2008; Kluft et al., 2002; Skouby et

al., 2002). In postmenopausal women oral estrogen use significantly raises levels of SAA, HDL and HDL-SAA, while transdermal estrogen reduces SAA and HDL-SAA levels (Abbas et al., 2004). In premenopausal women, COCs use has been found to increase SAA levels slightly (van Rooijen et al. 2006). It is not known how COCs or ERT use affects PTX3 levels, but one study has shown that PTX3 mRNA expression in human endometrial stromal cells is regulated by progesterone and estrogen (Popovici et al., 2008).

Unfavorably habits like low physical activity, smoking and alcohol consumption have been associated with increased CRP levels (Kushner et al., 2006). Physical activity and smoking have also been related to SAA levels (Bergmann and Siekmeier, 2009; Rothenbacher et al., 2003) but not alcohol consumption (De Bacquer et al., 2006). PTX3 levels have been associated with smoking (Dubin et al., 2010) but no data are available about a relation to physical activity or alcohol consumption. Other habits have also been shown to affect inflammatory markers, including factors like diet, education and stress (Kushner et al., 2006). However, it is appropriate to mention that the independent effect of these variables is partly unknown.

In addition to the correlations presented above, these inflammatory factors have been shown to intercorrelate. SAA concentrations have been reported to correlate closely with CRP (van Dielen et al. 2001). PTX3 levels also correlate with CRP but not as closely as SAA and CRP (Suliman et al. 2008; Jenny et al. 2009). No correlation between PTX3 and SAA has been reported.

2.5. Early vascular changes and inflammatory markers

Modern ultrasound examinations are capable of detecting atherosclerotic changes in arteries well before any clinical symptoms and these early vascular changes have been linked with increased CVD incidence and mortality in later years (Blacher et al., 1998; Bots et al., 1997; Chambless et al., 1997; Chan et al., 2003). Most of the studies have

investigated the relation between CRP levels and early vascular changes and many of them have shown an association between CRP and impaired vascular function. CRP concentrations have been associated with increased carotid artery intima-media thickness (IMT) (Hulthe et al., 2001; Juonala et al., 2006) but a Mendelian randomization study did not support the causality of this relation (Kivimaki et al., 2007). CRP levels haven been associated with endothelial dysfunction and inversely with brachial artery flow-mediated dilation (FMD) (De Haro et al., 2008; Fichtlscherer and Zeiher, 2000; Juonala et al., 2005; Yudkin et al., 1999). In addition, genetic polymorphisms of CRP have been associated with increased carotid artery distensibility (Cdist) in young adults (Eklund et al., 2008). The term carotid artery compliance (CAC) has also been used in addition to carotid artery distensibility. The latter term is nowadays recommended in order to avoid confusion with coronary artery calcification, which also can be abbreviated as CAC.

Only few studies have investigated the relation between early vascular changes and SAA. In a cohort consisting of elderly Swedish men, there was no correlation between IMT and SAA (Wohlin et al. 2007), whereas in a Finnish cohort consisting of type 2 diabetics, SAA levels correlated with IMT, but were not an independent determinant for IMT in a multivariate model (Leinonen et al., 2004). In a small Japanese cohort SAA values also correlated with IMT, but these results were not adjusted for all the typical atherosclerotic risk factors (Uurtuya et al., 2009). In a genetic approach, polymorphisms of SAA gene have been associated with IMT values (Xie et al.; Carty et al. 2009). However there was significant ethnic variation in these reports. There are no studies available about an association between SAA, Cdist and FMD. However, in an animal model SAA has been related to endothelial dysfunction (Wang et al., 2008).

In chronic kidney disease patients PTX3 levels have been shown to correlate directly with IMT and inversely with FMD (Suliman et al. 2008). It was further shown that an association between PTX3 and FMD remained after adjustment for typical atherosclerotic risk factors such as age, blood pressure, insulin sensitivity index and CRP levels. These results were confirmed in another chronic kidney disease cohort in the case of FMD but not IMT (Yilmaz et al., 2011). A direct correlation between IMT and PTX3 was also

observed in elderly hypertensive patients (Yano et al., 2010). In a study consisting of metabolic syndrome patients, PTX3 levels correlated with IMT but had no independent effect in the multivariate model (Zanetti et al. 2009).

2.6. Inflammatory mediators in the pathogenesis of atherosclerosis

2.6.1. C-reactive protein

Various mechanisms have been proposed for how inflammatory mediators contribute to the pathogenesis of atherosclerosis. *In vitro* CRP can bind to modified LDL cholesterol, especially to non-esterified LDL (Taskinen et al., 2002). CRP may function as a chemotactic substance for monocytes to infiltrate into the intima (Torzewski et al., 2000). CRP may lead to endothelial dysfunction via upregulation of cellular adhesion molecules, decrease in nitrix oxide expression, increase in endothelin-1 release and upregulation of Lectin-like oxidized LDL receptor-1 (Li et al., 2004; Pasceri et al., 2000; Verma et al., 2002). Additionally CRP may be involved in atherothombosis by increasing plasminogen activator inhibitor-1 availability and stability in endothelium (Devaraj et al., 2004). However, overexpression of CRP in human and animal models has not been shown to aggravate atherosclerosis (O'Brien and Chait, 2006).

2.6.2. Serum Amyloid A

In vitro SAA mediates HDL cholesterol binding to differentiated macrophages and endothelial cells (Hayat and Raynes, 2000; Kisilevsky and Tam, 2002). HDL bound SAA impairs the capacity of HDL to promote cholesterol efflux from macrophages (Artl et al., 2000). SAA may also play a role in HDL cholesterol retention in atherosclerotic lesions (O'Brien et al., 2005). In addition, SAA can stimulate the expression of matrix-degrading metalloproteinases and promote chemotaxis and adhesion of monocytes and lymphocytes

(O'Brien and Chait, 2006). SAA may also induce the expression of IL-1β in tissues (Niemi et al., 2011).

2.6.3. Pentraxin-3

PTX3 probably has a dualistic effect on atherosclerosis. On the other hand, there is evidence that PTX3 is expressed in atherosclerotic plaques, and PTX3 positive neutrophils can infiltrate into atherosclerotic plaques (Rolph et al., 2002; Savchenko et al., 2008). On the other hand, PTX3 may have cardioprotective functions by inhibiting inflammatory responses and macrophage accumulation (Norata et al., 2009). PTX3 can also bind to activated circulating platelets and impair platelets proinflammatory and prothrombotic actions (Maugeri et al., 2011). In addition, PTX3 can inhibit angiogenesis and promote restenosis (Camozzi et al., 2005; Inforzato et al., 2011).

3. Inflammation and adipose tissue

3.1. Obesity and inflammation in adipose tissue

Excess gain of nutrition compared to energy consumption leads to weight gain. The World Health Organization defines obesity as having of a body mass index ≥30 kg/m². In addition persons with waist circumference greater than 102 cm for men and 88 cm for women can be considered obese (2002). It is estimated that 300 million adults in the world meet these criteria of obesity. In men the fat accumulates typically centrally and in women accumulation is typically gluteal and femoral. This distribution difference is due to sex steroid hormones. The central or upper fat accumulation is the greatest health risk (Canoy, 2008). This excess nutrition is stored in adipose tissue in the form of lipids. Traditionally adipose tissue was considered merely as a lipid deposit organ. However, nowadays it is known that adipocytes have a more complex role (Attie and Scherer, 2009). One could say that adipose tissue is an endocrine organ with the capability to produce numerous inflammatory mediators.

Adipose tissue contains adipocytes and macrophages. In obese people, these macrophages are activated and produce cytokines such as IL-1, IL-6, TNF-α. In obese people, these cytokines are released into the circulation and elevated cytokine levels have been found in them (Wang and Nakayama, 2010). Cytokines further stimulate the hepatic production of CRP leading to chronic systemic inflammation *i.e.* systemic low grade inflammation. It has been shown that even the number of macrophages in adipose tissue is increased in obesity (Weisberg et al., 2003). Other unfavorable substances are also produced in adipose tissue: a procoagulant substance like plasminogen activator inhibitor-1, vasoactive substances like angiotensinogen and endothelin. Visceral fat especially has been shown to express significant amounts of cytokines and other deleterious substances (Rodriguez et al., 2007).

Macrophages in adipose tissue have the same characteristics as macrophages in atherosclerotic lesions in regulation and impression. Active and resident (alternatively activated) macrophages, *i.e.* more and less inflammatory, can be distinguished from each other and chemokines may also have a role in macrophage infiltration and expression in adipose tissue (Rocha and Libby, 2009). One intriguing finding was the discovery of peroxisome proliferator-activated receptor γ (PPARγ) – a nuclear receptor required for maturation of alternatively activated macrophages. Deficiency of PPARγ in mouse models has led to increased obesity and insulin resistance (Odegaard et al., 2007). Th2 cell derived anti-inflammatory cytokines IL-4 and IL-13 induce PPARγ activation (Kang et al., 2008). Glitazones, drugs designed to stimulate PPARγ activation, have been shown to decrease glucose levels as well as decrease systemic inflammation in humans (Rocha and Libby, 2009). T lymphocytes are present in adipose tissue and their number is increased in obesity (Wu et al., 2007a). T cell accumulation appears even earlier than macrophage accumulation (Kintscher et al., 2008). Th1-activated adaptive immunity is probably involved in obesity, leading to excess TNF-α and IFN-γ production (Rocha and Libby, 2009).

3.2. Metabolic syndrome

The term metabolic syndrome describes the group of disturbances including glucose intolerance, central obesity, dyslipidemia and hypertension. Metabolic syndrome is a growing problem in western lifestyle countries and is mainly due the high energy diet, low physical activity and stress (Monteiro and Azevedo, 2010). People who have metabolic syndrome have an increased risk of developing CVD, type 2 diabetes and cancer (Monteiro and Azevedo, 2010). Different exact definitions for metabolic syndrome have been proposed. The initial definition by the World Health Organization stated that a patient must have either diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance; and additionally two of the following: elevated blood pressure, dyslipidemia, central obesity, or microalbuminuria (Alberti and Zimmet, 1998; Balkau and Charles, 1999). The International Diabetes Foundation has

stated that patient must have central obesity and two of the following: raised triglycerides, low HDL cholesterol, hypertension, raised fasting plasma glucose. The United States National Cholesterol Education Program stated that diagnose requires at least three of the following: central obesity, raised triglycerides, low HDL cholesterol, blood pressure and increased fasting plasma glucose (2001). Other definitions have also been presented. Probably the most useful definition for the future is the recent joint statement from different institutes (Alberti et al., 2009a). Criteria include at least three of the five of the following: population specific waist circumference limits, hypertriglyceridemia (≥1.7 mmol/L), reduced HDL cholesterol (males: <1.0 mmol/L, females: <1.3 mmol/L), hypertension (systolic blood pressure ≥130 mmHg and diastolic blood pressure ≥85 mmHg) and hyperglycaemia (fasting blood glucose ≥100 mg/dL).

Central obesity and especially visceral fat has been shown to be highly predictive of metabolic syndrome, insulin resistance and other deleterious conditions associated with obesity (Elks and Francis, 2010). Systemic low-grade inflammation is an obvious feature of metabolic syndrome. Increased inflammatory parameters, including CRP levels, have been measured from these patients (Nakamura et al., 2008).

3.3. Adipokines

Adipose tissue is an endocrine organ that secretes bioactive peptides and proteins, which are collectively named adipokines. These substances have a substantial role in energy and vascular homeostasis. Many of the currently identified adipokines are dysregulated in people with obesity, metabolic syndrome or type 2 diabetes (Maury and Brichard, 2010). Adipokines have been shown to modulate the function of the cardiovascular system, insulin sensitivity, insulin secretion, inflammation and fat mass (Maury and Brichard, 2010). For example, adipokines called leptin, adiponectin, resistin, visfatin, chemerin, omentin, apelin and retinol-binding protein have been shown to be associated with these modulations (Maury and Brichard, 2010). Only the first two are discussed in more details in the scope of this dissertation.

3.3.1. Leptin

Leptin is a 16 kDa hormone secreted by adipocytes. Typically obese people have higher levels of leptin and signs of leptin resistance. In addition, leptin levels correlate with adipose tissue mass and decrease in body weight is related to decreased leptin values (Ahima, 2008). The current view is that leptin is a hormone that signals energy defiance in the body, and not signaling to lose weight as was initially thought (Maury and Brichard, 2010). Leptin acts in various organs, *e.g.* hypothalamus, cortex, limbic area, pancreas, liver and the immune system. The main action of leptin is to regulate energy homeostasis and reproductive functions in hypothalamus (Badman and Flier, 2007). There leptin stimulates the secretion of anorexigenic peptides and decreases the secretion of oroxigenic peptides, thereby leading to decreased food intake (Ahima, 2008). In a common form of obesity it has been shown that the hypothalamus becomes resistant to the action of leptin which prevents these favourable effects (Maury and Brichard, 2010). In the periphery, leptin further stimulates fatty acid oxidation, glucose uptake and prevents lipid accumulation in adipose and other tissues (Maury and Brichard, 2010). Administration of leptin to obese people in order to cure obesity initially yielded disappointing results (Heymsfield et al., 1999). This was probably due to the leptin resistance seen in obese people. However, more recent studies have combined leptin administration with medications that sensitize the body to the actions of leptin and this approach has yielded more promising tentative results (Ozcan et al., 2009; Roth et al., 2008).

3.3.2. Adiponectin

Adiponectin is a 30 kDa hormone that is produced in adipose tissue. In contrast to leptin, adiponectin levels are decreased in obesity and correlate negatively with BMI (Arita et al., 1999). Adiponectin levels are also decreased in type 2 diabetes and low adiponectin levels are associated with CVD (Ouchi and Walsh, 2007). Adiponectin receptors are expressed at least in muscles and liver. Adiponectin exhibits insulin sensitizing, fat burning, anti-atherogenic, anti-inflammatory and anti-oxidant properties (Maury and

Brichard, 2010). These features make adiponectin differ from leptin and it is one plausible therapeutic target in order to achieve favourable effects in the body. Interestingly, glitazones have been shown to increase adiponectin levels (Kubota et al., 2006).

4. Autonomic nervous system and inflammation

Traditional wisdom says that the mind has control over the body. This is also a valid expression in the regulation of inflammation in the body. It has become evident that a vagus nerve related inflammatory reflex mechanism regulates inflammatory responses (Huston and Tracey, 2011). The vagus nerve innervates the organs of the reticuloendothelial system which includes heart, lungs, liver, spleen and gastrointestinal tract, of which the spleen is the most critical (Thayer, 2009). Vagus nerve stimulation leads to the release of neurochemical mediators, acetylcholine molecules, which interact with alpha 7 nicotinic acetylcholine receptors (α7nAChR) on monocytes and macrophages (Wang et al., 2003). This activation leads to the inhibition of proinflammatory cytokines but not anti-inflammatory cytokines such as IL-10. The autonomic nervous system's control over the immune system has been termed the cholinergic anti-inflammatory pathway (Tracey, 2002).

Under normal body homeostasis, the cholinergic anti-inflammatory pathway exerts a tonic inhibitory impulse on innate immune responses. Interference with this pathway, by either damaging the vagus nerve or knocking out the gene of α7nAChR, leads to excess proinflammatory cytokine response and exaggerate response to bacterial products and injuries (Borovikova et al., 2000; Wang et al., 2003). The signal transduction mechanism involving the α7nAChR is not fully understood. The current view is that α7nAChR ligand interaction activates JAK-STAT dependent inhibition of the nuclear translocation of nuclear factor –κB, which leads to decreased transcription of cytokine genes (de Jonge et al., 2005; Huston and Tracey, 2011). A few mechanisms by which the cholinergic anti-inflammatory pathway alleviates inflammation have been identified. Vagus nerve stimulation significantly decreases TNF-α synthesis in spleen and this way the systemic inflammation is decreased (Huston et al., 2006). Vagus nerve stimulation also downregulates circulating neutrophil activation by attenuating the expression of CD11b, a surface molecule required for cell adhesion and chemotaxis (Huston et al., 2009).

The most widely used method to assess this vagus nerve activity in humans is to measure heart rate variability (HRV). Control of the heart rate is under the influence of the autonomic nervous system with the vagus nerve being the most important parasympathetic mediator. Under resting conditions, the parasympathetic nervous system is a more potent regulator of heart rate and during exercise the sympathetic nervous system is more important. The heart rate is not constant even at rest. Fluctuations between successive R waves can be recorded in electrocardiogram (ECG) tracings. From these recordings HRV can be assessed either with reference to time (time domain analysis) or frequency (frequency domain analysis). From time domains the following variables can be derived: the standard deviation of R-R intervals (SDNN), the square root of the mean squared difference of successive R-Rs (RMSSD), the number of pairs of successive R-Rs that differ by more than 50 ms (NN50) and the proportion of NN50 divided by total number of R-Rs (pNN50). From frequency domains the following variables can be derived: ultra-low frequency (ULF), very low frequency (VLF), low frequency (LF), high frequency (HF) and total power (TP). Profound mathematics is behind all these variables, which is beyond the scope of the study at hand, and computer based analysing software is used to derive these variables.

Indices of HRV have been associated with increased morbidity and mortality in various diseases (Huston and Tracey, 2011). Decreased HRV is associated with increased risk of CVD events (Dekker et al., 2000), diabetes (Urbancic-Rovan et al., 2007) and hypertension (Mussalo et al., 2001). HRV is also associated with autoimmune diseases like rheumatoid arthritis (Bruchfeld et al., 2010) and interestingly with depression (Kemp et al., 2010). This is thought to be one plausible mechanism behind unfavourable changes in immune parameters among depressed patients. Many studies have shown an association between decreased HRV and increased low-grade systemic inflammation measured as CRP, IL-6 or TNF levels (Haensel et al., 2008; Lampert et al., 2008; Sajadieh et al., 2004). Interestingly one study has shown a synergistic effect of reduced HRV and increased CRP on the risk for CVD (Sajadieh et al., 2006).

5. Infections and atherosclerosis

In the late 1980's a Finnish research group found evidence that seropositivity to *Chlamydia pneumoniae* infection was associated with increased CVD risk (Saikku et al., 1988). Since then numerous microbes have been investigated as possible risk factors for CVD, including hepatitis A, Epstein-Barr, herpes simplex viruses, cytomegalovirus, *Mycoplasma pneumoniae, Helicobacter pylor,* some dental bacteria and many others, as well (Leinonen and Saikku, 2002). Currently, no consensus on the matter has been reached whether microbe infections are a risk factor for CVD indices; both positive and negative associations have been reported (Danesh et al., 1997). Therefore, instead of studying single pathogens, the total infectious burdens of these pathogens have been also studied. The total infectious burden has been shown to be associated with CVD more strongly than the single pathogens (Smieja et al., 2003). In addition to these known pathogenic microbes with a tendency to cause chronic infections, there is increasing evidence that bacteria of the normal human gut, oral cavity and airways are related to obesity and atherosclerosis (Caesar et al., 2010). Adenoviruses and some other viruses have also been associated with obesity (Pasarica and Dhurandhar, 2007).

5.1. Cytomegalovirus

Cytomegalovirus (CMV) is a widespread virus in human populations. CMV is a member of the herpes family of viruses and contains double-stranded deoxyribonucleic acid (DNA) as their genome. Evidence of CMV infection has been found in all the world's populations examined (Soderberg-Naucler, 2006). The infection is typically acquired during childhood, with an incidence of 30-40% within the first year of life. Incidence rises over the lifespan and in cohorts of elderly people over 90% are seropositive to the virus (Roberts et al., 2010). Typically CMV infection is mild or even asymptomatic in healthy individuals and after the initial immune response, the virus becomes latent and resides in the cells of myeloid lineage (Taylor-Wiedeman et al., 1991). However, it seems

that CMV is not merely a silent and latent virus in the body after primary infection, but rather persists in the body with features of a chronic productive infection or as a latent infection with periodic reactivations. Evidence of CMV shedding was detected in the urine and plasma samples of the majority of healthy seropositive young women during a 30-month follow-up (Arora et al., 2010). In the same study cohort, one-third of the seropositive women also showed evidence of CMV reinfection with new CMV strains, which were identified by the appearance of new antibodies specific for CMV envelope glycoproteins (Ross et al., 2010). CMV shedding was observed in women with evidence of reinfection and in those without it (Arora et al., 2010). Additionally, shedding of CMV into urine has been associated with increased plasma CMV antibody titers in astronauts (Mehta et al., 2000).

CMV has an established role in many diseases and the possible role of CMV as a mediator in other disease has also been hypothesized. In immunocompromised people, the CMV infection causes significant symptoms. Patients may encounter symptoms such as spiking fever, leucopenia, malaise, hepatitis, interstitial pneumonia, gastrointestinal disease and retinitis. Typical risk groups are organ transplant patients and HIV-infected patients (Bailey et al., 1995; Soderberg-Naucler, 2006). The foetus is at risk of having birth defects, typically hearing loss and mental retardation, if CMV infection occurs during pregnancy (Fowler et al., 1992). In addition to these established pathologies, CMV has been associated with other chronic inflammatory diseases. The virus has been detected in many autoimmune diseases, psoriatic plaques, lesions of inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus and Sjögren's syndrome (Soderberg-Naucler, 2006). The virus has also been detected in different kinds of cancers, tumours of colon and prostate, and glioblastomas (Soderberg-Naucler, 2006).

In addition to all the other implications, there is a notable relation between CMV, atherosclerosis and CVD. CMV can infect human endothelial cells and it has been shown to aggravate the development of atherosclerosis (Cheng et al., 2009; Vliegen et al., 2004). CMV infection leads to neutrophil transendothelial migration, smooth muscle cell migration, intracellular adhesion molecule expression, leukocyte and thrombocyte

adhesion, CXC chemokine expression and cytokine secretion (Soderberg-Naucler, 2006). In addition CMV infection has been shown to cause increased blood pressure values in mice via stimulating renin synthesis (Cheng et al., 2009). In humans CMV DNA copy number and CMV related microRNA expression has been associated with hypertension (Li et al., 2011). In heart transplant patients the CMV infection has been shown to lead to higher incidence of graft rejection and cardiac allograft vascular disease (Grattan et al., 1989). The use of antiviral treatment with ganciclovir has been shown to decrease rejection and allograft vascular disease risk in CMV infected transplant patients (Potena et al., 2006). In immunocompromised people the role of CMV as a risk factor for CVD is controversial. Some studies have shown significant associations between CMV infection and CVD, when other studies have reported non-significant results. (Adler et al., 1998; Haider et al., 2002; Smieja et al., 2003; Sorlie et al., 2000). However, studies carried out on elderly people have shown consistently that high levels of antibodies against CMV are associated with increased cardiovascular and total mortality (Roberts et al., 2010; Strandberg et al., 2009; Wang et al., 2010). CMV infection has also been related to early vascular changes *i.e.* FMD and IMT (Grahame-Clarke et al., 2003; Nieto et al., 1996; Simmonds et al., 2008). However, non-significant results have also been reported (Khairy et al., 2003; Oshima et al., 2005).

Aims of the study

The aim of the dissertation was to study the role of different inflammatory markers in early atherosclerosis. The specific aims were to

1. Investigate the effect of combined oral contraceptives use on CRP levels, determinants and genetic regulation in healthy young women.
2. Examine SAA level distribution, determinants and predictive value for early atherosclerotic changes in healthy young adults.
3. Analyse the effect of cholinergic autonomic pathway estimated by short-term HRV, on CRP and SAA levels in healthy young adults.
4. Investigate PTX3 concentrations distribution, determinants and associations with early atherosclerotic changes in a middle aged cohort.
5. Study whether high CMV antibody titers are associated with early atherosclerotic changes, blood pressure and other traditional atherosclerotic risk factors in healthy young adults.

Subjects and methods

1. Subjects

1.1. Studies I,II,III and V

The study population consisted of participants in the Cardiovascular Risk in Young Finns Study, which is an ongoing multi-centre follow-up study involving five university hospitals in Finland. The study began in 1980, when 3,596 participants between the ages of 3 and 18 were randomly selected from the national population registers. The 21-year follow-up was conducted in 2001, when 2,283 participants were between 24 and 39 years of age. Cardiovascular risk factor measurements, including BMI, serum lipids, blood pressure values, CRP, SAA, leptin, adiponectin, alcohol consumption and smoking habits, were recorded during this follow-up.

1.2. Study IV

The study population was a subpopulation drawn from a Finnish cross-sectional health examination survey, the Health 2000 Survey, carried out 2000-2001. The overall study cohort was a two-stage stratified cluster sample (8,028 persons) representing the entire Finnish population aged 30 years and above. In order to study cardiovascular risk and diabetes more thoroughly, a supplementary study was carried out (sample size 1,867 and participation rate 82%). The participants in the study consisted of those supplementary study subjects from whom the data about the clinical and metabolic cardiovascular risk factors were available. The study subjects (n=1,236; n=666 women, n=570 men) were

between 46 and 76 years of age and the study was conducted in the catchment of the five Finnish university hospitals.

2. Methods

2.1. Measurements of inflammation markers

In Studies I, II, III and V; CRP measurements were taken from fasting plasma. CRP concentrations were analysed using a highly-sensitive latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany) with a detection limit of 0.06 mg/L. Serum SAA concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) kit with a detection limit of <0.004 mg/L (Human SAA, Biosource International, Camarillo, CA).

In Study IV, PTX3 concentrations were determined in EDTA-plasma using a commercial ELISA kit according to the manufacturer's instructions (Quantikine DPTX 30; R&D Systems Inc., Minneapolis, USA). The mean detection limit for the assay was 0.025 ng/mL and the assay exhibited no cross-reactivity with either CRP or serum amyloid P. Plasma CRP concentrations were determined using a chemiluminescent immunometric assay (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA).

In Study V, CMV IgG antibody titers were analysed using a commercial enzyme immunoassay (Enzygnost Anti-CMV/IgG, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). According to the manufacturer, the test sensitivity was 99.3% and the specificity was 98.2%. Seropositivity for CMV was defined as a serum anti-CMV IgG titer of ≥230.

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

DNA was extracted from whole blood using a commercially available kit (Qiagen Inc., Hilden, Germany) in 2001. Genotyping of *CRP* gene polymorphisms -717A>G (rs2794521), -286C>T>A (rs3091244), +1059G>C (rs1800947), +1444C>T (rs1130864) and +1846G>A (rs1205) were 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 SNPs -717, +1444 and +1846 were genotyped using Assays By Design from Applied Biosystems under standard conditions. The triallelic tagSNP -286 was genotyped with designed primers and probes as previously described (Carlson et al., 2005), except for the genotype calling, which was done manually from the PCR run component tab.

2.3. Analysis of typical atherosclerotic risk factors

In Studies I,II,III and V, blood pressure measurements were taken using a random zero sphygmomanometer (Hawksley & Sons Ltd, Lancing, UK) and the mean of three measurements was used in the analysis. BMI was calculated from measured height and weight values. Waist and hip circumferences were measured in standard positions and waist-hip ratio was calculated from these measurements. CRP, SAA, leptin, insulin, HDL cholesterol, triglyceride and total cholesterol levels were obtained from fasting blood plasma samples. LDL-cholesterol was calculated with the Friedewald formula. Homeostasis assessment of insulin resistance (HOMA-IR) was calculated according to the formula: HOMA-IR = fasting glucose (mmol L^{-1}) × fasting insulin (mU L^{-1}) / 22.5. Information on smoking habits, alcohol consumption, oral contraceptive use, type of oral contraceptives used, medications used, physical activity, pregnancy and breastfeeding were collected by administering a questionnaire.

In Study IV, BMI and waist-hip ratio measurement were done as described in previous paragraph. The blood pressure was measured with the automatic Omron M4 manometer (Omron Matsusaka Co., Japan, Omron Healthcare Europe B.V., Hoofddrop, the Netherlands) and the mean of three measurements was used in the analysis. Pulse pressure was calculated as the difference between the mean systolic and the mean diastolic pressure. Current smoking, CVD history and events were evaluated with a questionnaire. Those who were current smokers were defined as smokers and the rest of subjects as non-smokers. Venous blood samples were drawn after an overnight fast. HDL cholesterol, total cholesterol, triglyceride and plasma glucose concentrations were determined enzymatically with a clinical chemistry analyzer (Olympus, AU400, Hamburg, Germany).

2.4. Ultrasound measurements of arteries

In Studies II and V, Carotid ultrasound measurements were performed with a Sequoia 512 high-resolution ultrasound system (Acuson, CA). Cdist, which shows the ability of the large arteries to expand under cardiac pulse pressure, was assessed from the formula $Cdist=([D_s-D_d]/D_d)/(P_s-P_d)$, where D_s is the systolic diameter, D_d is the diastolic diameter, P_s is the systolic blood pressure and P_d is the diastolic blood pressure. Mean IMT was derived from a minimum of four measurements of the posterior wall of the left carotid artery (at ~10mm proximal to the bifurcation). In Study V, the brachial FMD was also measured. The left brachial artery diameter was measured both at rest and during reactive hyperaemia. Increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 250 mmHg for 4.5 minutes, followed by release. Three measurements of arterial diameter were performed at end-diastole at a fixed distance from an anatomic marker at rest and at 40, 60 and 80 seconds after cuff release. The vessel diameter in scans after reactive hyperaemia was expressed as the percentage relative to the resting scan. The average of three measurements at each time point was used to derive the maximum FMD (the greatest value between 40 and 80 seconds)

In Study IV, high-resolution B-mode carotid ultrasound examination of the right carotid artery was performed according to a standardized protocol using a 7.5 MHz linear array transducer. The examinations were performed by centrally trained and certified sonographers at six study locations around Finland. Carotid IMT measurements were performed off-line with automated imaging processing software and one reader was responsible for reading all the ultrasound images. Three summary measures of the carotid IMT were calculated: the mean of the three average IMTs of the common carotid artery (mean CCA IMT), the mean of the three average IMTs of the carotid bulb (mean bulb IMT), and the mean of these two means (mean IMT). Mean IMT was used in the study. The mean Cdist in Study IV was assessed analogously compared to description on Cdist measurements in Studies II and V.

2.5. Analysis of heart rate variability measurements

In Study III, a single-channel chest-lead ECG, 3 minutes in length, was recorded. Prior to the recording, subjects were comfortably seated in supine positions for a minimum of 15 minutes during a vascular ultrasound scan. The signals were converted from analogue to digital with a sampling rate of 200 Hz; the respective time-series of R-R intervals were generated. ECG signals were manually revised. The stationary period was identified during a 3-minute period of metronome controlled breathing at frequency of 0.25 Hz and was used to compute the time and frequency-domain HRV indices. The study subjects were trained for this procedure beforehand in order to avoid emotional arousal. The mean duration of the stationary period was analysed 173 seconds and the mean number of analysed R-peaks was 196. These indices were analysed using a commercial WinCPRS program (Absolute Aliens, Turku, Finland).

The following parameters were identified from time and frequency-domain measurements: HF (0.15-0.40 Hz), LF (0.04-0.14 HZ), TP, RMSSD, and SDNN. The ratio between low frequency and high frequency oscillations (LF/HF × 100%) was calculated. Under resting conditions vagal activity of cardiovascular autonomic

regulation prevails (Levy, 1971). The vagal activity is the major contributor of the HF component of spectral HRV (1996). The LF component may represent either sympathetic modulation or combination of both sympathetic and vagal influences. LF/HF is considered to reflect sympathovagal balance or sympathetic modulations (1996; Pagani et al., 1986). RMSSD is considered as a marker of vagal function and correlates strongly with HF.

Short-term HRV indices depend on the length of the time-series, and shorter time-series may result in less variability; therefore, HRV indices were recollected from 75 subjects in order to compare HRV indices on 3 and 5 minute ECG recordings. In addition, the reproducibility of short-term HRV indices was assessed from 51 subjects after an average of 4.4 months from the first measurement. Both the reproducibility and the comparability of these measurements were satisfactory and no statistically significant differences were observed in these measurements (Koskinen et al., 2009).

2.6. Statistical analyses

In all studies, the data was analysed by SPSS for Windows statistical software (versions 14.0, 15.0 and 17.0, SPSS Inc., Chicago, IL, USA). For non-parametric variables Mann-Whitney's test and Kruskall-Wallis test were used in statistical analyses. For normally distributed variables t-test and one-way ANOVA were used. Correlation between skewed variables was estimated with Spearman's test and the correlation between normally distributed variables was estimated using Pearson's test. Comparisons of the variables between sexes or according to COCs use were performed with Student's t-test, Mann-Whitney's test and Chi-squared test as appropriate. For linear regression analysis and for analysis of covariance (ANCOVA) the non-parametric values were log transformed prior to analysis. In all analyses, the level of $p<0.05$ was considered statistically significant.
In Study I, the haplotypes were constructed from the five *CRP* SNPs using the PHASE v2.0.2. program. It calculates from the genotype data the most probable haplotype pairs for each individual using a Bayesian statistical method.

2.7. Ethics

In Studies I, II, III and V; the study was approved by local ethics committees and was conducted following the guidelines of the Declaration of Helsinki. All participants also gave their written informed consent. In Study IV, the protocol was approved by the Epidemiology Ethics Committee of the Helsinki and Uusimaa Hospital District. All participants also gave their signed informed consent.

Results

1. Effect of using different COCs on CRP determinants and genetic regulation

It was previously known that COCs use has a marked effect on women's CRP levels. In this study we aimed to find out if COCs use alters the usually seen determinants of CRP in women and if these changes are somehow related to different COCs used. In multivariate linear regression model, it was shown that in women who use COCs, CRP levels were related to BMI (B=0.020, SE=0.009, p=0.029), leptin (B=0.524, SE=0.124, p<0.001) and triglyceride (B=0.358, SE=0.156, p=0.023) levels, while in non-users CRP levels were only related to BMI (B=0.028, SE=0.005, p<0.001) and leptin (B=0.557, SE=0.080, p<0.001). Further it was seen that the relation of CRP and triglycerides in COC users was associated with use of high dosage of progestagen or cyproterone. Interestingly, the genetic haplotypes previously shown to associate with CRP levels in this cohort had an effect on CRP levels only in those women who did not use COCs.

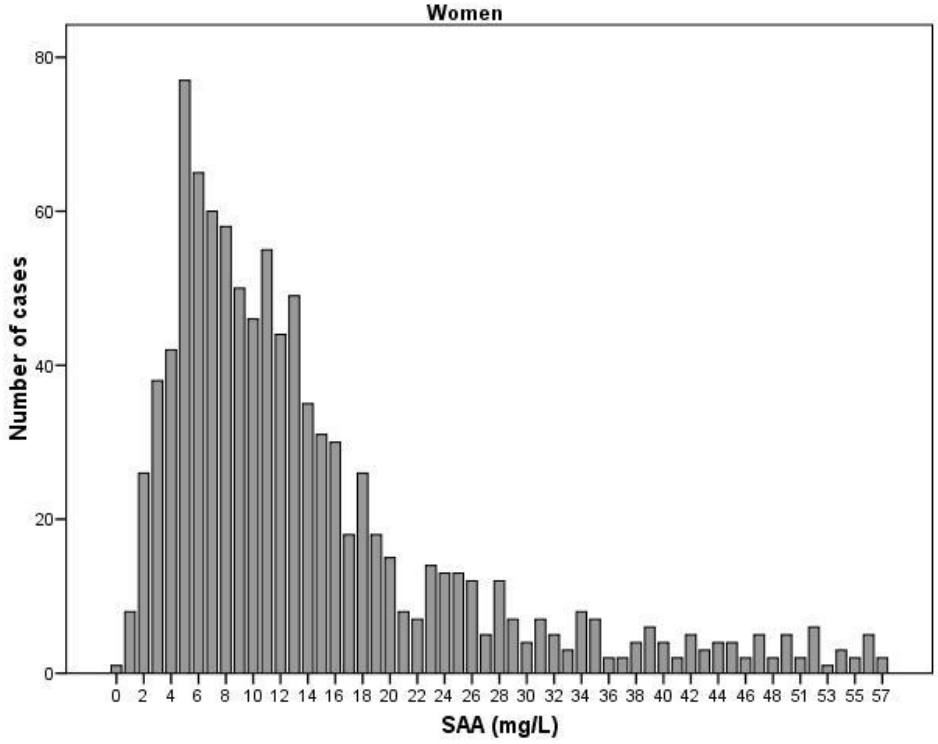
2. SAA values in young adults

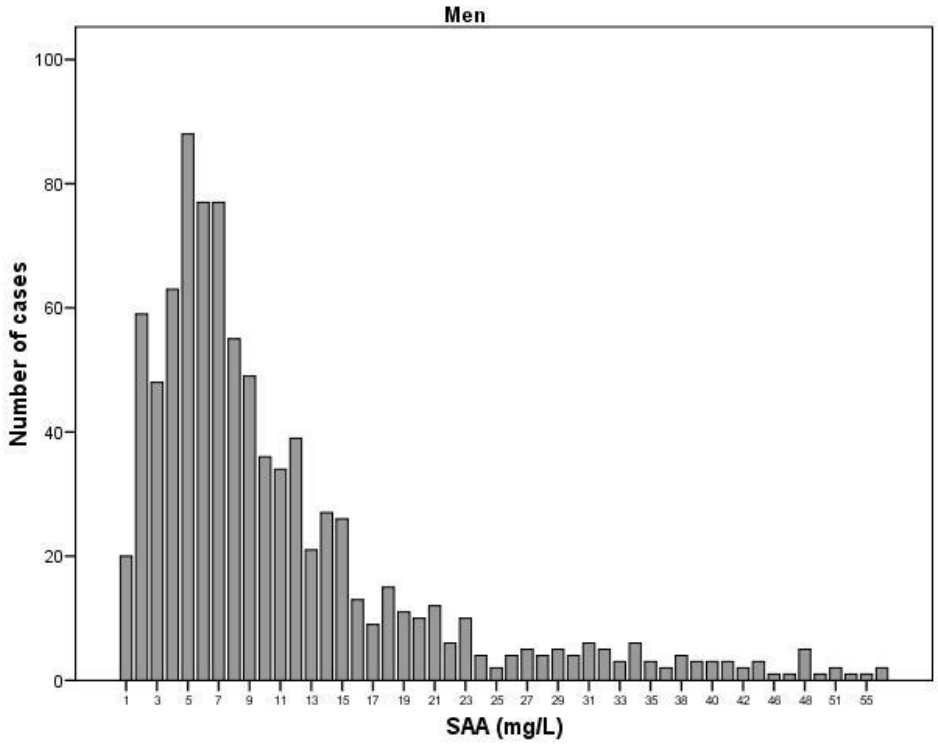
In Study II, the aim was to ascertain the distribution of SAA levels in healthy young adults and identify associations between SAA, atherosclerotic risk factors and early vascular changes. Distributions of SAA concentrations in healthy young women and men are presented in Figure 1. At baseline women had higher SAA levels (p<0.001) but this association was attenuated to null when the analysis was adjusted with leptin, adiponectin

or ApoA1. Women using COCs had higher SAA levels than non-users (median 13.60 mg/L vs. 10.70 mg/L, p<0.001). Whereas in women using a levonorgestrel-releasing intrauterine device (IUD) had lower SAA values compared to non-users (median 8.75 mg/L vs. 10.7 mg/L, p=0.034). SAA levels correlated directly with indices of adipose tissue mass (BMI, waist circumference), lipid, blood pressure, insulin, HOMA-IR, leptin and CRP values in both sexes. Interestingly many of these associations diminished after adjustment for BMI.

In multivariate regression model, independent determinants for SAA were CRP (women: B=0.479, SE=0.028, p<0.001; men: B=0.610, SE=0.025, p<0.001), leptin (women: B=0.121, SE=0.048, p=0.012; men: B=0.112, SE=0.041, p=0.007) and ApoA1 (B=0.270, SE=0.054, p<0.001) in women and HDL cholesterol (B=0.097, SE=0.040, p=0.017) in men. We also did alternative multivariate models without CRP. In that model independent determinants for SAA were leptin (B=0.424, SE=0.070, p<0.001), ApoA1 (B=0.270, SE=0.054, p<0.001) and BMI (B=0.011, SE=0.004, p=0.013) in women; and leptin (B=0.340, SE=0.071, p<0.001) and waist circumference (B=0.001, SE=0.001, p=0.006) in men. Serum SAA concentrations correlated significantly with early vascular changes *i.e.* IMT and Cdist (termed as carotid artery compliance in the original publication). However, in multivariate model, SAA levels had no independent effect on these variables.

Figure 1. Distribution of serum SAA levels in healthy young women and men.





3. HRV measurements and inflammatory markers in young adults

HRV indices and inflammatory markers, CRP and SAA, were measured from 1,601 subjects after the exclusion criteria. We aimed to ascertain the relations of these inflammatory markers and HRV indices. CRP levels correlated with the following HRV indices: heart rate ($r=0.112$, $p<0.001$), LF ($r=-0.065$, $p<0.01$), TP ($r=-0.074$, $p<0.01$), SDNN ($r=-0.080$, $p<0.01$) and RMSSD ($r=-0.072$, $p<0.01$). SAA levels correlated with heart rate ($r=0.113$, $p<0.001$), HF ($r=-0.049$, $p<0.005$), LF ($r=-0.057$, $p<0.05$), TP ($r=-0.054$ $p<0.01$), SDNN ($r=-0.070$, $p<0.01$) and RMSSD ($r=-0.075$, $p<0.01$). Multivariate regression models were created to assess the independent effect of HRV indices on inflammatory markers. Heart rate ($B=0.006$, $SE=0.003$, $p=0.040$), LF ($B=-0.070$, $SE=0.031$, $p=0.024$), TP ($B=-0.087$, $SE=0.032$, $p=0.007$), SDNN ($B=-0.186$, $SE=0.066$, $p=0.005$) and RMSSD ($B=-0.108$, $SE=0.048$, $p=0.024$) remained independent determinants for CRP after adjustment with typical atherosclerotic risk factors including leptin. However, none of the HRV indices were associated independently with SAA levels.

4. PTX3 levels determinants and associations with early atherosclerotic markers

Aim of Study IV was to ascertain the distribution and determinants of PTX3 values, and its relation to early vascular changes. PTX3 levels did not differ between men and women (median 1.04 ng/L vs. 0.98 ng/L, $p=0.107$). Considering all the subjects in Study IV, PTX3 levels correlated significantly ($p<0.05$) with age, systolic and diastolic blood pressure, pulse pressure, total cholesterol (inverse), LDL-cholesterol (inverse), glucose, insulin, HOMA-IR, indoleamine 2,3-dioxygenase (IDO), IMT and Cdist (inverse) (Table 1). In stepwise linear regression model, LDL-cholesterol ($B=-0.038$, $SE=0.011$, $p=0.001$), pulse pressure ($B=0.002$, $SE=0.001$, $p=0.004$) and IDO ($B=0.003$, $SE=0.001$, $p=0.017$)

were independent determinants of PTX3 levels. In multivariate analysis, PTX3 had no independent effect on IMT (p=0.659) nor Cdist (p=0.878).

However, earlier studies have yielded different results on PTX3 determinants among people with various clinical conditions. In order to homogenize our study groups, subjects were divided in four subclasses: insulin-resistant, hypercholesterolaemic, hypertensive and healthy subjects. In insulin-resistant subjects, PTX3 levels correlated directly with age, pulse pressure and IDO, and correlated inversely with total and LDL cholesterol. In hypercholesterolaemic subjects, PTX3 levels correlated directly with HDL cholesterol, systolic blood pressure and pulse pressure. In hypertensive subjects, PTX3 levels correlated directly with systolic blood pressure, pulse pressure and IDO. No significant correlations were observed in healthy subjects. After the subclass division, there were no significant correlations between PTX3 and early vascular changes. In multivariate regression model, LDL cholesterol (B=-0.054, SE=0.019, p=0.005) and IDO (B=0.004, SE=0.002, p=0.015) were independently associated with PTX3 levels in insulin-resistant subjects. In hypercholesterolaemic and hypertensive subjects only pulse pressure was independently associated with PTX3 levels.

Table 1. Pearson's correlations for PTX3 in Health 2000 study subjects.

	r	p
Age (years)	0.116	**<0.001**
Body mass index (kg/m^2)	-0.032	0.272
Waist circumference (cm)	0.010	0.739
Hip circumference (cm)	0.003	0.917
Waist-hip ratio	0.010	0.724
Heart rate (beats/min)	0.010	0.731
HDL-cholesterol (mmol/l)	0.014	0.628
LDL-cholesterol (mmol/l)	-0.084	**0.004**
Total cholesterol (mmol/l)	-0.064	**0.026**
(log)Triglycerides (mmol/l)	0.011	0.713
Glucose (mmol/l)	0.063	**0.029**
(log)Insulin (mmol/l)	0.086	**0.003**
(log)HOMA-IR	0.091	**0.002**
Systolic blood pressure (mmHg)	0.123	**<0.001**
Diastolic blood pressure (mmHg)	0.067	**0.020**
Pulse pressure (mmHg)	0.132	**<0.001**
(log)CRP (mg/l)	0.023	0.430
IDO	0.114	**0.002**
Carotid IMT (mm)	0.073	**0.012**
Cdist (%/10mmHg)	-0.076	**0.012**

5. Relation of high CMV antibody titers to atherosclerotic risk factors, blood pressure and early vascular changes

In Study V, we aimed to ascertain if CMV antibody titers were associated with typical atherosclerotic risk factors, blood pressure and early vascular changes. CMV antibody titers were significantly higher in women than in men. There were significantly ($p<0.001$) more women with CMV antibody titers over 14,000 than men (72.5% vs. 27.5%). In men, high CMV antibody titers were directly associated with age ($p<0.001$), systolic ($p=0.053$) and diastolic ($p=0.002$) blood pressure elevation, and inversely associated with flow-mediated dilation ($p=0.015$). In women, CMV antibody titers were not associated

with any of the parameters analysed. In a multivariate regression model, which included traditional atherosclerotic risk factors, CMV antibody titers were independent determinants for systolic (p=0.030) and diastolic (p=0.004) blood pressure elevation and flow-mediated dilation (p=0.015) in men. These findings were also repeatable in a subgroup consisting solely of seropositive subjects.

Discussion and conclusions

1. COCs use alters metabolic determinants and genetic regulation of CRP

In Study I, we investigated the effect of COCs use on CRP determinants and genetic regulation. It was shown that triglycerides were an independent determinant of CRP only in women who used COCs. This association especially was related to use of high dosage of progestagen or cyproterone. However, CRP levels were not significantly different in these groups compared to users of other progestagens. These findings raise the possibility that lipids and CRP are not related in all women as has been thought. The relation between CRP and triglycerides could be a result of simultaneous expression of these substances in liver stimulated by COCs usage. This hypothesis is supported by the finding that COCs use increase CRP levels without increase in IL-6 levels (van Rooijen et al., 2006), indicating that COCs stimulate hepatocytes directly.

Another important finding in Study I was that CRP genetic polymorphisms seem to have an effect on CRP levels only in those women who do not use COCs. This finding highlights the importance of thorough adjustment with known internal and external factors affecting CRP levels, for example in genetic studies. The mechanism by which COCs induce CRP production in liver is unknown. No obvious steroid hormone binding site has been identified in CRP gene.

The risk of CVD events is slightly increased in women who use COCs (Battaglioli and Martinelli, 2007; Tanis et al., 2001). CVD risk is also associated with the increased levels of CRP (Kaptoge et al., 2010; Pearson et al., 2003; Ridker et al., 2000). We conclude that use of COCs is associated with higher CRP and triglyceride levels. Additionally known

genetic regulation of CRP levels can be overwhelmed by the strong COCs effect. Further studies are still needed to ascertain whether there is cell based interplay between these factors. It is a clinically relevant question so that women can have even safer and more efficient means of contraception in the future.

2. SAA concentrations are associated with cardiometabolic risk factors but not with early vascular changes

In Study II, we demonstrated that SAA levels were associated with several cardiometabolic risk factors. Although in multivariate model only few variables had independent associations with SAA. Predictably indices of adipose tissue *i.e.* BMI, waist circumference and leptin were closely related to SAA levels. This supports the hypothesis that SAA is expressed mainly in adipose tissue and hence could be a possible link between obesity and inflammation (Yang et al., 2006). Additionally HDL cholesterol in men and ApoA1 in women were independently associated with SAA levels. These findings were expected because of the capability of SAA to displace ApoA1 on the surface of HDL particles (Kisilevsky and Tam, 2002). SAA levels were also associated with insulin levels and with insulin sensitivity index, although these associations were adjusted to null in multivariate models. Our results therefore indicate that insulin sensitivity has no independent relation to SAA.

Our results do not support the role of SAA as an independent predictor of early atherosclerotic changes. SAA levels correlated with IMT and CAC indices but had no independent predictive value for these early vascular changes. Despite these results, the relation between SAA and vascular changes remains controversial. Our results and the results by Wohlin et al. support the notion that SAA is not an independent predictor of IMT in European populations (Wohlin et al., 2007). In cohorts consisting of Asian or African-American subjects there is evidence that SAA levels and SAA genetics may be associated with IMT (Carty et al., 2009; Uurtuya et al., 2009; Xie et al., 2010). Thus there

may be some ethnic diversity in relation of SAA and CVD risk that needs to be more thoroughly investigated in the future.

We were also able to demonstrate that SAA levels are increased among COCs using healthy young women and decreased in those women using IUD. The association between COCs use and increased SAA levels has also been seen in one other study (van Rooijen et al., 2006). Another study showed there was positive association between ERT use and SAA levels in postmenopausal women (Miller et al., 2004), but this association may be diminished by oral administration of an androgenic progestin, medroxyprogesterone acetate (Wakatsuki et al., 2002). The association between IUD use and decreased SAA levels is a novel finding that has not been reported elsewhere. It has previously been shown that transdermal administration of ERT reduces SAA levels (Abbas et al., 2004). The mechanism by which transdermal ERT reduces SAA levels is unknown but it may be related to anti-inflammatory effects of estrogen because subcutaneous estradiol administration has been shown to reduce leukocyte infiltration into injured vessels in animals (Miller et al. 2004). This mechanism could partly explain the inverse association between IUD use and SAA levels, but IUDs do not release estrogen. It is possible that progestagen may also have some unknown anti-inflammatory mechanism.

In conclusion, serum SAA levels are associated with indices related to adipose tissue mass. However, SAA levels had no independent effect on early vascular changes. Further studies are still needed to determine whether early SAA measurements can have prognostic value on future CVD events. It is important to concede that only free SAA levels of serum were measured. Therefore these results may only give a limited view of the meaning of SAA in early atherosclerosis. Thus other forms of SAA may be more relevant *i.e.* locally produced SAA, HDL bound SAA or LDL-SAA complex.

3. HRV is independently associated with CRP but not with SAA

In Study III, we measured HRV, a way to assess autonomic nervous system function, and inflammatory markers CRP and SAA in healthy young adults. The CRP and SAA levels correlated significantly with the most of the HRV indices. However, in multivariate regression model, only the association between CRP and HRV indices remained significant while the association between SAA and HRV was attenuated to null after adjustment for typical atherosclerotic risk factors, including leptin. It has been previously been shown that leptin levels correlate significantly with HRV indices and there is a strong relation between leptin and inflammatory markers (Paolisso et al., 2000; Viikari et al., 2007). None of the previous studies had taken all these three aspects into consideration; hence we could conclude for the first time that the known association between CRP and HRV indices is independent of leptin. However, there were no independent associations between SAA and HRV. One plausible explanation for this difference could be the different sites of expression. In normal conditions the majority of circulating SAA is expressed in adipose tissue while in the case of CRP the main source is liver (Sjoholm et al., 2005; Yang et al., 2006). The liver is under the innervation of the vagus nerve but this is not the case with adipose tissue. Therefore it is plausible that the autonomic nervous system can only regulate CRP production but not SAA expression.

Our findings support the link between abnormalities in autonomic nervous system function and changes in inflammatory parameters. This further supports the hypothesis that the cholinergic anti-inflammatory pathway may be involved in the pathogenesis of chronic inflammatory diseases such as CVD.

4. PTX3 levels are related to atherosclerotic risk factors but not to subclinical atherosclerosis in middle-aged individuals with high risk for CVD

In Study IV, we demonstrated that PTX3 levels were associated with atherosclerotic risk factors among middle-aged subjects with high risk for CVD. After categorizing these people into three subgroups: insulin-resistant, hypercholesterolemic and hypertensive subjects; not all of these associations were seen in the whole population remained. In multivariate models done either in whole population or in these high CVD risk subgroups, the same determinants were seen: LDL cholesterol, pulse pressure and IDO. However, all these associations were rather weak and these factors could explain only 1.1-5.7% of the variation in PTX3 values. Additionally no correlations between PTX3 and markers of subclinical atherosclerosis were seen in high CVD risk subgroups or in the multivariate model of whole population. Therefore it is plausible that PTX3 represents some unknown aspect of atherosclerosis. PTX3 has a possible dualistic role; in mouse models, PTX3 has been shown to have a cardioprotective function (Norata et al., 2009; Salio et al., 2008). Our finding of a positive relation between PTX3 and IDO also supports this idea. IDO is a known immunoinflammatory downregulator of Th1 cell responses (Xu et al., 2008). Hence it is plausible that PTX3 is expressed by cells in order to achieve a protective physiological response. Further studies are still needed to ascertain the PTX3 possible cardioprotective role in atherosclerosis and CVD.

Contradictory results have been reported about the determinants of PTX3. Our results support a correlation between PTX3, age, and cholesterol and blood pressure values. The direct correlation between PTX3 and age is seen quite unanimously (Jenny et al., 2009; Matsui et al., 2010; Yamasaki et al., 2009). Differing results have been reported about the correlation between PTX3 and cholesterol values (Ogawa et al., 2010; Yamasaki et al., 2009; Zanetti et al., 2009). Our results indicate that there may be an inverse relation between these variables. Further studies are needed to clarify this discrepancy. Earlier studies have reported non-significant correlations between PTX3 and blood pressure

(Alberti et al., 2009b; Suliman et al., 2008b; Zanetti et al., 2009). Our study was the first also to take pulse pressure into consideration and had decidedly more subjects than in these earlier studies. Therefore the relation of PTX3 and blood pressure may have not been seen in them. However, further studies are still needed to confirm our results and to determine the factors involved in the regulation of PTX3.

5. CMV IgG antibodies titers are directly associated with blood pressure and inversely with FMD in healthy young men

In Study V, we discovered that CMV IgG antibodies titers were associated with increased blood pressure values and inversely with FMD values in young men. These results were not seen in women. However, women had significantly higher CMV titers than men. Cheng et al. showed in mice that CMV infection increased blood pressure values in a dose dependent manner (Cheng et al., 2009). They could also demonstrate that CMV infection stimulates renin synthesis in cell cultures and increased renin values were measured from these mice after CMV infection. In Chinese cohort, hypertension were shown to associate with increased CMV DNA copy number in plasma (Li et al., 2011). Our results were the first to demonstrate that high CMV antibody titers are associated with increased blood pressure in humans. The relation between CMV titers and blood pressure is a new mechanism which could explain the deleterious effects of CMV. High CMV antibody levels as well as seropositivity indicating past or present CMV infection are known to be associated with mortality (Roberts et al., 2010; Simanek et al., 2011; Strandberg et al., 2009; Wang et al., 2010).

The inverse association between CMV titers and FMD is another possibly deleterious mechanism caused by CMV. *In vitro*, CMV infection has been shown to stimulate the P38-MAPK signalling pathway and upregulate phosphatase and tensin homolog substances (Shen et al., 2006; Weis et al., 2004). Via this pathway, endothelial nitric

oxide synthase is inhibited and reduced nitric oxide production leads to endothelial dysfunction (Petrakopoulou et al., 2004). The association between CMV and endothelial dysfunction has been further hypothesised to be a possible pathologic mechanism of hypertension (Zhang et al., 2011). This mechanism is one possible explanation for the demonstrated association between CMV titers and reduced FMD in our study, and it is also a possible alternative explanation for the demonstrated association between CMV titers and increased blood pressure. Despite these promising novel results, further studies are still needed to confirm these results and longitudinal studies are needed to show if there is a causal relation between CMV infection, blood pressure and endothelial function.

Summary and limitations

In this dissertation we found that measured inflammatory parameters were related to various metabolic, lifestyle and physical findings. These factors appeared to relate with each other in a rather complex way, which well accords with the current understanding of many pathological mechanisms linking inflammation and atherosclerosis. Our results also support the existence of such multiple relations in healthy young adults and middle-aged subjects. However, we could only show that CMV antibody levels were independently associated with early vascular changes. It is still important to realize that we measured only a few of the hundreds of known inflammatory parameters in humans (Cytokines & Cells Online Pathfinder Encyclopedia, www.copewithcytokines.org). In the future, it will be important to continue the research on inflammatory parameters, also in people without apparent atherosclerotic manifestations, in order to detect markers of ongoing atherosclerotic inflammation in the body.

There were limitations in these studies. First of all, we must concede that despite a wide range of studies affirming the relation between inflammation and atherosclerosis, the causality of these two events has not been confirmed in humans. Also, inflammation in atherosclerosis is not limited merely to the early phases of atherosclerosis. The inflammatory process continues from the initial lesion to the plaque rupture. Within the limits of this dissertation, it was only possible to discuss the inflammation in the early phase of atherosclerosis. This setup also limits the generalizability of these results. The cross-sectional natures of these studies do not allow us to investigate the causality. Therefore it is possible that measured inflammatory marker levels in these early stages of atherosclerosis may have no relation to late atherosclerotic indices or they might equally have an even more significant relation to them. Eventually only the lifelong longitudinal follow-up of the young Finns Study will reveal the true significance of these findings. Finally, there are also many other pathological mechanisms that play a substantial part in atherosclerosis, such as coagulation, lipid accumulation, fibrosis and calcification.

Therefore this dissertation cannot be considered a comprehensive presentation of the pathogenesis of atherosclerosis.

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ORIGINAL ARTICLE

Use of combined oral contraceptives alters metabolic determinants and genetic regulation of C-reactive protein. The Cardiovascular Risk in Young Finns Study

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Background. Use of combined oral contraceptives (COCs) is known to increase concentrations of C-reactive protein (CRP), an important predictor of cardiovascular disease. The inflammatory nature of the disease is well acknowledged. The aim of this study was to find out whether the metabolic, lifestyle and genetic determinants of CRP differ between women who use COCs and those who do not use any hormonal contraceptives (non-users). ***Material and methods***. A total of 1,257 women (24–39 years) participated in the ongoing Cardiovascular Risk in Young Finns Study, a population based cross-sectional follow-up study. Use of hormonal contraceptives was determined by questionnaire. Plasma CRP and other cardiovascular risk factors were measured; five *CRP* gene polymorphisms were genotyped (−717A>G, −286C>T>A, +1059G>C, +1444C>T and +1846G>A) and *CRP* haplotypes were constructed. ***Results***. Multivariate regression analysis revealed that BMI and leptin were the main determinants of CRP in non-users, whereas in COC users the main determinants were BMI, leptin and triglycerides. The median CRP and triglyceride values were significantly higher in COC users than in non-users. The correlations between triglyceride and CRP were tested separately in different COC users in accordance with progestagen content and dosage, the analysis revealing significant association only in women using a high dosage of progestagen or cyproterone. The haplotypes of *CRP* gene had no significant association with CRP concentration in COC users, while independent effects on CRP were found in non-users. ***Conclusion***. Our study suggests that use of COCs alters the metabolic determinants and genetic regulation of CRP.

Keywords: Body mass index; haplotypes; leptin; progestins; triglycerides

Introduction

C-reactive protein (CRP) is an acute phase protein that has been widely used as a marker of acute inflammation. It is now known that even a minor elevation of CRP, i.e. low-grade inflammation, is associated with an increased risk of cardiovascular disease (CVD) morbidity and mortality [1,2]. Many demographic, social, metabolic and lifestyle factors are known to have an effect on CRP concentration, e.g. age, sex, ethnicity, socio-economic status, birthweight, dietary pattern, physical activity, alcohol consumption, diabetes mellitus, insulin concentrations, glucose concentrations, blood pressure, body mass index (BMI), HDL-cholesterol, triglycerides and oestrogen/progestogen use [3,4].

Genetics plays a part in determination of CRP concentration. Twin studies suggest that CRP plasma concentrations are over 40 % heritable, and there is an increasing amount of evidence showing an association between *CRP* genetics and CRP concentration [5,6]. For example, in an American population, haplotypes constructed from seven selected *CRP* gene SNPs have been associated with different CRP concentrations [7]. In Finns, data from the ongoing Cardiovascular Risk in Young Finns Study, the population used in the present study, have shown that at least five *CRP* SNPs are associated with different CRP concentrations [8]. Haplotypes based on those five SNPs have been associated with lifelong differences in average CRP concentrations, and seem to explain about 5 % of circulating CRP concentrations [9].

Combined oral contraceptives (COCs) have been widely used as a safe and efficient method for preventing unwanted pregnancies. Over the years, the amounts of oestrogen and progestagen in COCs

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have been decreased and chemical formations have been modified in order to reduce the risk of thrombosis. The progestagens can be divided into second-generation (norgestrel, levonorgestrel, norgestrione) and third-generation (desogestrel, gestodene) compounds. Some progestagens are not classifiable into second or third generation (e.g. cyproterone, norgestimate). Despite these developments, with use of COCs there is still an increased the risk of developing myocardial infarction, especially in women with other cardiovascular risk factors [10]. Similarly, the risk of thromboembolic disease is still increased [11].

We have previously shown in this population that women using oral contraceptives have higher CRP concentrations than non-users [12]. It is usually considered that increased CRP is mostly due the oestrogen component in COCs, although there are some studies suggesting that CRP concentration might also depend on progestagen content in COCs [13–15]. In the present analysis, our aim was to find out whether the metabolic, lifestyle and genetic determinants of CRP differ between women who use COCs and those who do not use any hormonal contraceptives.

Methods

Subjects

The subjects in the study were participants in the ongoing Cardiovascular Risk in Young Finns Study, a five-centre follow-up study involving five university hospital cities in Finland. The study began in 1980, when 3,596 participants aged 3, 6, 9, 12, 15 and 18 were randomly selected for the study [16]. The most recent follow-up was conducted in 2001, when 1,257 women and 1,026 men 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 [17]. The study was approved by local ethics committees.

Clinical and chemical analyses

BMI was calculated from measured height and weight. A random zero sphygmomanometer (Hawksley & Sons Ltd, Lancinn UK) was used to measure blood pressure and a mean of three measurements was used in the analysis. From fasting plasma sample CRP, insulin, leptin, total cholesterol, HDL-cholesterol and triglyceride concentrations were drawn. LDL-cholesterol concentration was calculated using the Friedewald formula (detailed description in [17,18]). Smoking habits, alcohol consumption, hormonal contraceptive use, physical activity [19], history of recent infection, diabetes and chronic rheumatic disease were elicited by questionnaire.

The study included women who did not use any hormonal contraceptives (non-users) (n=811) and those who used COCs (COC users) (n=305), progestin only pills (n=12), intrauterine devices (n=119) or subcutaneous capsules (n=3). This data was unavailable in 7 subjects. The COCs contained the following substances: ethinylestradiol (n=291), oestradiol valerate (n=14), gestodene (n=136), desogestrel (n=78), cyproterone (n=50), levonorgestrel (n=47), norethisterone (n=4), lynestrenol (n=2). All intrauterine devices released levonorgestrel.

Fasting plasma CRP concentrations were analysed using a high-sensitive latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany); detection limit 0.06 mg/L. DNA was extracted from whole blood using a commercially available kit (Qiagen Inc., Hilden, Germany) in 2001. *CRP* gene polymorphisms −717A>G (rs2794521), −286C>T>A (rs3091244), +1059G>C (rs1800947), +1444C>T (rs1130864) and +1846G>A (rs1205) were genotyped using the ABI Prism 7900HT Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, Foster City, Calif., USA). For SNP +1059, a commercial kit from Applied Biosystems was used (Assay On Demand, C_177490_10 CRP). The SNPs −717, +1444 and +1846 were genotyped using Assays By Design from Applied Biosystems under standard conditions. The triallelic tagSNP −286 was genotyped as previously described [7], except for genotype calling, which was done manually from the PCR run component tab.

Statistical analyses

The haplotypes were constructed using the PHASE v. 2.0.2 program [20] from the five *CRP* SNPs. This program calculates from the genotype data the most likely haplotype pairs for each individual using a Bayesian statistical method. The haplotypes are shown in the order −717, −286, +1059, +1444 and +1846. The three most common haplotypes (frequency >0.10) in this cohort were: A-T-G-T-G (frequency 0.350), A-C-G-C-A (0.301), G-C-G-C-G (0.207) (previously published in [8]).

The data were analysed with SPSS for Windows statistical software (versions 14.0 and 15.0; SPSS Inc., Chicago, IL., USA). We excluded subjects whose CRP concentrations were above 10 mg/L (n=51), triglycerides above 4 mmol/L (n=4), who had a history of recent infections (n=83), diabetes (n=11) or chronic rheumatic disease (n=25), who were

pregnant ($n=62$) or lactating ($n=54$). Haplotyping was unsuccessful in four subjects. No separate analyses were performed for subjects using pills containing progestin only, subcutaneous capsules, oestradiol valerate, norethisterone or lynestrenol, because the number of subjects in these groups was low ($n<10$). As even vaginal administration of hormones can affect protein synthesis of hepatocytes [21], the intrauterine device users were excluded from the non-user group. The total number of subjects after the exclusions was 841.

Since the distributions of CRP, insulin, leptin and triglyceride values were skewed, the non-parametric Mann-Whitney test was used in statistical analysis. For linear regression analysis and for analysis of covariance (ANCOVA), the values were log-transformed prior to analysis. Correlation between skewed variables was estimated with Spearman's test. For normally distributed variables, the *t*-test for independent samples was used to detect differences in mean values among different groups.

The effects of metabolic and lifestyle factors on CRP concentration were analysed using a linear regression model, and therefore scale variables or dummy variables were used. The regression model for logarithmic CRP was constructed from the following variables: BMI, waist circumference, age, HDL-cholesterol, LDL-cholesterol, (log)triglycerides, diastolic blood pressure, systolic blood pressure, (log)insulin, glucose, (log)leptin, physical activity index, alcohol consumption and smoking. The variables were tested in a univariate model and values that were associated with the dependent variable ($p<0.15$) were selected for the multivariate model. From co-linear variables (e.g. BMI and waist circumference) only the one that showed a stronger association with (log)CRP was selected for the model. All non-significant variables ($p>0.05$) were excluded one by one from the multivariate model, starting from the least significant.

The effects of the haplotypes on CRP concentration were compared between carriers and non-carriers using the Mann-Whitney test. The difference between groups after adjustment by the variables showing significance in the regression model ($p<0.05$) was calculated with the ANCOVA method.

Results

Characteristics of the study subjects are given in Table I. Women using COCs had higher median CRP concentrations ($p<0.001$), triglyceride concentrations ($p<0.001$), insulin concentrations ($p=0.032$), mean BMI ($p=0.002$) and HDL-cholesterol ($p<0.001$) than non-users. The COC users also had significantly lower waist circumference ($p<0.001$), LDL-cholesterol concentrations ($p<0.001$), glucose concentrations ($p=0.009$) and were significantly younger ($p<0.001$). The intrauterine device users did not differ significantly from the non-users according to the parameters in data characteristics (data not shown).

To determine whether metabolic and lifestyle determinants of CRP differ between non-users and COC users, we built separate linear regression models for these groups (Table II). In the multivariate analysis of non-users, variables that remained significant in the model were BMI and (log)leptin. In the

Table I. Characteristics of study subjects.

	Non-users ($n=591$)		COC users ($n=250$)		
Variable	Mean	SD	Mean	SD	*p* for difference*
Body mass index (kg/m^2)	24.21	±4.49	26.21	±3.32	0.002
Waist circumference (cm)	79.37	±11.65	75.67	±8.62	<0.001
Age (years)	32.17	±4.99	29.36	±4.73	<0.001
HDL-cholesterol (mmol/L)	1.34	±0.28	1.54	±0.30	<0.001
LDL-cholesterol (mmol/L)	3.19	±0.75	2.93	±0.71	<0.001
Systolic blood pressure (mmHg)	116.13	±12.71	118.03	±12.49	0.053
Diastolic blood pressure (mmHg)	71.85	±8.74	72.52	±8.79	0.318
Glucose (mmol/L)	4.93	±0.43	4.84	±0.42	0.009
Physical activity index	16.50	±14.75	17.90	±14.05	0.258
Smoking (daily) (%)	20.07 %		20.99 %		
	Median	Quartiles	Median	Quartiles	*p* for difference**
CRP (mg/L)	0.54	0.26–1.33	1.66	0.81–3.30	<0.001
Triglycerides (mmol/L)	0.90	0.70–1.20	1.20	1.00–1.60	<0.001
Insulin (mU/L)	6.00	5.00–9.00	7.00	5.00–9.00	0.032
Leptin (mU/L)	12.59	7.59–19.95	12.88	8.66–19.50	0.538

**T*-test for difference between non-users and COC users. **Mann-Whitney test for difference between non-users and COC users.

Table II. Univariates and adjusted multiple linear regression model of (log)CRP in women without hormonal contraceptives and with COCs.

	Non-users (n=591)						COC users (n=250)					
	Univariate			Multivariate			Univariate			Multivariate		
Variable	B	SE	p	B	SE	p	B	SE	p	B	SE	p
Body mass index (kg/m^2)	0.053	±0.004	<0.001	0.028	0.005	<0.001	0.048	±0.008	<0.001	0.020	0.009	0.029
Waist circumference (cm)	0.002	±0.001	<0.001				0.002	±0.001	<0.001			
Age (years)	0.002	±0.004	0.542				0.000	±0.006	0.956			
HDL-cholesterol (mmol/L)	−0.294	±0.070	<0.001				−0.040	±0.097	0.680			
LDL-cholesterol (mmol/L)	0.101	±0.026	<0.001				0.014	±0.042	0.742			
(log) Triglycerides (mmol/L)	0.797	±0.107	<0.001				0.666	±0.169	<0.001	0.358	0.156	0.023
Systolic blood pressure (mmHg)	0.007	±0.002	<0.001				0.006	±0.002	0.006			
Diastolic blood pressure (mmHg)	0.010	±0.002	<0.001				0.009	±0.003	0.003			
(log) Insulin (mU/L)	0.697	±0.081	<0.001				0.440	±0.128	0.001			
Glucose (mmol/L)	0.163	±0.045	<0.001				0.063	±0.065	0.331			
(log) Leptin (mU/L)	0.869	±0.058	<0.001	0.557	0.080	<0.001	0.760	±0.101	<0.001	0.524	0.124	<0.001
Physical activity index	−0.004	±0.001	0.003				−0.005	±0.002	0.050			
Smoking (daily)	−0.007	±0.046	0.879				−0.123	±0.069	0.076			
Alcohol (drinks per week)	−0.001	±0.003	0.863				−0.007	±0.005	0.214			
				R^2=0.309						R^2=0.225		

multivariate analysis of COC users, variables that remained significant were BMI, (log)leptin and (log)triglycerides. To find out whether the effect of triglycerides was dependent on a certain progestagen formulation in COCs, we calculated correlation between triglycerides and CRP inside different progestagen-containing subgroups (Table III). The correlation was significant only in subjects using cyproterone (r=0.508, p=0.001). To assess the progestagen dosage effect, we calculated the correlation in women using continuous COCs with low dosages of progestagen (<3150 μg/month) and in women using continuous COCs with high dosages of progestagen (≥3150 μg/month). Cyproterone users were not included in this analysis. The correlation was significant only in women using continuous high dosages of progestagen (r=0.298, p=0.012). Differences in median CRP values between different progestagen users did not reach statistical significance.

The effects of the haplotypes on CRP concentrations were analysed separately in women according to contraceptive use before and after adjustment of metabolic and lifestyle factors. The results are presented in Table IV (ANCOVA). In non-users, all haplotypes had significant effects on CRP

Table III. Median CRP and triglycerides levels compared to the type of progestagen included in the COCs.

		CRP		Triglycerides		Correlation*	
Progestagen compound	n	Median	Quartiles	Median	Quartiles	r	p
Gestodene	116	1.56	0.89–3.33	1.20	1.00–1.40	0.128	0.171
Desogestrel	60	1.90	0.79–3.26	1.30	1.00–1.70	0.210	0.106
Levonorgestrel	37	1.29	0.54–2.34	1.10	0.90–1.50	0.290	0.082
Cyproterone	41	2.00	0.52–4.06	1.60	0.90–2.00	0.508	0.001
Low-dosage continuous	96	1.56	0.89–3.31	1.20	1.00–1.50	0.042	0.686
High-dosage continuous (without cyproterone)	71	2.02	0.82–3.43	1.30	1.00–1.80	0.298	0.012

*Spearman's test for correlation between CRP and triglycerides.

Table IV. Effect of CRP haplotype carriage on CRP levels before and after adjustment of metabolic and lifestyle factors.

Carriage of haplotype	Non-users					COC users				
	n	Median	Quartiles	*p**	Adjusted *p***	*n*	Median	Quartiles	*p**	Adjusted *p***
ATGTG+	308	0.59	0.28–1.36			124	1.65	0.72–3.44		
ATGTG−	210	0.43	0.23–0.96	0.008	0.004	94	1.71	0.82–3.27	0.776	0.688
ACGCA+	261	0.48	0.24–1.05			103	1.67	0.80–3.33		
ACGCA−	257	0.56	0.28–1.35	0.131	0.041	115	1.67	0.89–3.25	0.572	0.391
GCGCG+	195	0.41	0.20–0.86			87	1.87	0.89–3.18		
GCGCG−	323	0.66	0.28–1.49	<0.001	<0.001	131	1.54	0.79–3.33	0.584	0.419

Haplotypes are composed of SNPs −717A>G, −286C>T>A, +1059G>C, +1444C>T, 1846G>A. **Mann-Whitney test for difference between carrier and non-carrier. *ANCOVA test with (log)CRP values: Non-users after adjustment of BMI, (log)leptin. COC users after adjustment of BMI, (log)triglycerides, (log)leptin.

concentrations, the *p*-values ranging from <0.001 to 0.041. In COC users, the haplotypes had no significant effects on CRP concentration. Further analysis in relation to progestagen/oestrogen content or dosage did not change the result.

Discussion

CRP has various roles in the development of atherosclerosis. It can bind to modified LDL-cholesterol particles, especially to the non-esterified cholesterol in LDL [22], after which CRP-opsonized LDL can be taken up by macrophages via CRP receptor CD32 [23]. This can lead to increased foam-cell formation in atherosclerotic plaques. CRP can also induce the expression of adhesion molecules [24] and inhibit nitric oxide expression in the human endothelial cells [25], both of which facilitate the atherosclerosis processes further. In addition, CRP promotes apoptosis of endothelial progenitor cells that are responsible for vascular regenerative potential [26].

Although the risk of both myocardial infarction (MI) and venous thromboembolism (VTE) is generally low in young women, COC use is known to increase the risk of both. This is especially true in the case of women who have other cardiovascular risk factors – environmental and genetic. However, the risk of MI might be lower in women who use third-generation oral contraceptives [10], while the risk of VTE diseases might be higher in women who use third-generation COCs [11]. Because CRP is one possible pathological agent behind CVD, and COC usage affects CRP levels, we assessed the effects of COC usage and the different progestagen effects on CRP in young Finnish women.

The data shown in this report demonstrate that CRP and triglyceride levels are higher in COC users than in non-users. Also, the determinants of plasma CRP concentration are different in COC users than in non-users. The most striking difference was seen in triglycerides, i.e. in COC users there was a positive association between triglyceride and CRP concentrations, while in non-users there was no association at all. In COC users, the association was found only in users of COCs containing cyproterone or other high progestagen dosage COCs. To the best of our knowledge, these findings are now described for the first time.

It has previously been shown that COC use increases both plasma triglyceride concentrations [21,27] and CRP concentrations [28]; this was also observed in the present study. It is known that COCs increases CRP concentrations without increasing IL-6 concentrations [29], suggesting that COCs stimulates hepatocytes directly to synthesize CRP, and not via IL-6-mediated inflammation. The mechanism of how COCs stimulates hepatocytes protein synthesis is still not known. However, the role of oestrogen might be more important than that of progestagen, because studies in postmenopausal women have shown that oestrogen alone can induce CRP concentrations [14,30], and in premenopausal women it has been observed that pills containing only progestagen do not elevate CRP concentrations [28]. The role of progestagens on CRP concentration is still unclear. Studies comparing different progestagen effects on CRP suggest that the effect might depend on specific progestaten content [13–15]. Our findings support the idea that progestagens do have a role in CRP regulation. In particular, the amount of progestagen seems to be important. Although there were differences in correlation between CRP and triglycerides depending on the progestagens that had been used, there were no significant differences in CRP concentrations. The cyproterones are usually prescribed to women with androgen excess, e.g. polycystic ovary syndrome. This underlying disorder affects the CRP [31] and triglyceride [32] levels, so we cannot rule out that correlation between triglycerides and CRP

among cyproterone users is due to the underlying disorder, because its frequency is unknown in this cohort. To avoid this bias, all the analyses were also performed without cyproterone users, but it did not change the results. The other significant determinants of plasma CRP in our data were BMI and leptin. They had similar effects on CRP in COC users and non-users, so it seems that the IL-6 mediated stimulation of CRP by BMI [33] and leptin [34] is not affected by COC use.

In addition to the changes in the role of metabolic factors in the regulation of CRP concentrations, we found that the contribution of genetic control on CRP concentrations was entirely different between COC users and non-users. Haplotypes of the *CRP* gene were associated with CRP concentrations in non-users, but not in COC users. At present, the molecular background of this is not known. However, hepatic induction of *CRP* gene via IL-6 and without IL-6 is different; the *CRP* gene IL-6 responsive elements are probably involved in the former but not in the latter. In other words, the CRP production induced or enhanced by COCs is presumably regulated by different transcription factors from the IL-6 mediated CRP production. Of the 5 SNPs used for construction of the haplotypes, two are promoter region polymorphisms (−717 and −286), one is exonic (+1059) and two are in the 3′UTR region (+1444 and +1846), and there is no obvious steroid receptor binding sequences in these positions. Therefore, understanding the molecular mechanism of this observed difference requires a more thorough analysis of the transcriptional control of this gene. The haplotypes analysed can explain only about 5 % of the circulating CRP [9], so it is possible that the genetic effect is overwhelmed by the strong COC effect.

The most limiting factor in this study was its cross-sectional design. There was a relatively large variation in the use of different COCs with different contents and amounts of oestrogen and progestagens, which made it difficult to create comparable and representative subgroups in relation to COC usage. In order to keep comparison clear and representative in both groups, the main comparison was done between COC users and non-users. There were also other disadvantages. The use of contraceptives was elicited only by questionnaire; COC users and non-users might not be comparable in every respect. For example, there might be some infertile women who do not need COCs and also women wishing to become pregnant and therefore not using COCs. There could also be other reasons possibly altering the use of COCs that we could not account for, such as temporary intermission due to broken relationships, inconstant use of COCs (time and brand, etc.). Unfortunately we were not able to analyse all of these confounders, but still we believe that these cases are low in number and do not affect our results. Despite these limitations, we believe that this study included a relatively large and representative number of young women (n=841) and that our conclusions are valid.

In conclusion, our findings support a direct role for COCs in CRP determination by affecting its metabolic and genetic regulation. These findings highlight the importance of thorough adjustment of CRP concentration with confounding variables in the analysis of genetic polymorphism, i.e. COC usage diminishes the effect of *CRP* genetics on CRP concentration. Future research of COC usage-related changes in CRP determination and genetic regulation are needed, especially random controlled trials, and the clinical relevance of the findings has to be seen.

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Original Article

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Serum amyloid A is independently associated with metabolic risk factors but not with early atherosclerosis: the Cardiovascular Risk in Young Finns Study

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Abstract. Jylhävä J, Haarala A, Eklund C, Pertovaara M, Kähönen M, Hutri-Kähönen N, Levula M, Lehtimäki T, Huupponen R, Jula A, Juonala M, Viikari J, Raitakari O, Hurme M (University of Tampere and Tampere University Hospital, Tampere; and University of Turku, Health Care District of Southwest Finland and National Public Health Institute, Turku; Finland). Serum amyloid A is independently associated with metabolic risk factors but not with early atherosclerosis: the Cardiovascular Risk in Young Finns Study. *J Intern Med* 2009; **266:** 286–295.

Background. Serum amyloid A (SAA) is a sensitive marker of inflammation and its elevation has been implicated in obesity and in cardiovascular disease, yet data on its regulation in young adults or on its role in early atherosclerosis is scarce. We investigated which factors explain the variation in SAA and analysed whether SAA could be associated with preclinical atherosclerosis.

Methods. Serum amyloid A levels were measured in participants of the Cardiovascular Risk in Young Finns Study (n = 2280, n = 1254 women, n = 1026 men). Correlates and determinants of SAA were analysed and the effect of SAA on subclinical atherosclerosis, measured as intima-media thickness (IMT) and carotid artery compliance, was evaluated with risk-factor adjusted models.

Results. Serum amyloid A correlated directly and independently of BMI with C-reactive protein (CRP), waist circumference and leptin in both sexes, with total cholesterol, LDL cholesterol and Apolipoprotein-A1 (ApoA1) in women and with triglycerides, insulin levels and insulin resistance in men. Use of combined oral contraceptives and intrauterine device was also associated with SAA levels. Determinants for SAA included CRP, leptin and ApoA1 in women, and CRP, leptin and HDL cholesterol in men. SAA levels correlated with carotid compliance in both sexes and with IMT in men, yet SAA had no independent effect on IMT or carotid compliance in multivariable analysis.

Conclusions. Serum amyloid A was associated with several metabolic risk factors but was not an independent predictor of IMT or carotid artery compliance. Further longitudinal studies will show whether SAA holds a prognostic value as a risk marker, analogously to CRP.

Keywords: early atherosclerosis, low-grade inflammation, metabolic risk factors, serum amyloid A.

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Introduction

Inflammation has been proposed to have an essential pathophysiological role in atherosclerosis, metabolic syndrome and diabetes [1, 2]. The biochemical measurements used to detect the low-grade inflammatory state in these disorders has mainly focused on C-reactive protein (CRP), which frequently – yet not consistently – has been associated with severity or poorer prognosis in these disorders [1–3]. Another acute phase reactant, serum amyloid A (SAA), has also proven to be a suitable and sensitive indicator of the inflammation involved in various stages of these diseases [3, 4]. However, it is yet to be resolved whether CRP and SAA can act as functional risk factors or if they are merely risk markers, i.e. indicators of the systemic nature of the low-grade inflammatory conditions. Nevertheless, elevation in SAA has been shown to predict cardiovascular events analogously with or even better than CRP [5–7] and in this sense, it has been speculated that SAA could be one of the links or even a proatherogenic risk factor between the inflammatory metabolic disorders and cardiovascular disease (CVD) [8, 9].

The human genome encompasses four SAA genes, of which three encode functional proteins [4]. SAA1 and SAA2 are highly homologous reactants whose concentration can increase up to 1000-fold upon infection or trauma, whereas SAA3 is a pseudogene and SAA4 is a constitutively expressed minor constituent of nonacute-phase HDL [10]. During the acute phase, liver is considered to be the major site of SAA synthesis, whilst in nonacute low inflammatory conditions, such as obesity, the role of adipocytes as the secretory site of SAA has recently been firmly established [11–13]. Expression of SAA is induced in response to proinflammatory cytokines such as IL-1β, IL-6 and TNF-α and circulating SAA associates predominantly with HDL particles, in which it displaces ApoA1 and thus alters the reverse cholesterol transport [10].

Because of its established correlation with CRP, leptin, body mass index (BMI) and other obesity indices [9, 11, 14, 15] as well as with cardiovascular events [5, 6], SAA has been a subject of several recent studies focusing on subjects with chronic diseases or obesity. However, little is known about its baseline distribution and regulation in younger healthy adults, likewise its association with early atherosclerosis. The aim of this study was therefore to establish the correlates and determinants of SAA in ostensibly healthy young adults as well as to evaluate the associations of SAA and CRP with early atherosclerosis.

Methods

Subjects

The study population consisted of participants of the Cardiovascular Risk in Young Finns study, which is an ongoing multicentre follow-up study on atherosclerosis risk factors in Finnish children and young adults. The first study was conducted in 1980, when the study was initiated and when the participants (n = 3596), who were randomly chosen from the national population registers of Helsinki, Tampere, Turku, Oulu, Kuopio and their rural surroundings, were 3, 6, 9, 12, 15 and 18 years of age. The study design has been described in more detail elsewhere [16, 17]. The follow-up, on which the data of the current study is based, was carried out in 2001, when the participants had reached 24, 27, 30, 33, 36 and 39 years of age. The parameters of early atherosclerosis, i.e. carotid artery compliance and intima-media thickness (IMT), as well as serum lipids, proteins and hormones, obesity indices, smoking habits, blood pressure values, alcohol consumption, physical activity, the presence of diabetes and rheumatic diseases were also recorded in this follow-up [18]. The study was approved by the local ethics committees and was conducted following the guidelines of the Declaration of Helsinki. All participants gave their written informed consent.

Clinical characteristics and biochemical measurements

Height and weight as well as waist and hip circumferences were measured and BMI and waist-hip ratio were calculated. Blood pressure was measured using a random zero sphygmomanometer and the mean of three measurements was used in the analyses. Venous

blood samples for the determination of SAA, leptin, adiponectin, CRP, serum lipids (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides ApoA1 and ApoB), glucose and insulin were drawn after 12 h fasting. Serum SAA concentrations were measured with an ELISA kit with a detection limit of <0.004 mg L^{-1} (Human SAA, Biosource International, Camarillo, CA, USA). The inter-assay coefficient of variation (CV) was 10.6% at a mean level of 0.5988 mg L^{-1} and 12.6% at a mean level of 0.0613 mg L^{-1} and the intra-assay coefficient of variation was 5.8% at the mean level of 13.54 mg L^{-1}. Serum CRP was determined using a high-sensitivity latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany) with a detection limit of 0.06 mg L^{-1}. The CV for repeated measurements was 3.3% at the mean level of 1.52 mg L^{-1} and 2.65% at the mean level of 2.52 mg L^{-1}. Serum adiponectin was measured with radioimmunoassay (Human Adiponectin RIA kit; Linco Research, Inc., MO, USA) with an inter-assay CV of 11.9%. More detailed descriptions of the other analytical procedures and physical examination have been reported previously [18, 19]. Insulin resistance index was assessed with homeostasis assessment of insulin resistance (HOMA-IR), which was calculated based on fasting insulin and glucose values according to the formula: HOMA-IR = fasting glucose (mmol L^{-1}) × fasting insulin (mU L^{-1})/22.5 [20]. Physical activity, alcohol consumption, smoking habits, use of combined oral contraceptives (COCs) or intrauterine device (IUD), rheumatic diseases, diabetes and recent infections were elicited by questionnaire [18, 19].

Physical activity was assessed as a metabolic equivalent (MET) index, in which one MET is the consumption of 1 kcal of a person per weight kilogram per hour in rest. MET was calculated from the product of intensity × frequency × duration and commuting physical activity. In the estimation of the physical activity during commuting to work place, the length of the journey and the means (i.e. whether it was travelled by foot or by bicycle) were taken into account. The coefficients for the variables were estimated from the previously established tables [21].

Subjects with diabetes (n = 22, SAA median 17.90 mg L^{-1}), chronic rheumatic disease (n = 34, SAA 17.10 mg L^{-1}), history of recent infection (n = 113, SAA 12.10 mg L^{-1}) as well as pregnant women (n = 61, SAA 14.30 mg L^{-1}) were excluded. Lactating women (n = 41) were not excluded as their plasma SAA (13.10 mg L^{-1}) as well as CRP levels (0.7 mg L^{-1}) were comparable to the study population (P = 0.090 for SAA and P = 0.849 for CRP). Subjects with triglycerides above 4 mmol L^{-1} (n = 30, SAA 12.95 mg L^{-1}) were also excluded as the Friedwald formula used in LDL calculation could not be applied. In addition, we excluded women using COCs (n = 279, SAA 13.80 mg L^{-1}) or levonorgestrel-releasing IUD (n = 104, SAA 8.70 mg L^{-1}) from the analyses concerning the correlates, determinants and cardiovascular associations of SAA, as their SAA values deviated markedly ($P < 0.001$ and P = 0.03 respectively) from those of the nonusers. Rest of the excluded subjects (n = 128) were due to missing information in one or several measured variables. After subtracting these excluded individuals, we ended up with a population of n = 1509 subjects (n = 618 women and n = 891 men) for which we analysed the population data characteristics.

Carotid artery ultrasound measurements

Carotid ultrasound measurements were performed with a Sequoia 512 high-resolution ultrasound system (Acuson, CA, USA). Carotid artery compliance, which depicts the ability of the large arteries to expand under cardiac pulse pressure, was assessed from the formula $([D_s-D_d]/D_d)/(P_s-P_d)$, where D_s is the systolic diameter, D_d is the diastolic diameter, P_s is the systolic blood pressure and P_d is the diastolic blood pressure [22]. Mean IMT, a structural marker of vascular changes and a predictor of future cardiovascular events, was derived from a minimum of four measurements of the posterior wall of the left carotid artery (at approximately 10 mm proximal to the bifurcation) [22]. Both carotid compliance and IMT are continuous variables and they followed the normal distribution in our study population.

Statistical analyses

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). All the analyses were carried out separately for men and women due to their markedly deviating SAA values ($P < 0.001$). Variables with skewed distribution (SAA, CRP, leptin, adiponectin, insulin and triglycerides) were log-transformed prior to analyses when normal distribution was required. Comparisons of the variables between sexes were performed with Student's *t*-test, Mann–Whitney's test and Chi-squared test as appropriate. In addition, Mann–Whitney's test was used to test the heterogeneity of SAA levels between smokers and nonsmokers as well as between COC or IUD users and nonusers. The correlations between (log)SAA and clinical parameters as well as other serum markers were assessed with Pearson's correlation coefficients, additionally adjusted for BMI. Determinants, i.e. the variables that explained the variation in SAA, were analysed with stepwise multivariable regression analysis in which all the variables that correlated significantly ($P < 0.05$) with SAA were included. Finally, multivariable linear regression analysis was used to evaluate whether SAA and CRP had an effect on preclinical atherosclerosis markers, carotid compliance and IMT. To exclude confounding effects, SAA and CRP were entered into the model as an added factor and the analysis was adjusted in a stepwise manner with the established risk-factors of atherosclerosis, i.e. age, BMI, LDL cholesterol, (log)triglycerides, glucose, systolic blood pressure (only IMT), daily smoking and physical activity. In all analyses, the level of $P < 0.05$ was considered statistically significant.

Results

Characteristics of the study population are shown in Table 1. The majority of the risk factors differed significantly between men and women, the only variables not deviating were age, physical activity index, insulin, insulin resistance index (HOMA-IR) and CRP (Table 1). A significant difference in SAA levels between men and women was observed ($P < 0.001$), but divergence in SAA levels was not, however, attenuated (data not shown) when the analysis was adjusted for leptin, adiponectin or ApoA1, which are the factors more elevated in women than in men.

Median SAA levels of daily smokers and nonsmokers did not differ amongst either sex ($P = 0.546$ for women and $P = 0.642$ for men). Women using COCs had significantly higher SAA values than nonusers [SAA median 13.60 mg L^{-1}, (IQR 7.67–25.90) vs. SAA median 10.70 mg L^{-1}, (IQR 6.33–18.68), $P < 0.001$], whereas women using levonorgestrel-releasing IUD had lower SAA values than nonusers [SAA median 8.75 mg L^{-1}, (IQR 4.91–15.78) vs. 10.70 (IQR 6.33–18.68 mg L^{-1}), $P = 0.034$]. To examine whether the difference in SAA levels could be attributed to the higher CRP concentration in COC using women [23], the analysis was adjusted for plasma CRP. This adjustment, however, did not abolish the observed differences.

SAA was found to correlate significantly ($P < 0.05$) and directly with BMI, waist circumference, waist-hip ratio, total cholesterol, LDL cholesterol, triglycerides, blood pressure, insulin, insulin resistance index (HOMA-IR), leptin, CRP and ApoB in both genders. However, significant and direct correlation was observed with glucose and physical activity index only in men, whilst a direct correlation with ApoA1 and adiponectin was observed only in women. Variables not correlating with SAA were age, glucose, use of alcohol, ApoB and homocysteine in women and use of alcohol, ApoA1 and homocysteine in men. All the significant correlations ($P < 0.05$) were further adjusted for BMI. Correlates that remained significant after BMI adjustment are presented in Table 2.

All variables correlating significantly ($P < 0.05$) with SAA were included in the multiple linear regression model. According to the stepwise multivariable linear regression analyses, the determinants for SAA in women were CRP ($B = 0.479$, $P < 0.001$), ApoA1 ($B = 0.270$, $P < 0.001$) and leptin ($B = 0.121$, $P = 0.012$) which together explained 44.9% of the total variation in SAA (Table 3a). In men, the determinants for SAA were CRP ($B = 0.610$, $P < 0.001$), leptin ($B = 0.112$, $P = 0.007$) and HDL cholesterol ($B = 0.097$, $P = 0.017$), together explaining 49.7% of the

Table 1 Characteristics of the study population

	Women (n = 618)		Men (n = 891)		
Variable	Mean	SD	Mean	SD	P for difference
Age (years)	32.05	4.94	31.66	5.02	0.134
BMI (kg m^{-2})	24.35	4.60	25.61	3.81	<0.001
Waist circumference (cm)	79.39	11.46	89.30	10.55	<0.001
Waist-hip ratio	0.80	0.06	0.89	0.06	<0.001
Systolic blood pressure (mmHg)	115.54	12.54	128.96	13.59	<0.001
Diastolic blood pressure (mmHg)	71.49	8.69	74.83	9.09	<0.001
Total cholesterol (mmol L^{-1})	5.01	0.89	5.24	1.00	<0.001
HDL cholesterol (mmol L^{-1})	1.35	0.27	1.17	0.28	<0.001
LDL cholesterol (mmol L^{-1})	3.19	0.77	3.43	0.89	<0.001
Triglycerides (mmol L^{-1})[a]	0.90	0.70–1.20	1.20	0.90–1.80	<0.001
ApoA1 (g L^{-1})	1.50	0.22	1.41	0.21	<0.001
ApoB (g L^{-1})	0.97	0.23	1.12	0.26	<0.001
Insulin (mU L^{-1})[a]	6.00	4.00–8.00	6.00	4.00–9.00	0.403
Glucose (g L^{-1})	4.91	0.41	5.15	0.41	<0.001
HOMA-IR	1.59	1.31	1.70	1.19	0.081
Leptin (ng mL^{-1})[a]	12.59	7.58–19.50	4.07	2.40–6.46	<0.001
Adiponectin (μg mL^{-1})[a]	10.00	7.80–13.50	6.80	5.10–9.20	<0.001
Homocysteine (μmol L^{-1})	9.27	3.57	10.79	3.89	<0.001
CRP (mg L–)[a]	0.56	0.26–1.36	0.58	0.29–1.34	0.679
SAA (mg L^{-1})[a]	10.70	6.33–18.68	8.00	5.00–15.00	<0.001
Physical activity index	16.59	14.74	16.16	17.40	0.649
Alcohol (drinks per week)	3.72	6.44	8.76	10.40	<0.001
Smoking daily (% of total)[b]	20		29		<0.001
Carotid compliance (%/10 mmHg)	2.34	0.78	2.02	0.66	<0.001
IMT (mm)	0.58	0.09	0.59	0.10	0.003

t-test for difference between groups.
[a]Median values and interquartile range (IQR), Mann–Whitney's test for difference between groups.
[b]Chi-Squared test for difference between groups.
ApoA1, apolipoproteinA1; ApoB, apolipoproteinB; BMI, body mass index; CRP, C-reactive protein; HOMA-IR, homeostasis assessment of insulin resistance; IMT, intima-media thickness; SAA, serum amyloid A.

variation in SAA (Table 3a). However, due to the strong intercorrelation and similar proinflammatory regulation of SAA and CRP we also wished to analyze the determinants of SAA without the effect of CRP. In this model, the determinants for SAA in women were leptin (B = 0.424, P < 0.001), ApoA1 (B = 0.270, P < 0.001) and body mass index (B = 0.011, P = 0.013) and in men they were leptin (B = 0.340, P < 0.001) and waist circumference (B = 0.001, P = 0.006) (Table 3b). These models, however, explained only 18.5% (in women) and 12.6% (in men) of the variation in SAA.

Stepwise multiple linear regression analyses were used to assess the effect of SAA and CRP on the early atherosclerosis markers carotid compliance and IMT. The effect of SAA and CRP was found to be very similar, yet dependent on other risk factors, mainly BMI and serum lipids (Table 4). In univariable analysis (model 1, Table 4) both SAA and CRP correlated inversely with carotid compliance in both sexes and directly with IMT in men (Table 4). Neither SAA, nor CRP correlated with IMT in females (Table 4). A similar trend was also observable after adjusting for age, smoking and physical activity

Table 2 Correlates of clinical and biochemical parameters with (log)SAA in women and men

	Women (n = 618)		Adjusted for BMI		Men (n = 891)		Adjusted for BMI	
Variable	r^a	P	r^a	P	r^a	P	r^a	P
Body mass index (BMI)	0.328	<0.001			0.281	<0.001		
Waist circumference	0.334	<0.001	0.093	0.021	0.312	<0.001	0.138	<0.001
Waist-hip ratio	0.214	<0.001	0.034	0.405	0.250	<0.001	0.092	0.006
Age	0.025	0.732			0.074	0.028	0.026	0.434
Total cholesterol	0.189	<0.001	0.147	<0.001	0.099	0.003	0.032	0.348
HDL cholesterol	0.056	0.163			−0.066	0.049	0.010	0.776
LDL cholesterol	0.144	<0.001	0.094	0.021	0.071	0.035	0.010	0.766
(log)Triglycerides	0.170	<0.001	0.059	0.144	0.180	<0.001	0.077	0.022
ApoA1	0.121	<0.001	0.205	<0.001	0.010	0.766		
ApoB	0.219	<0.001	0.066	0.152	0.165	<0.001	0.060	0.073
Systolic blood pressure	0.163	<0.001	0.048	0.235	0.080	0.017	−0.006	0.869
Diastolic blood pressure	0.157	<0.001	0.052	0.202	0.104	0.002	0.028	0.438
(log)Insulin	0.183	<0.001	0.014	0.765	0.206	<0.001	0.077	0.032
Glucose	0.022	0.593			0.068	0.042	0.024	0.512
(log)Leptin	0.390	<0.001	0.236	<0.001	0.342	<0.001	0.217	<0.001
HOMA-IR	0.191	<0.001	0.002	0.968	0.200	0.001	0.075	0.025
(log)Adiponectin	0.100	0.016	−0.021	0.611	−0.051	0.131		
Homocysteine	−0.009	0.830			0.023	0.492		
Physical activity index	−0.036	0.438			−0.072	0.043	−0.068	0.058
Alcohol	−0.015	0.707			−0.001	0.978		
(log)CRP	0.650	<0.001	0.585	<0.001	0.703	<0.001	0.672	<0.001

The significant correlations were adjusted for BMI.
[a]Pearson's correlation.
ApoA1, apolipoproteinA1; ApoB, apolipoproteinB; BMI, body mass index; CRP, C-reactive protein; HOMA-IR, homeostasis assessment of insulin resistance; SAA, serum amyloid A.

(model 2 in Table 4). However, after adjusting for BMI, (log)triglycerides, glucose, LDL cholesterol and systolic blood pressure (only for IMT), the effects of SAA and CRP on carotid compliance and IMT were attenuated to the null (model 3 in Table 4). Only the association of CRP with carotid compliance in males remained of borderline significance (model 3 in Table 4).

Discussion

The results of this large cross-sectional study demonstrate as a novel finding that amongst ostensibly healthy adults, SAA levels correlate directly with several metabolic risk factors in women and in men, all correlations being independent of BMI. CRP was found to be the main determinant for SAA in both sexes, yet the additional model, in which we assessed the determinants for SAA without CRP, indicated that we might actually lack the best biological regulator(s) of SAA, as the additional model did not explain the variation on SAA very well.

Our results concerning the correlation of SAA with obesity indices, leptin and CRP corroborate earlier reports, which have shown that SAA levels correlate directly with CRP, leptin, obesity indices, body fat percentage and adipocyte size [9, 11, 14, 15]. However, others have not consistently documented the BMI-independent correlations between SAA and triglycerides, ApoA1 and HDL cholesterol [11, 13, 15]. These divergences may be due to the fact that the other studies have generally been performed on older and obese individuals in smaller study populations, in

Table 3 Determinants for serum amyloid A (SAA) in adjusted multiple linear regression models in women and in men with (a) and without (b) the effect of C-reactive protein (CRP)

	Women (n = 618)			Men (n = 891)		
Variable	B	SE	P	B	SE	P
(a)						
(log)CRP	0.479	±0.028	<0.001	0.610	±0.025	<0.001
(log)Leptin	0.121	±0.048	0.012	0.112	±0.041	0.007
ApoA1	0.270	±0.054	<0.001			
HDL cholesterol				0.097	±0.040	0.017
	R^2 = 0.449			R^2 = 0.497		
(b)						
(log)Leptin	0.424	±0.070	<0.001	0.340	±0.071	<0.001
ApoA1	0.270	±0.054	<0.001			
BMI	0.011	±0.004	0.013			
Waist circumference				0.001	±0.001	0.006
	R^2 = 0.185			R^2 = 0.126		

Variables correlating significantly with (log)SAA ($P < 0.05$) were entered into the model in a stepwise manner.
Variables entered for women were BMI, waist circumference, waist-hip ratio, total cholesterol, LDL cholesterol, (log)triglycerides, systolic blood pressure, diastolic blood pressure, (log)adiponectin, (log)insulin, glucose, insulin resistance (HOMA-IR), (log)leptin, (log)CRP (only in model a), ApoA1 and ApoB. Variables entered for men were BMI, waist circumference, waist-hip ratio, age, total cholesterol, HDL cholesterol, LDL cholesterol, (log)triglycerides, systolic blood pressure, diastolic blood pressure, (log)insulin, glucose, insulin resistance (HOMA-IR), (log)leptin, physical activity index, (log)CRP (only in model a) and ApoB.
ApoA1, apolipoproteinA1; BMI, body mass index; CRP, C-reactive protein.

Table 4 Serum amyloid A (SAA) and C-reactive protein (CRP) as markers of IMT and carotid compliance in multivariable stepwise regression model

		Model 1				Model 2				Model 3			
		n	B	SE	P	n	B	SE	P	n	B	SE	P
(log)SAA													
Women	IMT	609	−0.001	0.009	0.881	466	0.002	0.010	0.833	466	−0.008	0.010	0.427
	Carotid compliance	609	−0.206	0.081	0.011	466	−0.227	0.092	0.014	466	−0.100	0.094	0.289
Men	IMT	883	0.022	0.008	0.005	773	0.015	0.008	0.065	771	0.004	0.008	0.620
	Carotid compliance	880	−0.170	0.051	0.001	771	−0.149	0.052	0.004	771	−0.071	0.053	0.177
(log)CRP													
Women	IMT	609	0.002	0.007	0.797	466	0.003	0.008	0.740	466	−0.012	0.009	0.172
	Carotid compliance	609	−0.142	0.064	0.026	466	−0.163	0.072	0.023	466	0.014	0.081	0.861
Men	IMT	883	0.027	0.007	<0.001	773	0.021	0.007	0.003	771	0.008	0.008	0.275
	Carotid compliance	880	−0.215	0.045	<0.001	771	−0.199	0.046	<0.001	771	−0.100	0.050	0.045

Model 1. Only (log)SAA or (log)CRP as added factor.
Model 2. The effect of (log)SAA or (log)CRP adjusted for age, physical activity and smoking.
Model 3. The effect of (log)SAA or (log)CRP adjusted for age, physical activity, smoking, BMI, LDL cholesterol, glucose, systolic blood pressure (only for IMT) and (log)triglycerides.
BMI, body mass index; IMT, intima-media thickness.

which SAA levels have also fallen drastically, concomitantly with weight loss. The BMI-independent correlations observed here therefore demonstrate that SAA can be regarded as an indicator of the metabolic status in lean and younger individuals as well. Along these lines, as all the determinants for SAA in our

cohort, i.e. CRP, leptin and ApoA1/HDL cholesterol are related to lipid metabolism, we speculate that adipose tissue, regardless of its extent, contributes to SAA regulation via these factors. This hypothesis is plausible in light of the knowledge that excess/dysfunctional adipose tissue is a source of several pro-inflammatory reactants and that SAA and CRP are upregulated by pro-inflammatory stimuli, such as IL-1β, TNF-α and IL-6 [1, 4]. On the other hand, SAA, CRP and leptin can also induce pro-inflammatory cytokines [1, 9, 24], suggesting a positive feedback mechanism and a complex interconnection between these three factors. In addition, SAA has been shown to have a long-term effect in stimulating basal lipolysis [9] thus perhaps contributing to insulin resistance via increased release of free fatty acids into circulation. Our results corroborate this finding as we observed that in male subjects SAA correlates directly with insulin resistance index and triglycerides, independently of BMI. However, the molecular mechanisms interconnecting obesity and inflammation are still rather unknown and our data do not permit any conclusions on the direction or causality of this regulation scheme.

We observed that women had significantly elevated SAA levels compared with men; Lappalainen *et al.* (2008) also observed a similar sex difference amongst obese subjects [11]. Concurrent observations have also been reported for SAA mRNA production in adipose tissue [11, 13], though the reason for the gender difference is not known. We sought to explain the gender difference by the higher leptin and ApoA1 concentrations in women, but adjustment with these factors did not change the result, indicating that the divergence is related to other factors, possibly body composition or hormonal profile. Unfortunately, we have no data available on body fat percentage or sex hormones in our study population.

Nevertheless, hormonal contribution to SAA regulation has been reported in premenopausal women using COCs and also in postmenopausal women receiving oestrogen replacement therapy (ERT); SAA levels were significantly elevated amongst those receiving oral-conjugated estrogens [25, 26]. In addition, it has recently been demonstrated that ERT-associated elevation in SAA was counteracted by oral administration of an androgenic progestin, medroxyprogesterone acetate [27]. In this study, we observed that premenopausal women using COCs had significantly higher SAA levels compared with nonusers, whereas women using levonorgestrel-releasing IUD had lower median SAA than nonusers. Although not directly comparable, our results lend support to these observations on hormonal regulation of SAA. First, the higher SAA levels amongst the COC using women were not merely secondary to their higher CRP concentrations [23] as the difference remained unchanged after adjusting for plasma CRP. Second, the IUD used by our study women contained levonorgestrel which is an androgenic, testosterone-derived progestin [28], thus likely to excrete anti-inflammatory effects analogous to medroxyprogesterone acetate. The mode of action of these hormonal components most probably deals with the first-pass hepatic effect, as transdermal ERT has an opposite effect on SAA levels compared with oral ERT [25]. However, whether systemic inflammatory reaction is involved in this regulation of SAA is still somewhat uncertain [25, 26] and we were unfortunately unable to evaluate the matter as we did not measure the IL-6 or TNF-α levels in our cohort.

Whilst acute-phase SAA is almost entirely of liver origin, mounting evidence implies that adipose tissue is the main source of circulating SAA during the non-acute conditions, [9, 11–13, 15]. Also, endothelial cells, smooth muscle cells, monocytes and macrophages in atherosclerotic lesions have been reported to account for the extrahepatic production of SAA, as the presence of both SAA mRNA and protein products has been detected in these cell types [29–31]. Moreover, SAA is able to alter vascular proteoglycans in a proatherogenic manner [32] and to stimulate the production of various inflammatory mediators, such as TNF-α, IL-1β, IL-8, plasminogen activator inhibitor-1 and tissue factor in cultured vascular endothelial cells, neutrophils and monocytes [9, 24, 33]. In addition, activated neutrophils can induce formation of SAA-LDL complexes via lipoprotein oxidation *in vitro* [34]. Elevation in circulating SAA has also been related to acute cardiovascular events, with a better or

equal prognostic value compared with CRP [5–7]. It therefore follows that, as CRP is already a widely used marker in prognosis of CVD [3, 7], these observations have raised the possibility that SAA could also be a proatherogenic risk factor and not merely a marker of systemic or local inflammation. However, data are still lacking with regard to the clinical relevance of minor elevation in SAA and also the cut-off value for low-grade elevation in SAA has not been established yet.

Our results, however, indicate that the association of SAA, as well as that of CRP, on the early atherosclerosis is mediated through BMI and serum lipids as the associations with carotid compliance and IMT were attenuated to the null when the multivariable model was adjusted for BMI and serum lipids. These results are in accordance with those of Wohlin *et al.* (2007) who observed no independent association between SAA and IMT in older men [35], yet Schillinger *et al.*, (2005) reported that both SAA and CRP are associated with the progression of active but initially asymptomatic atherosclerosis in both sexes [36]. Therefore, functional studies are required to establish the role, if any, of SAA in atherogenesis, both in conditions conferring a risk of atherosclerosis *per se* – such as obesity – as well as in healthy and lean individuals. Although SAA is not an independent determinant for subclinical atherosclerosis in healthy young adults, we suggest that it is a marker of metabolic status and it remains to be discovered whether it contributes to the pathogenesis of early atherosclerosis via factors related to lipid metabolism. In conclusion, the results of this study demonstrate that SAA is associated with several metabolic risk factors in both genders regardless of BMI, and also with the use of COCs or IUD in premenopausal women, independently of CRP.

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Conflict of interest statement

None declared.

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ORIGINAL ARTICLE

Heart rate variability is independently associated with C-reactive protein but not with Serum amyloid A. The Cardiovascular Risk in Young Finns Study

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ABSTRACT

Background Increased levels of C-reactive protein (CRP) and serum amyloid A (SAA) are associated with an increased risk of cardiovascular disease. It is hypothesized that dysregulation of the autonomic nervous system (ANS) leads to increased inflammation via the cholinergic anti-inflammatory pathway. Heart rate variability (HRV) is a marker of ANS function. HRV has been shown to be associated with CRP levels. Currently, there are no studies addressing the relationship between HRV and SAA.

Design The purpose of this study was to compare the associations between HRV, CRP and SAA in healthy young adults. CRP and SAA concentrations and short-term HRV indices [high frequency (HF), low frequency (LF), total spectral component of HRV, root mean square differences of successive R-R intervals, the standard deviation of all R-R intervals and ratio between LF and HF) were measured in 1601 men and women aged 24–39 taking part in the Cardiovascular Risk in Young Finns study.

Results A significant inverse correlation ($P < 0{\cdot}05$) between HRV indices and inflammatory markers was observed. However, in linear regression analyses, only inverse association between HRV indices and CRP levels remained significant ($P < 0{\cdot}05$), while association between HRV indices and SAA levels was attenuated to the null ($P > 0{\cdot}05$) after adjusting for age, sex, body mass index, cholesterol levels, leptin and other common traditional cardiovascular risk factors.

Conclusions Reduced HRV indices are independently associated with increased CRP levels, but not with SAA levels. This association supports the hypothesis that dysregulation of the ANS may lead to increased inflammation early in adulthood.

Keywords Autonomic nervous system, C-reactive protein, heart rate variability, inflammation, leptin, Serum amyloid A.

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Introduction

Heart rate variability (HRV) is the oscillation of consecutive heart beats over time. The HRV measurement is a non-invasive method used to assess autonomic nervous system (ANS) control of the heart [1]. Under resting conditions, the parasympathetic nervous system is a more potent regulator of heart rate (HR) while, during exercise, the sympathetic nervous system is more important. Reduced HRV is associated with an increased risk of cardiovascular disease (CVD) [2,3], diabetes [4] and

hypertension [5]. In addition, many studies have also reported the simultaneous presence of increased inflammatory parameters.

Previous studies have shown that acetylcholine can suppress the production of pro-inflammatory cytokines *in vitro*, while extrinsic stimulation of the vagus nerve *in vivo* has been shown to inhibit the release of pro-inflammatory cytokines and to prevent inflammation [6,7]. The pathway related to ANS control of the immune system has been termed the cholinergic anti-inflammatory pathway [8]. Several studies have shown an inverse relationship between HRV and inflammatory markers levels, such as C-reactive protein (CRP) and interleukin-6, in individuals suffering from CVD [9], as well as in healthy populations [10–13]. The current view is that reduced HRV indices reflect an imbalance in the ANS, which results in increased inflammation and stimulation of CRP synthesis via the cholinergic anti-inflammatory pathway. This pathway represents a possible link between ANS dysfunction, inflammation and CVD. Inflammation has an important role in the pathogenesis of atherosclerosis [14]. CRP has been used as a marker of low-grade inflammation, and it has been associated with an increased risk of CVD [15,16]. One study has reported that reduced HRV and increased CRP levels have a synergistic effect on the risk for CVD [17].

Like CRP, serum amyloid A (SAA) is an acute phase protein that is associated with CVD [15,18] but not with subclinical atherosclerosis [19]. Unlike CRP, SAA is mainly produced in adipose tissue under noninflammatory conditions. In addition, serum concentrations of SAA correlate strongly with adipose mass [20,21]. It has been suggested that SAA may be a link between obesity and inflammatory conditions [21]. Both CRP and SAA have been shown to associate strongly with leptin levels in noninflammatory conditions [19,22]. Also, it has been previously demonstrated that leptin levels correlate with HRV indices [23]. Currently, there are no studies addressing the association between HRV indices and SAA levels, and none of the previous studies have investigated whether the association between HRV and inflammatory markers is independent of leptin [10–13]. In this study, we measured short-term HRV indices and CRP, SAA and leptin levels in a population of healthy young adults. We sought to determine whether HRV indices are independent of the inflammatory markers CRP and SAA in a large population consisting of healthy young adults.

Methods

Subjects

The study population consisted of participants in the Cardiovascular Risk in Young Finns study, which is an ongoing multicentre follow-up study involving five university city hospitals in Finland. The study began in 1980, when 3596 participants between the ages of 3 and 18 were randomly selected from the national population registers. The design of this study has been described in more detail elsewhere [24–26]. The 21-year follow-up was conducted in 2001, when the participants were between 24 and 39 years of age. Cardiovascular risk factor measurements, including body mass index (BMI), serum lipids, blood pressure values, CRP, SAA, leptin, alcohol consumption and smoking habits, were recorded during this follow-up. This study was approved by local ethics committees. All participants provided a written informed consent.

Clinical and chemical analyses

Blood pressure measurements were taken using a random zero sphygmomanometer (Hawksley & Sons Ltd, Lancin, UK), and the mean of three measurements was used in the analysis. BMI was calculated from measured height and weight values. CRP, SAA, leptin, insulin, triglycerides and total cholesterol levels were obtained from blood plasma samples drawn during fasting. Information on smoking habits, alcohol consumption, oral contraceptive use, physical activity, pregnancy and breastfeeding was collected by administering a questionnaire.

Fasting plasma CRP concentrations were analysed by using a high-sensitive latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany) with a detection limit of 0·06 mg L^{-1}. Serum SAA concentrations were measured with an ELISA kit with a detection limit of < 0·004 mg L^{-1} (Human SAA, Biosource International, Camarillo, CA, USA). Serum leptin concentrations were analysed with a RIA (Human leptin RIA kit; Linco Research, St. Charles, MO, USA). Other analytical and physical examination procedures were performed as previously reported [22,25].

HRV measurements

A single-channel chest-lead electrocardiogram (ECG), of 3 min in length, was recorded in 2001. Prior to the recording, subjects were comfortably seated in supine positions for a minimum of 15 min during a vascular ultrasound scan. The signals were converted from analogue to digital with a sampling rate of 200 Hz; the respective time series of R-R intervals were generated. ECG signals were manually revised by T.K. The stationary period was identified during 3-min period of metronome-controlled breathing at frequency of 0·25 Hz and was used to compute the time- and frequency-domain HRV indices. The study subjects were trained to this procedure beforehand to avoid emotional arousal. The mean duration of the analysed stationary period was 173 s, and the mean number of analysed R-peaks was 196. These indices were analysed by using a commercial WinCPRS program (Absolute Aliens, Turku, Finland). More detailed descriptions of these methods are described elsewhere [27].

The following parameters were identified from time- and frequency-domain measurements: the high-frequency oscillation (HF, 0·15–0·40 Hz), the low-frequency oscillation (LF, 0·04–0·14 Hz), the total spectral component of HRV (TP), mean root square differences of successive R-R intervals (RMSSD) and the standard deviation of all R-R intervals (SDNN). The ratio between LF and HF oscillations (LF/HF × 100%) was calculated. Under resting conditions, vagal activity of cardiovascular autonomic regulation prevails [28]. The vagal activity is the major contributor of the HF component of spectral HRV [1]. The LF component may represent either sympathetic modulation or combination of both sympathetic and vagal influences. LF/HF is considered to reflect sympathovagal balance or sympathetic modulations [1,29]. RMSSD is considered as a marker of vagal function, and it correlates strongly with HF.

Short-term HRV indices depend on the length of the time-series, and shorter time-series may result in less variability; therefore, 5-min recordings have been recommended [1]. For this study, HRV indices were recollected from 75 subjects to compare HRV indices on 3- and 5-min ECG recordings. In addition, the reproducibility of short-term HRV indices was assessed from 51 subjects after an average of 4·4 months from the first measurement. Both the reproducibility and the comparability of these measurements were satisfactory, and no statistically significant differences were observed in these measurements [27].

Statistical analysis

Electrocardiogram signals were recorded from 2151 subjects. Ectopic beats can bias both time- and frequency-domain measurements; therefore, subjects with three or more ectopic beats (n = 87) were excluded, and recordings with less than three ectopic beats were manually interpolated. Data recordings or saving of the data posed some problems for 21 of the study subjects. We excluded subjects who had a recent history of infection (n = 129), diabetes (n = 26) or chronic rheumatic disease (n = 36); subjects on antihypertensive (n = 65), lipid-lowering (n = 8), antidepressive (n = 69) or antipsychotic (n = 26) drugs; and subjects who were pregnant (n = 62) or lactating (n = 54), had impaired fasting glucose levels (> 6·0 mM, n = 62) or who suffered from hypertension and were not on medication (n = 12). Subjects with missing data or with one or more exclusion criteria were not included in the analysis. The total number of subjects included in the study was then 1601.

The data were analysed with SPSS (version 15.0; SPSS Inc., Chicago, IL., USA). The distributions of HF, LF, TP, LF/HF, SDNN and RMSSD indices were skewed; therefore, variables were logarithmically (natural logarithm) transformed. In addition, natural logarithm values were used for CRP, SAA, leptin, triglycerides and insulin levels when normal distribution was required. The correlation between inflammatory variables and HRV indices was estimated using the Pearson's test. Multivariate linear regression models were performed to assess the independent effects of HRV indices as determinants of CRP and SAA levels. HRV indices were added to the model using the enter method; the stepwise method was used to adjust the model to the clinical and metabolic determinants. Of the colinear variables (*e.g.* BMI and waist–hip ratio), only those which showed the strongest correlation with the inflammatory variables were selected for the model. Reporting of the study conforms to STROBE Statement [30].

Results

The characteristics of the study population are presented in Table 1. Pearson correlations between inflammatory markers levels, leptin and HRV indices are shown in Table 2. CRP and SAA levels correlated significantly with HR, lnLF, lnTP, lnSDNN and lnRMSSD indices. Additionally, SAA levels correlated significantly with lnHF indices. However, CRP and SAA levels did not correlate with the lnLF/HF ratio. Leptin levels correlated significantly with HR, lnLF, lnTP, lnLF/HF, lnSDNN and lnRMSSD.

Linear regression models were created to assess the independent effects of these associations (Table 3). The model was adjusted for sex, age, smoking habits, diastolic blood pressure, BMI, lnleptin, lninsulin, lntriglycerides, total cholesterol, oral contraceptive use, physical activity index, alcohol consumption and HR. In this model, CRP levels showed significant association with HR, lnLF, lnTP, lnSDNN and lnRMSSD indices. There was no statistical association between SAA levels and HRV indices.

Associations were also assess without exclusion criteria (n = 2042). In those models, lnHF correlated significantly also with CRP and leptin. In linear regression model, CRP had significant association only with HR, lnLF and lnSDNN. Otherwise, results were identical compared to analysis with exclusion criteria.

Discussion

In this study, we have shown that HRV indices correlate with CRP levels in a large cohort consisting of healthy young adults. The associations remained significant after adjusting for common CVD risk factors, such as age, sex, blood pressure, metabolic markers and life style factors. These findings are consistent with previous reports. Sajadieh *et al.* [10] were the first to show that decreased HRV indices are associated with increased CRP levels in healthy middle-aged and elderly subjects. Sloan *et al.* [11] reported an inverse relationship between HRV indices and CRP levels in a population consisting of 757 healthy subjects. Thayer and Fischer [12] reported that the

Table 1 Data characteristics

	N	Mean	SD
Sex (% of women)	1601	52	
Daily smokers (% of total)	1568	26	
Oral contraceptive use (% of women)	1599	29	
Body mass index (kg m^{-2})	1555	24·7	4·0
Waist–hip ratio	1599	0·84	0·08
Age (years)	1601	31·6	5·0
Total cholesterol (mML^{-1})	1601	5·11	0·93
Triglycerides (mML^{-1})	1601	1·30	0·82
Glucose (mML^{-1})	1601	4·98	0·40
Insulin ($mU\ L^{-1}$)	1600	7·32	4·80
Systolic blood pressure (mmHg)	1592	121·9	13·8
Diastolic blood pressure (mmHg)	1592	72·9	8·6
Physical activity index	1346	16·2	15·9
Alcohol (drinks per week)	1585	6·3	8·8
Leptin ($ng\ mL^{-1}$)	1601	10·26	8·89
CRP ($mg\ L^{-1}$)	1601	1·70	3·43
SAA ($mg\ L^{-1}$)	1600	22·23	77·65
HR (beat min^{-1})	1601	67·26	10·59
HF (ms^2)	1601	1052·9	1557·7
LF (ms^2)	1601	480·1	537·9
TP (ms^2)	1601	2347·3	2419·1
LF/HF (%)	1601	94·7	133·8
SDNN (ms)	1601	51·8	23·4
RMSSD (ms)	1601	49·9	32·6

CRP, C-reactive protein; HF, high frequency; HR, heart rate; LF, low frequency; LF/HF, ratio between LF and HF × 100%; RMSSD, root mean square differences of successive R-R intervals; SAA, serum amyloid A; SDNN, standard deviation of all R-R intervals; TP, total spectral component of HR variability.

relationship between HRV indices and CRP levels remains significant after controlling for urine norepinephrine levels, a marker of sympathetic ANS activity, in 611 healthy subjects. Lampert *et al.* [13] showed that decreased HRV indices are associated with high CRP levels in 264 healthy middle-aged male twins.

An important finding of this study is that HRV indices were found to be associated with CRP levels, independently of leptin levels. It has been shown that leptin levels associate strongly with CRP levels during noninflammatory conditions [22]. In multivariable models, leptin levels appear to be the strongest

Table 2 Correlation between inflammatory markers, leptin and HR variability indices

	Correlation*		
	lnCRP	lnSAA	lnLeptin
HR (beat min^{-1})	0·112†	0·113†	0·250†
lnHF (ms^2)	−0·048	−0·049‡	−0·006
lnLF (ms^2)	−0·065§	−0·057‡	−0·186†
lnTP (ms^2)	−0·074§	−0·054*	−0·076§
lnLF/HF (%)	−0·005	0·002	−0·163†
lnSDNN (ms)	−0·080§	−0·070§	−0·090†
lnRMSSD (ms)	−0·072§	−0·075§	−0·073§

*Pearson correlation, †<0·0001, ‡<0·01, §<0·05.
CRP, C-reactive protein; HF, high frequency; HR, heart rate; LF, low frequency; LF/HF, ratio between LF and HF × 100%; RMSSD, root mean square differences of successive R-R intervals; SAA, serum amyloid A; SDNN, standard deviation of all R-R intervals; TP, total spectral component of HR variability.

Table 3 Linear relationship between inflammatory markers and HR variability indices

Dependent	Variable	*B*	SE	*P*
CRP	HR (beat min^{-1})	0·006	0·003	**0·040**
	lnHF (ms^2)	−0·046	0·025	0·068
	lnLF (ms^2)	−0·070	0·031	**0·024**
	lnTP (ms^2)	−0·087	0·032	**0·007**
	lnLF/HF (%)	−0·017	0·030	0·577
	lnSDNN (ms)	−0·186	0·066	**0·005**
	lnRMSSD (ms)	−0·108	0·048	**0·024**
SAA	HR (beat min^{-1})	0·002	0·002	0·339
	lnHF (ms^2)	−0·015	0·023	0·526
	lnLF (ms^2)	−0·028	0·028	0·317
	lnTP (ms^2)	−0·034	0·029	0·240
	lnLF/HF (%)	−0·006	0·026	0·823
	lnSDNN (ms)	−0·088	0·060	0·143
	lnRMSSD (ms)	−0·046	0·044	0·298

Multivariate model, the effect of HR variability indices adjusted for sex, smoking, age, diastolic blood pressure, body mass index, lnleptin, lninsulin, lntriglycerides, total cholesterol, oral contraceptive use, physical activity index, alcohol use and HR.
Models were performed independently to each HR variability indices.
CRP, C-reactive protein; HF, high frequency; HR, heart rate; LF, low frequency; LF/HF, ratio between LF and HF × 100%; RMSSD, root mean square differences of successive R-R intervals; SAA, serum amyloid A; SDNN, standard deviation of all R-R intervals; TP, total spectral component of HR variability. Bold indicates statistical significant $P < 0·05$ values.

predictor of CRP levels. Also, it has been previously shown that leptin levels correlate with HRV indices [23]. We also found this association. However, other current studies have not shown a leptin-independent association between CRP levels and HRV indices. In this study, we showed that the association between CRP levels and HRV indices remained significant even after adjusting for leptin levels and other risk factors.

We also examined the association between HRV indices and SAA levels. Both CRP and SAA are acute phase proteins, and their physiological roles in infection are analogous. SAA is one of the less frequently studied acute phase proteins, although SAA levels have been shown to be associated with an increased risk for CVD [15,18]. The correlation between HRV indices and SAA levels was analogous to the relationship between HRV indices and CRP levels. However, in multivariable analysis, the association between HRV indices and SAA levels became non-significant when adjusted for age and sex, and cardiovascular risk factors including smoking, oral contraceptive use, physical activity, alcohol consumption, diastolic blood pressure, BMI, leptin, insulin, triglycerides and total cholesterol levels, while the association between HRV indices and CRP levels remained significant. The association between SAA levels and HRV indices was diminished after adjusting for leptin levels or diastolic blood pressure, indicating that the possible effect of the ANS on these inflammatory markers is different. One possible explanation for this difference is that most of the vagal nervous endings are found in the gastrointestinal tract [31]. Under noninflammatory conditions, CRP is mainly produced in the liver, while SAA is produced in adipose tissue [20,21]. Therefore, the ANS can possibly regulate CRP levels more directly, via the cholinergic anti-inflammatory pathway, while the effect on SAA levels is not direct. Reduced HRV indices have been shown to be associated with metabolic syndrome [32] and hypertension [5], conditions, which are also associated with increased inflammation. Thus, the association between HRV indices and SAA levels could be because of the possible effect of the ANS on both the metabolic and vascular systems.

It is important to note that there are limitations to this study. Because of time restrictions, only 3 min of ECG recordings were collected. At least 5 min of recordings have been previously recommended [1]. The mean duration of stationary R-R time series was 173 s in our study, which should be adequate for HRV components evaluation. Also, we have demonstrated earlier that there was no statistically significant difference between these 3-min ECG measurements when compared to 5-min recordings, and the reproducibility of these measurements was satisfactory [27]. However, we recognize that the amount of subjects in the reproducibility and the comparability studies was quite low. A single-channel chest-lead ECG was recorded during a 3-min period of metronome-controlled breathing at the frequency of 0·25 Hz. Using of controlled breathing may have led to enhancement of HF component [29]. The HRV index is one of several tools used to assess the ANS. Additional methods have been used to control the sympathetic ANS. The sympathetic ANS has been shown to modulate both pro- and anti-inflammatory functions [31]. We were not able to control sympathetic ANS modulation of inflammation; hence, we did not have additional information about the study subject's sympathetic ANS activity, *e.g.* norepinephrine levels. Therefore, it is also possible that the association observed here between HRV indices and inflammation markers levels may reflect an effect of the sympathetic ANS or a combined effect of both components of the ANS, although LF/HF, which has suggested to be the most reliable HRV-derived marker of sympathetic ANS activity [1], was not associated with inflammatory markers. Blood pressure measurements were taken with a random zero sphygmomanometer. Using this method might have lead to inaccuracies in blood pressure measurements, because this method has been shown to underestimate blood pressure values [33]. Because numerous variables affect significantly on HRV variables and inflammatory markers values, many exclusion criteria were used. This lowered the number of study subjects significantly (1601 vs. 2042). Therefore, we also analysed results without exclusion criteria. This made minor changes to results, but the main results and conclusions remained the same. These results cannot be generalized to subjects with hypertension, diabetes, chronic rheumatic disease pregnant and breastfeeding women, and subjects with certain medications, because such individuals were excluded from the analysis.

Conclusion

In summary, the present study showed that SAA and CRP levels correlate analogously with HRV indices, but only the association between HRV indices and CRP levels remains significant after adjusting for risk factors, including leptin levels. The independent association between HRV and CRP supports the hypothesis that dysregulation of the ANS may lead to increased inflammation via the cholinergic anti-inflammatory pathway in early adulthood. Understanding these mechanisms is clinically relevant to better manage and understand chronic inflammatory diseases such as atherosclerosis in the future.

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Pentraxin 3 (PTX3) is associated with cardiovascular risk factors: the Health 2000 Survey

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Summary

Pentraxin 3 (PTX3) is a novel candidate immunoinflammatory marker that has been reported to be associated with cardiometabolic risk factors and to predict adverse outcomes in individuals with cardiovascular disease (CVD). Despite being a member of the same pentraxin protein family as C-reactive protein (CRP), PTX3 probably reflects different aspects of CVD pathogenesis. In this study, we assessed plasma PTX3 correlates and determinants in the Health 2000 Survey population, which comprised $n = 403$ insulin-resistant subjects, $n = 845$ hypercholesterolaemic subjects and $n = 311$ hypertensive subjects, all aged between 46 and 76 years. In insulin-resistant subjects the PTX3 concentration was found to correlate directly with age, pulse pressure and indoleamine 2,3-dioxygenase (IDO) enzyme activity and inversely with total and low-density lipoprotein (LDL) cholesterol. In hypercholesterolaemic subjects, the PTX3 concentration correlated directly with HDL cholesterol, systolic blood pressure and pulse pressure, whereas in hypertensive subjects, the PTX3 concentration correlated directly with systolic blood pressure, pulse pressure and IDO activity. No correlation was observed between the concentrations of PTX3 and CRP, adiposity indicators or indicators of subclinical atherosclerosis in any of the subject groups. PTX3 concentration variations were attributed to variations in LDL cholesterol and IDO activity in insulin-resistant subjects and to pulse pressure in hypercholesterolaemic and hypertensive subjects. These results indicate that, in individuals at high risk of CVD, the PTX3 concentration is associated with cardiovascular risk factors but not with subclinical atherosclerosis.

Keywords: acute phase proteins, atherosclerosis, inflammation/inflammatory mediators including eicosanoids

Introduction

Immune response and inflammatory factors are known to be involved in the development of atherosclerosis from the point of endothelial injury to acute clinical manifestations. According to current understanding, chronic low-grade inflammation in the arterial wall can accelerate the accumulation of asymptomatic atherosclerotic changes, whereas an abrupt and more vigorous inflammatory activation precedes end-point plaque rupture [1]. However, it is unclear whether inflammatory mediators can act as causal agents in the pathogenesis of cardiovascular disease (CVD) or whether they merely emerge as indicators of ongoing vascular damage. Various players of the innate immune system, such as the traditional C-reactive protein (CRP) and a newer candidate of the same pentraxin family, pentraxin 3 (PTX3), have been presented as potential atherosclerotic biomarkers, as their low-level increase in plasma has been associated with cardiovascular events in a number of studies [2–7].

PTX3 is an acute-phase reactant that shares structural and functional homology with CRP, as it activates the complement system, binds microbial surfaces and apoptotic cells and aids in their clearance [8]. However, unlike CRP, which is synthesized mainly in the liver, PTX3 is produced at the site of inflammation by macrophages, dendritic cells, neutrophils, fibroblasts, endothelial cells and smooth muscle cells

(SMCs) [8]. Synthesized PTX3 can also be stored in neutrophil granules that, upon stimulation, release PTX3 rapidly into circulation [9]. Production of PTX3 is induced by interleukin (IL)-1, tumour necrosis factor (TNF)-α, oxidized low-density lipoprotein (ox-LDL) and microbial moieties, but is not induced by IL-6 [8]. It has been suggested that PTX3 plays the same role in the periphery that CRP does in circulation [10]. However, in the case of myocardial infarction (MI), the PTX3 concentration has been reported to peak more rapidly than the CRP concentration [2], which could indicate the higher sensitivity of PTX3 in response to vascular damage. Alternatively, the PTX3 concentration may reflect different and uncharacterized aspect(s) of inflammation because in mouse models of atherosclerosis and MI, PTX3 has been demonstrated to exert a cardioprotective function [11,12].

Despite the incompletely understood role of PTX3 in vascular biology, several lines of epidemiological and experimental evidence imply that PTX3 could be involved intimately in the pathogenesis of CVD. Elevated PTX3 plasma levels have been associated with adverse cardiovascular outcomes [2–4,6,7], and PTX3 expression has also been detected in atherosclerotic plaques [13,14]. Furthermore, PTX3-positive neutrophils can infiltrate atherosclerotic lesions, and their presence has been observed in coronary arterial thrombi originating from culprit lesions in MI patients [14]. However, no consensus currently exists as to whether plasma PTX3 is an independent risk factor in the pathophysiology of atherosclerosis or whether it is just an auxiliary signalling marker between vascular inflammation and tissue repair. To determine the usefulness of PTX3 as a marker of subclinical atherosclerosis in individuals free of clinical CVD, we studied the relationship between plasma PTX3 and the following factors: cardiovascular risk factors, indices of subclinical atherosclerosis and inflammatory measures, namely CRP and indoleamine 2,3-dioxygenase (IDO) enzyme activity.

Materials and methods

Study population

The study population was a subpopulation drawn from the Health 2000 Survey, a large Finnish cross-sectional health examination survey carried out from 2000 to 2001 [15]. The overall study cohort was a two-stage stratified cluster sample (8028 individuals) representing the entire Finnish population aged 30 years and older. To study cardiovascular risk and diabetes more thoroughly, a supplemental study was carried out (sample size 1867, 82% participation rate). The participants in this study consisted of those supplementary study subjects for whom clinical and metabolic cardiovascular risk factor data were available. The study population consisted of the following subject groups, based on clinical condition: 403 insulin-resistant subjects, 845 hypercholesterolaemic subjects, 311 hypertensive subjects and 108 healthy subjects, all aged between 46 and 76 years, and without a history of CVD, MI, stroke or heart failure. Please see the inclusion criteria for the subject groups in the following section (Clinical and biochemical analyses). Among the 403 insulin-resistant subjects, 355 individuals also had hypertension and/or hypercholesterolaemia; among the 845 hypercholesterolaemic subjects, 521 individuals also had insulin resistance and/or hypertension; and among the 311 hypertensive subjects, 284 individuals had also hypercholesterolaemia and/or insulin resistance. The study was conducted in five Finnish University Hospitals. The Health 2000 Survey protocol was approved by the Epidemiology Ethics Committee of the Helsinki and Uusimaa Hospital District. All participants gave their written informed consent.

Clinical and biochemical analyses

Height and weight were measured, and body mass index (BMI) was calculated. Waist and hip circumferences were measured in the standing position using the standards created for population health studies. Waist circumference was not measured in the supplemental study; therefore, the waist circumference values measured for the Health 2000 Survey were used. Blood pressure was measured after at least 10 min of rest with the automatic Omron M4 manometer (Omron Matsusaka Co., Japan; Omron Healthcare Europe BV, Hoofddrop, the Netherlands) and the mean of three measurements was used in the analysis. Pulse pressure was calculated as the difference between the mean systolic pressure and the mean diastolic pressure.

Current smoking, diabetes, CVD history and CVD events, including coronary artery disease (CAD), were evaluated with a questionnaire. Those who were current smokers were defined as smokers, and the rest of the subjects were defined as non-smokers. Insulin resistance was assessed according to the International Diabetes Federation criteria [16], and hypercholesterolaemia (LDL cholesterol >3 mmol/l or total cholesterol >5 mmol/l) and hypertension (diastolic blood pressure >90 mmHg and systolic blood pressure ≥140) were assessed according to the Finnish Current Care guidelines, formulated on the basis of the European guidelines on cardiovascular disease prevention [17]. Subjects were classified as healthy based on the absence MI, CAD, heart failure, stroke, diabetes, hyperlipidaemia, hypercholesterolaemia and hypertension.

Venous blood samples were drawn after an overnight fast. High-density lipoprotein (HDL) cholesterol, total cholesterol, triglyceride and plasma glucose concentrations were determined enzymatically with a clinical chemistry analyser (Olympus, AU400, Hamburg, Germany). LDL cholesterol was calculated with the Friedewald formula. Plasma insulin concentrations were determined using a radioimmunoassay (Phadeseph Insulin RIA, Pharmacia Sweden). Homeostasis assessment of insulin resistance (HOMA-IR) was calculated

according to the formula: HOMA-IR = fasting glucose (mmol/l) × fasting insulin (mU/l)/22·5 [18]. Detailed descriptions of the methods used have been published elsewhere [19]. Plasma CRP concentrations were determined using a chemiluminescent immunometric assay (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA). PTX3 concentrations were determined in ethylenediamine tetraacetic acid (EDTA)-plasma using a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Quantikine DPTX 30; R&D Systems Inc., Minneapolis, USA). According to the manufacturer, the mean detection limit for the assay is 0·025 ng/ml and it exhibits no cross-reactivity with either CRP or serum amyloid P. The enzymatic activity of IDO was assessed (data available in 759 individuals) using the plasma kynurenine/tryptophan ratio (μmol/mmol), as described previously [20].

Carotid artery studies

High-resolution B-mode carotid ultrasound examination of the right carotid artery was performed according to a standardized protocol using a 7·5-MHz linear array transducer. The examinations were performed by centrally trained and certified sonographers at six study locations throughout Finland. Carotid IMT measurements were performed off-line with the use of automated image processing software, and one researcher was responsible for reading all of the ultrasound images. Three summary measures of the carotid IMT were calculated: (i) the mean of the three average IMTs of the common carotid artery (mean CCA IMT), (ii) the mean of the three average IMTs of the carotid bulb (mean bulb IMT) and (iii) the mean of these two means (mean IMT). Mean IMT was used in the present study. This method has been described in detail previously [21]. Arterial elasticity was assessed as carotid artery compliance (CAC) according to the following formula:

$$\mathrm{CAC}\,(\%/10\ \mathrm{mmHg}) = 100 \times 10 \times [(\mathrm{ADC}/\mathrm{DAD})/\mathrm{PP}],$$

where ADC is the arterial diameter change, DAD is the diastolic arterial diameter and PP is pulse pressure. Arterial diameters were calculated as the mean of three average systolic and diastolic arterial diameters.

Statistical analyses

Individuals with CRP values above 10 mg/ml were excluded from the analyses ($n = 66$) due the possibility of an acute infection. The distributions of the PTX3, CRP, insulin, HOMA-IR and triglyceride values were skewed, hence the variables were transformed logarithmically prior to the analyses. Student's *t*-tests and Mann–Whitney's tests were used to analyse differences between sexes in the test variables, and χ^2 analyses with Fisher's exact tests were used to assess differences in smoking between sexes and prevalence of CVD events between sexes. The correlates for plasma PTX3 were estimated separately for each subgroup (insulin-resistant subjects, hypercholesterolaemic subjects, hypertensive subjects and healthy subjects) using Pearson's tests. Stepwise multivariate linear regression modelling was performed to assess the independent determinants for plasma PTX3 concentration. Regression modelling for the determinants of PTX3 was not, however, performed for the healthy subjects due to the lack of statistically significant correlates for the PTX3 concentration (Table 2). All statistical analyses were performed using SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL, USA) and *P*-values < 0·05 were considered statistically significant.

Results

The characteristics of the study population are presented in Table 1. The majority of the variables differed significantly between men and women; the only variables that did not deviate between the sexes were age, LDL cholesterol level, pulse pressure, CRP level, PTX3 level and IDO activity. Additionally, the prevalences of hypertension, hypercholesterolaemia, CAD, heart failure and stroke did not differ between the sexes. In insulin-resistant subjects, the PTX3 concentration correlated directly with age, pulse pressure and indoleamine 2,3-dioxygenase (IDO) enzyme activity and correlated inversely with total and LDL cholesterol (Table 2). In hypercholesterolaemic subjects, the PTX3 concentration correlated directly with HDL-cholesterol, systolic blood pressure and pulse pressure (Table 2). In hypertensive subjects, the PTX3 concentration correlated directly with systolic blood pressure, pulse pressure and IDO activity (Table 2). No significant correlations with the PTX3 concentration were observed in healthy subjects (Table 2). Smoking was not associated with plasma PTX3 concentration ($P = 0{\cdot}546$). The determinants, i.e. factors that explained the variation in plasma PTX3, were LDL cholesterol and IDO activity in insulin-resistant subjects, and pulse pressure in hypercholesterolaemic and hypertensive subjects (Table 3).

Discussion

In this study, we demonstrated that the plasma PTX3 concentration correlates with several cardiovascular risk factors in individuals at high risk of developing CVD. In insulin-resistant subjects plasma PTX3 correlated directly with age and pulse pressure, and inversely with total and LDL cholesterol. In hypercholesterolaemic subjects, we found that plasma PTX3 correlated directly with age, HDL cholesterol, systolic blood pressure and pulse pressure, whereas in hypertensive subjects PTX3 correlated directly with diastolic blood pressure and pulse pressure. In addition, we observed that PTX3 correlated directly with another inflammatory marker, IDO, in insulin-resistant and hypertensive subjects. The factors explaining the variation in plasma PTX3 were found

Table 1. Characteristics of the study population.

	All		Men		Women		*P* for difference between sexes
	Mean	s.d.	Mean	s.d.	Mean	s.d.	
Age (years)	58·21	8·03	57·90	7·75	58·47	8·246	0·223
BMI (kg/m^2)	27·13	4·35	27·49	3·97	26·82	4·62	0·006
Waist circumference (cm)	93·36	13·11	99·31	11·31	88·23	12·36	<0·001
Hip circumference (cm)	102·19	9·08	101·27	7·98	102·97	9·86	<0·001
Waist-to-hip ratio	0·91	0·09	0·98	0·06	0·85	0·06	<0·001
Heart rate (beats/min)	66·51	10·14	65·49	10·51	67·35	9·75	0·002
Systolic blood pressure (mmHg)	138·47	21·71	141·63	20·46	135·71	22·41	<0·001
Diastolic blood pressure (mmHg)	84·42	10·56	87·50	10·79	81·80	9·62	<0·001
Pulse pressure (mmHg)	54·02	15·00	54·15	13·76	53·91	15·99	0·798
Total cholesterol (mmol/l)	5·59	0·94	5·50	0·96	5·66	0·91	0·002
HDL cholesterol (mmol/l)	1·58	0·43	1·43	0·38	1·71	0·43	<0·001
LDL cholesterol (mmol/l)	3·40	0·87	3·41	0·88	3·38	0·86	0·626
Triglycerides (mmol/l)*	1·20	0·90–1·60	1·30	1·00–1·80	1·10	0·80–1·50	<0·001
Glucose (mmol/l)	5·85	1·19	6·13	1·35	5·61	0·97	<0·001
Insulin (mmol/l)*	7·80	5·70–11·10	8·50	5·93–12·28	7·30	5·40–10·20	<0·001
HOMA-IR*	1·98	1·37–2·96	2·23	1·49–3·36	1·80	1·29–2·69	<0·001
CRP (mg/l)*	1·44	0·79–2·87	1·47	0·82–2·81	1·38	0·76–2·95	0·332
PTX3 (ng/l)*	1·01	0·68–1·44	1·04	0·74–1·44	0·98	0·64–1·46	0·107
IDO (kyn/tryp)	31·65	8·27	31·36	8·02	31·89	8·48	0·379
IMT (mm)	0·93	0·23	0·96	0·24	0·90	0·21	<0·001
CAC (%/10 mmHg)	0·93	0·48	0·88	0·41	0·96	0·53	0·010
Smoking (%)†							
No	78·5		73·7		82·6		<0·001
Yes	21·5		26·3		17·4		
Hypertension (%)†							
No	71·8		71·5		72·0		0·845
Yes	28·2		28·5		28·0		
Insulin resistance (%)†							
No	58·1		52·4		62·7		0·001
Yes	41·9		47·6		37·3		
Hypercholesterolemia (%)†							
No	21·3		23·0		80·3		0·684
Yes	78·7		77·0		68·2		
Diabetes (%)†							
No	94·6		92·4		96·5		0·002
Yes	5·4		7·6		3·5		
CAD (%)†							
No	93·5		93·0		94·0		0·471
Yes	6·5		7·0		6·0		
Heart failure (%)†							
No	96·6		95·6		97·4		0·076
Yes	3·4		4·4		2·6		
MI (%)†							
No	96·0		93·9		97·7		0·001
Yes	4·0		6·1		2·3		
Stroke (%)†							
No	98·1		97·4		98·8		0·064
Yes	1·9		2·6		1·2		

*Median values and interquartile range (IQR): Mann–Whitney's *U*-test for difference between sexes. †χ^2 test for difference between sexes; *t*-test for difference between sexes. BMI: body mass index; CAC: carotid artery compliance; CAD: coronary artery disease; CRP: C-reactive protein; PTX3: pentraxin 3; HDL: high-density lipoprotein; HOMA-IR: insulin resistance index; IDO: indoleamine 2,3-dioxygenase (IDO) enzyme; IMT: intima-media thickness; LDL: low-density lipoprotein; MI: myocardial infarction; s.d.: standard deviation.

Table 2. Pearson's correlations for (log)pentraxin 3 (PTX3).

	Insulin-resistant subjects $n = 403$		Hypercholesterolaemic subjects $n = 845$		Hypertensive subjects $n = 311$		Healthy subjects* $n = 108$	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age	0·105	**0·034**	0·085	**0·013**	0·103	0·070	0·033	0·734
Body mass index (kg/m)	–0·059	0·236	–0·058	0·093	0·034	0·546	–0·069	0·475
Waist circumference (cm)	–0·014	0·783	–0·017	0·613	0·028	0·621	0·032	0·744
Hip circumference (cm)	–0·037	0·464	–0·009	0·805	0·074	0·192	–0·035	0·722
Waist-to-hip ratio	0·021	0·680	–0·019	0·574	–0·030	0·604	0·068	0·485
Heart rate (beats/min)	–0·023	0·652	0·038	0·273	0·079	0·167	–0·060	0·541
HDL cholesterol (mmol/l)	0·017	0·737	0·080	**0·019**	0·058	0·310	–0·048	0·623
LDL cholesterol (mmol/l)	–0·139	**0·006**	–0·061	0·079	0·040	0·487	–0·107	0·273
Total cholesterol (mmol/l)	–0·102	**0·040**	–0·012	0·721	0·063	0·270	–0·156	0·106
(log)Triglycerides (mmol/l)	0·034	0·498	–0·012	0·722	0·001	0·987	–0·019	0·844
Glucose (mmol/l)	0·041	0·408	0·053	0·126	0·018	0·748	0·074	0·448
(log)Insulin (mmol/l)	0·082	0·102	0·032	0·360	0·103	0·069	0·070	0·474
(log)HOMA-IR	0·081	0·103	0·042	0·225	0·091	0·110	0·079	0·414
Systolic blood pressure (mmHg)	0·089	0·073	0·117	**0·001**	0·143	**0·012**	0·159	0·102
Diastolic blood pressure (mmHg)	0·017	0·733	0·062	0·073	0·025	0·666	0·168	0·083
Pulse pressure (mmHg)	0·111	**0·025**	0·126	**<0·001**	0·171	**0·003**	0·110	0·261
(log)CRP (mg/l)	0·041	0·413	–0·006	0·868	0·041	0·467	0·083	0·390
IDO	0·161	**0·010**	0·010	0·828	0·152	**0·035**	0·031	0·795
Carotid IMT (mm)	0·057	0·260	0·041	0·243	0·032	0·578	0·044	0·656
Carotid artery compliance (%/10 mmHg)	–0·051	0·339	–0·062	0·090	–0·085	0·155	0·038	0·707

Statistically significant correlations ($P < 0·05$) are shown in bold type. *Subjects excluded for CVD, MI, stroke, heart failure, insulin resistance, hyperlipidaemia or hypertension. BMI: body mass index; CAC: carotid artery compliance; CRP: C-reactive protein; PTX3: pentraxin 3; HDL: high-density lipoprotein; HOMA-IR: insulin resistance index; IDO: indoleamine 2,3-dioxygenase (IDO) enzyme; IMT: intima-media thickness; LDL: low-density lipoprotein.

to be LDL cholesterol and IDO in insulin-resistant subjects and pulse pressure in hypercholesterolaemic and hypertensive subjects, although these factors accounted for only 1·1–5·7% of the variation in the PTX3 levels. Interestingly, no statistically significant correlations for plasma PTX3 were observed in healthy subjects. Moreover, the PTX3 concentration did not correlate with indicators of subclinical atherosclerosis, IMT and CAC or with CRP and adiposity indicators in any of the subject groups.

The association of the PTX3 concentration with cardiometabolic risk factors has been documented previously. In subjects with metabolic syndrome, Zanetti *et al.* (2009) observed that plasma PTX3 correlated directly with triglyceride levels and inversely with HDL cholesterol levels [22], whereas Yamasaki and colleagues (2009) have reported inverse correlations between plasma PTX3 and triglyceride levels and between plasma PTX3 and BMI [23]. Negative findings on the relationships between the levels of all plasma lipids and PTX3 in rheumatic and non-rheumatic patients with CVD have also been presented [24,25]. Intriguingly, Alberti *et al.* (2009) detected expression of PTX3 in adipose tissue from obese and lean subjects and reported that the age- and sex-adjusted expression of PTX3 in visceral adipose tissue correlated with BMI, HDL, HDL/LDL ratio, triglycerides, CRP, fibrinogen and adiponectin [26]. They did not, however, find a correlation between the levels of adipose

Table 3. Independent determinants for plasma (log)pentraxin 3 (PTX3) in a stepwise linear regression model.

	Insulin-resistant subjects $n = 403$			Hypercholesterolaemic subjects $n = 845$			Hypertensive subjects $n = 311$		
Determinats	B	s.e.	*P*	B	s.e.	*P*	B	s.e.	*P*
LDL cholesterol	–0·054	0·019	0·005						
Pulse pressure				0·002	0·001	0·019	0·003	0·001	0·007
IDO	0·004	0·002	0·015						
	$R^2 = 0·057$			$R^2 = 0·011$			$R^2 = 0·025$		

The model included age, LDL cholesterol, total cholesterol, glucose, insulin, HOMA-IR, systolic blood pressure, diastolic blood pressure, pulse pressure and IDO as dependent variables. HOMA-IR: insulin resistance index; IDO: indoleamine 2,3-dioxygenase (IDO) enzyme; LDL: low-density lipoprotein; s.e.: standard error.

tissue-derived PTX3 expression and LDL cholesterol, glucose, insulin or blood pressure. In contrast, Bosutti *et al.* (2007) demonstrated that plasma LDL-cholesterol is associated with PTX3 mRNA levels in adipose tissue and white blood cells in non-diabetic pacemaker-implanted patients [27].

Results on the associations of PTX3 with CRP [4,7,25,28] and sex [6,7,23] also seem to be contradictory. With regard to age, however, there seems to be a consensus that advancing age is associated with higher PTX3 levels [7,23–25], a notion that was also verified in our study among insulin-resistant and hypercholesterolaemic subjects.

Even though the potential role of PTX3 in vascular biology and CVD has been studied intensively, the causalities between PTX3 levels and cardiovascular outcomes are still unclear. Similarly, the functional role of PTX3, if any, in atherogenesis has not been established. Nevertheless, PTX3 has been reported to be associated with cardiac events in heart failure patients [4,6] and in hospitalized AMI patients [2,3,7] and the PTX3 concentration has also been demonstrated to be associated with all-cause and cardiovascular mortality among ostensibly healthy subjects [25]. Additionally, recent mouse studies have proposed that PTX3 could have an atheroprotective role [11,12], potentially ascribed to its relationship with HDL cholesterol [29]. In keeping with the protective capability of PTX3, Deban and colleagues (2010) reported that, in both localized and systemic inflammatory conditions, PTX3 can act as a negative feedback mediator by dampening excessive neutrophil recruitment, thereby limiting inflammation [30]. Our novel observation that plasma PTX3 correlates directly with IDO activity in insulin-resistant and hypertensive subjects could similarly reflect the immunosuppressive function of PTX3 because IDO, a product of antigen-presenting cells, is a known immunoinflammatory down-regulator of type 1 T helper cell responses [31]. Currently, however, the relationship between elevated plasma PTX3 levels and acute cardiovascular events and PTX3's putative suppressive or protective role are unclear.

As a limitation of this study, it must be acknowledged that in our study the subject groups, especially the healthy subjects, were rather small, and the observed correlations between plasma PTX3 and cardiovascular risk factors were, although significant, somewhat weak. Consequently, it appears that in our population, PTX3 plasma levels were determined largely by factors other than the traditional CVD risk factors, as our regression models explained only a minor part of the variation in the PTX3 concentration. Additionally, the frequency of subjects with a diagnosed CVD in our study population was too low to carry out statistical analysis on plasma PTX3 levels among these subjects.

Analogously to the results of Zanetti *et al.* (2009) [22], we observed no correlation between cardiometabolic risk factors and plasma PTX3 in the group of healthy subjects, indicating that plasma PTX3 may not be a suitable risk indicator in healthy subjects. Similarly, the lack of correlation between plasma PTX3 and subclinical CVD indicators in all the subject groups in this study, as well as in a recent study by Miyaki *et al.* (2010), suggests that plasma PTX3 might not reflect early atherosclerotic changes. Nevertheless, a direct correlation between PTX3 levels and IMT was observed in elderly hypertensive patients by Yano *et al.* (2010) [32], whereas Yilmaz *et al.* (2009) demonstrated an independent association between PTX3 levels and endothelial dysfunction in diabetic renal disease patients [33]. Moreover, Jenny *et al.* (2009) have shown that plasma PTX3 correlates directly with the ankle-brachial blood pressure index, but not with IMT, in CVD-free individuals [25].

Taken together, the results of this study indicate that, although plasma PTX3 levels were not associated with subclinical atherosclerosis, they correlated with several cardiovascular risk factors in individuals at high risk of developing CVD. Further research is required to validate the use of plasma PTX3 as an auxiliary and/or adiposity- and CRP-independent risk marker in CVD-free individuals.

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Disclosure

The authors declare that there is no conflict of interest.

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Relation of high cytomegalovirus antibody titres to blood pressure and brachial artery flow-mediated dilation in young men: the Cardiovascular Risk in Young Finns Study

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Summary

Human cytomegalovirus (CMV) infection is associated with a higher risk of cardiovascular disease in immunocompromised organ transplant patients. It has been linked with the pathogenesis of elevated arterial blood pressure. However, controversy exists as to whether CMV infection is associated with endothelial function, and little is known about its role as a potential risk factor for early atherosclerosis development at a young age. We aimed to discover if CMV antibody titres are associated with early vascular changes (carotid intima-media thickness, carotid artery distensibility and brachial artery flow-mediated dilation), blood pressure elevation or other traditional cardiovascular risk factors. CMV antibody titres were measured in 1074 women and 857 men (aged 24–39 years) taking part in the Cardiovascular Risk in Young Finns study. CMV antibody titres were significantly higher in women compared to men. In men, high CMV antibody titres were associated directly with age ($P < 0{\cdot}001$) and systolic ($P = 0{\cdot}053$) and diastolic ($P = 0{\cdot}002$) blood pressure elevation, and associated inversely with flow-mediated dilation ($P = 0{\cdot}014$). In women, CMV antibody titres did not associate with any of the analysed parameters. In a multivariate regression model, which included traditional atherosclerotic risk factors, CMV antibody titres were independent determinants for systolic ($P = 0{\cdot}029$) and diastolic ($P = 0{\cdot}004$) blood pressure elevation and flow-mediated dilation ($P = 0{\cdot}014$) in men. High CMV antibody titres are associated independently with blood pressure and brachial artery flow-mediated dilation in young men. This association supports the hypothesis that common CMV infection and/or an immune response to CMV may lead to impaired vascular function at a young age.

Keywords: atherosclerosis, blood pressure, cytomegalovirus, flow-mediated dilation, risk factors

Introduction

Human cytomegalovirus (CMV) is a member of the herpes virus family. It is a widely spread virus, and up to 40% of people acquire the infection during their first year of life [1]. The prevalence increases progressively with increasing age, and more than 90% of senior citizens are CMV seropositive [2]. Women are known to have higher CMV seroprevalence than men [2]. Additionally, the seroprevalence is higher among people with low socio-economic status (SES) [2]. In most immunocompetent people, the primary CMV infection is mild or even asymptomatic. CMV infection is typically a clinical problem in immunocompromised people, including patients with congenital infections or immunodeficiency syndrome, as well as patients receiving organ transplants [1].

CMV infection is associated with various chronic inflammatory diseases, including cardiovascular disease (CVD), autoimmune diseases and certain cancers [1]. The role of CMV as a risk factor for CVD is controversial. CMV infection has been shown to increase the risk of cardiac allograft vascular disease and graft rejection in immunocompromised organ transplant patients [3]. The use of anti-viral treatment with ganciclovir has been shown to decrease the risk of allograft vascular disease in transplant patients [4,5]. In immunocompetent people, the association between CMV infection and an increased risk of CVD is less clear. Significant associations have been reported between CMV

seropositivity and CVD risk [6,7], and also between high CMV antibody titres and increased CVD risk [8,9]. However, negative associations have also been reported [6,10,11]. Few relatively small studies exist that have shown an association between CMV and early atherosclerotic changes; none the less, CMV has been shown to be associated with endothelial function [12,13] and increased carotid intima-media thickness (IMT) [14]. Not all reports are unanimous, however [15,16]. Interestingly, recent publication has shown that essential hypertension is associated with increased CMV DNA copy number and CMV-encoded microRNA expression [17]. Also, CMV infection has been linked with the pathogenesis of increased arterial blood pressure via stimulation of renin and cytokine production and contributes to increased blood pressure values in mice [18].

Recent studies have linked high cytomegalovirus antibody titres with cardiovascular disease and total mortality among older people [19–21]. Therefore, we hypothesized that CMV antibody titres, rather than seropositivity, might be more relevant markers of deleterious effects of CMV infection in young adults. In this study, we sought to identify if intense humoral CMV-specific immunity, measured by CMV antibody titres, is associated with early vascular changes, IMT, carotid artery distensibility (Cdist), brachial artery flow-mediated dilation (FMD), blood pressure values or other traditional CVD risk factors in young healthy adults.

Materials and methods

The study population consisted of participants in the Cardiovascular Risk in Young Finns study, which is an ongoing multi-centre follow-up study in five university hospitals in cities with medical schools in Finland. The study began in 1980, when 3596 participants between the ages of 3 and 18 years were selected randomly from the national population registers. The study design has been presented in more detail elsewhere [22,23]. The 21-year follow-up was conducted in 2001, when the participants were between 24 and 39 years of age. Cardiovascular risk factor measurements, including body mass index (BMI), waist circumference, serum lipids, blood pressure values, levels of C-reactive protein (CRP), SES (occupation and education), alcohol consumption and smoking habits, were recorded during this follow-up. The study complies with the Declaration of Helsinki. The study was approved by local ethics committees and subjects gave informed consent.

Blood pressure measurements were taken using a random zero sphygmomanometer (Hawksley & Sons Ltd, Lancin, UK), and the mean of three measurements was used in the analysis. BMI was calculated from the measured height and weight. Waist circumference and hip circumference were measured, and the waist–hip ratio was calculated. Data regarding occupation, education, smoking habits, alcohol consumption and physical activity were gathered via questionnaires. Measurements of plasma lipids, glucose and insulin were performed by fasting plasma, as described previously [22]. CRP concentrations were analysed with a high-sensitivity latex turbidimetric immunoassay (Wako Chemicals GmbH, Neuss, Germany) with a detection limit of 0·06 mg/l. CMV IgG antibody titres were analysed using a commercial enzyme immunoassay (Enzygnost Anti-CMV/IgG; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). According to the manufacturer, the test sensitivity was 99·3% and the specificity was 98·2%. Seropositivity for CMV was defined as a serum anti-CMV immunoglobulin (Ig)G titre of $\geq$230.

Carotid ultrasound measurements were performed using a Sequoia 512 high-resolution ultrasound system (Acuson, Mountain View, CA, USA). Subjects were instructed to avoid smoking, high-calorie meals, coffee and other caffeine drinks on the day of the ultrasound measurements. Subjects also were instructed to avoid vigorous exercise and alcohol consumption on the previous evening of the measurements. Cdist, which depicts the ability of the large arteries to expand under cardiac pulse pressure, was assessed from the formula $\text{Cdist} = ([D_s - D_d]/D_d)/(P_s - P_d)$, where D_s is the systolic diameter, D_d is the diastolic diameter, P_s is the systolic blood pressure and P_d is the diastolic blood pressure [24]. Mean IMT was derived from a minimum of four measurements of the posterior wall of the left carotid artery (at ~10 mm proximal to the bifurcation) [24]. To assess brachial FMD, the left brachial artery diameter was measured both at rest and during reactive hyperaemia. Increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 250 mmHg for 4·5 min, followed by release. Three measurements of arterial diameter were performed at end-diastole at a fixed distance from an anatomic marker at rest and at 40, 60 and 80 s after cuff release. The vessel diameter in scans after reactive hyperaemia was expressed as the percentage relative to the resting scan. The average of three measurements at each time-point was used to derive the maximum FMD (the greatest value between 40 and 80 s) [25].

CMV antibody titres were measured successfully in 2133 subjects. We found that 648 subjects were seronegative (<230 antibody titres) and that 1485 subjects were seropositive ($\geq$230). Subjects with missing data on smoking, education, BMI, waist circumference, waist–hip ratio, blood pressure, insulin, alcohol consumption, IMT and/or Cdist values were not included in the analysis. Therefore, the total number of subjects included in the study was 1931 subjects (1074 women and 857 men). The subjects were then divided into four quartiles according to CMV antibody titres. CMV antibody titres were parameterized with a dummy variable comparing the highest quartile (Q4), with the bottom three quartiles combined (Q1–Q3). A *t*-test was used for normally distributed variables and the Mann–Whitney *U*-test was used for skewed variables (triglycerides, insulin and CRP). The χ^2 test was used for categorized variables. A

multivariable linear regression model was used to assess CMV significance as an independent determinant for blood pressure values and FMD. Log_{10}-transformed values were used for skewed variables. All analyses were also repeated in a subcohort consisting of only CMV seropositive subjects.

Results

In our study cohort, there were significantly ($P = 0{\cdot}003$) more seropositive women (72·3%) than seropositive men (65·9%). Additionally, there were significantly ($P < 0{\cdot}001$) more women with CMV antibody levels over 14 000 titres compared to men (72·5% *versus* 27·5%). Because of the strong sex difference in our study sample, all analyses were performed separately in men and women, including the quartile division.

Characteristics of the study subjects according to sex and CMV antibody titre quartiles are presented in Table 1. In men, high CMV antibody titres were associated significantly with diastolic blood pressure ($P = 0{\cdot}002$) and were associated borderline significantly with systolic blood pressure ($P = 0{\cdot}053$) compared to all other men. In men, age was also associated with the highest CMV antibody titre quartile ($P < 0{\cdot}001$). There were no significant associations between other traditional CVD risk factors (anthropometric, lipid, metabolic, inflammatory or lifestyle factors) and high CMV antibody titres. In men, subjects with high CMV antibody titres had significantly lower FMD values ($P = 0{\cdot}013$). IMT and Cdist values did not differ significantly between CMV antibody titres. In women, there were no significant associations between CMV antibody titres and early markers of atherosclerosis. However, there was a trend between high CMV antibody titre and higher FMD values ($P = 0{\cdot}070$).

A multivariable regression model was used to analyse whether CMV antibody titres are independent determinants for blood pressure and FMD. In men, high CMV antibody titres were associated independently with systolic blood pressure ($P = 0{\cdot}029$) and diastolic blood pressure ($P = 0{\cdot}004$) after adjusting for age, BMI, HDL cholesterol, triglycerides, insulin, CRP, smoking, occupational status and alcohol consumption (Table 2). In men, high CMV antibody titres were also associated independently and inversely with FMD ($P = 0{\cdot}014$) after adjusting for age, baseline brachial diameter, BMI, systolic blood pressure, HDL cholesterol, triglycerides, insulin, CRP, smoking, occupational status and alcohol consumption (Table 3). In women, high CMV

Table 1(a). Baseline characteristics of women according to cytomegalovirus immunoglobulin (Ig)G antibody titrers.

	Cytomegalovirus IgG antibody titre quartiles				
	1	2	3	4	
Characteristics	<230 titre ($n = 297$)	290–9 300 titre ($n = 234$)	9 400–15 000 titre ($n = 289$)	16 000–46 000 titre ($n = 254$)	*P-value
Age (years)*	31·2 (4·9)	30·8 (5·0)	32·4 (5·0)	32·1 (4·7)	0·120
Smokers, % daily‡	16·2	20·3	20·4	21·3	0·413
Occupational status, % manual‡§	21·0	25·9	21·7	25·0	0·424
Education, % comprehensive school‡	6·7	7·8	10·4	8·3	0·990
Body mass index (kg/m^2)*	23·9 (4·1)	24·0 (4·4)	24·4 (4·3)	24·5 (4·6)	0·200
Waist circumference (cm)*	77·7 (10·3)	77·8 (10·6)	79·9 (11·2)	79·6 (11·7)	0·166
Waist–hip ratio*	0·79 (0·06)	0·78 (0·06)	0·80 (0·06)	0·80 (0·06)	0·145
Systolic blood pressure (mmHg)*	115·3 (12·2)	116·4 (12·2)	116·9 (11·6)	115·2 (12·8)	0·288
Diastolic blood pressure (mmHg)*	71·3 (8·2)	71·4 (9·1)	72·4 (8·1)	71·2 (8·9)	0·360
Total cholesterol (mmol/l)*	5·00 (0·84)	5·03 (0·88)	5·07 (0·91)	5·05 (0·96)	0·780
HDL cholesterol (mmol/l)*	1·40 (0·29)	1·44 (0·29)	1·38 (0·30)	1·38 (0·30)	0·302
LDL cholesterol (mmol/l)*	3·09 (0·73)	3·08 (0·75)	3·18 (0·75)	3·16 (0·82)	0·453
Triglycerides (mmol/l)†	1·00 (0·80–1·30)	1·00 (0·80–1·30)	1·00 (0·75–1·40)	1·00 (0·78–1·30)	0·591
Glucose (mmol/l)*	4·8 (0·5)	4·8 (0·6)	4·9 (0·8)	4·8 (1·1)	0·453
Insulin (mU/l)†	6·0 (5·0–8·0)	6·0 (4·0–8·0)	7·0 (5·0–9·0)	6·0 (4·0–9·0)	0·766
C-reactive protein (mg/l)†	0·77 (0·30–1·90)	1·00 (0·38–2·50)	0·84 (0·33–2·22)	0·80 (0·34–2·29)	0·941
Physical activity index*‖	17·7 (15·9)	16·2 (13·6)	15·8 (15·6)	16·4 (14·5)	0·886
Alcohol (no. drinks per week)*	3·4 (4·3)	4·2 (5·5)	3·7 (4·9)	3·9 (7·9)	0·763
IMT (mm)*	0·56 (0·08)	0·57 (0·09)	0·58 (0·09)	0·58 (0·08)	0·221
Cdist (%/10 mmHg)*	2·38 (0·81)	2·32 (0·71)	2·30 (0·76)	2·33 (0·76)	0·996
FMD (%)*#	8·49 (4·55)	8·73 (4·43)	8·72 (4·60)	9·24 (4·52)	0·070

*Mean values and standard deviation, *t*-test for difference between highest CMV antibody titrers compared to other groups. †Median values and interquartile range (IQR), Mann–Whitney *U*-test for difference between highest CMV antibody titres compared to other groups. ‡Percentages and χ^2 test for difference between highest CMV antibody titres compared to other groups. §Data missing for 136 participants. ‖Data missing for 231 participants. #Data missing for 62 participants. HDL: high-density lipoprotein; LDL: low-density lipoprotein; IMT: carotid intima-media thickness; Cdist: carotid artery distensibility; FMD: flow-mediated dilation.

Table 1(b). Baseline characteristics of men according to cytomegalovirus (CMV) immunoglobulin (Ig)G antibody titres.

	Cytomegalovirus IgG antibody titre quartiles				
	1	2	3	4	
Characteristics	<230 titre ($n = 292$)	240–5 400 titre ($n = 139$)	5500–10 000 titre ($n = 206$)	11 000–35 000 titre ($n = 220$)	*P*-value
Age (years)*	30·8 (4·8)	31·6 (4·9)	31·5 (5·0)	33·0 (5·1)	<0·001
Smokers, % daily‡	23·4	39·6	30·6	28·2	0·796
Occupational status, % manual‡**	42·5	43·3	45·8	43·5	0·934
Education, % comprehensive school‡	7·2	11·5	5·3	5·9	0·451
Body mass index (kg/m^2)*	25·8 (4·1)	25·5 (3·8)	25·3 (3·6)	26·1 (4·0)	0·126
Waist circumference (cm)*	89·9 (11·4)	89·6 (10·5)	88·7 (10·0)	90·2 (10·7)	0·374
Waist–hip ratio*	0·89 (0·06)	0·90 (0·07)	0·89 (0·06)	0·90 (0·06)	0·347
Systolic blood pressure (mmHg)*	129·2 (13·2)	127·5 (12·1)	128·1 (11·9)	130·8 (16·4)	0·053
Diastolic blood pressure (mmHg)*	74·5 (8·8)	73·9 (8·1)	74·0 (7·8)	76·8 (10·8)	0·002
Total cholesterol (mmol/l)*	5·12 (0·99)	5·27 (1·11)	5·26 (0·96)	5·25 (1·00)	0·536
HDL cholesterol (mmol/l)*	1·16 (0·27)	1·17 (0·28)	1·17 (0·28)	1·15 (0·27)	0·282
LDL cholesterol (mmol/l)*	3·34 (0·90)	3·45 (0·97)	3·45 (0·88)	3·45 (0·88)	0·478
Triglycerides (mmol/l)†	1·20 (0·90–1·70)	1·30 (0·90–1·90)	1·20 (0·90–1·80)	1·30 (0·90–1·80)	0·460
Glucose (mmol/l)*	5·2 (0·5)	5·3 (1·6)	5·2 (0·5)	5·2 (0·5)	0·850
Insulin (mU/l)†	6·0 (4·0–9·0)	6·0 (4·0–9·0)	6·0 (4·0–9·0)	7·0 (4·3–9·0)	0·329
C-reactive protein (mg/l)†	0·56 (0·29–1·25)	0·70 (0·26–1·51)	0·52 (0·27–1·33)	0·62 (0·30–1·43)	0·236
Physical activity index*,††	18·3 (18·3)	13·1 (15·0)	15·3 (16·9)	15·1 (16·7)	0·436
Alcohol (no. drinks per week)*	8·2 (9·3)	10·6 (12·9)	8·5 (8·3)	7·6 (10·0)	0·123
IMT (mm)*	0·59 (0·10)	0·59 (0·09)	0·60 (0·10)	0·59 (0·11)	0·811
Cdist (%/10 mmHg)*	2·04 (0·67)	1·99 (0·64)	2·03 (0·65)	1·94 (0·66)	0·086
FMD (%)*‡‡	7·17 (3·96)	6·96 (4·03)	7·23 (4·42)	6·36 (3·69)	0·013

*Mean values and standard deviation, *t*-test for difference between highest CMV antibody titrers compared to other groups. †Median values and interquartile range (IQR), Mann–Whitney *U*-test for difference between highest CMV antibody titres compared to other groups. ‡Percentages and χ^2 test for difference between highest CMV antibody titres compared to other groups. **Data missing for 126 participants. ††Data missing for 91 participants. ‡‡Data missing for 79 participants. HDL: high-density lipoprotein; LDL: low-density lipoprotein; IMT: carotid intima-media thickness; Cdist: carotid artery distensibility; FMD: flow-mediated dilation.

antibody titres were not associated independently with FMD ($P = 0·112$).

We also carried out a subanalysis in CMV seropositive subjects. In men, high CMV antibody titres were associated with age ($P = 0·001$), systolic blood pressure ($P = 0·020$), diastolic blood pressure ($P = 0·001$), alcohol consumption ($P = 0·050$) and FMD ($P = 0·033$). High CMV antibody titres remained a significant determinant for systolic blood pressure ($P = 0·021$), diastolic blood pressure ($P = 0·005$) and FMD ($P = 0·022$) in the multivariable model. In seropositive women, no significant associations were found between CMV antibody titres and the risk factors or markers of early atherosclerosis.

Discussion

In this study, we showed that high CMV antibody titres are associated independently with blood pressure values and associated inversely with FMD in young men. To our knowledge, there are no prior studies demonstrating an association between CMV antibody titres and blood pressure in humans. Recently, in a Chinese cohort, Li *et al.* have shown that plasma CMV DNA copy number is associated with hypertension [17]. Additionally, they showed that CMV-encoded microRNA, hcmv-miR-UL112, was highly expressed in hypertensive patients. Further they showed that hcmv-miR-UL112 could target interferon regulatory factor 1, which is related to up-regulation of angiotensin II type 2 receptor [26]. This pathway is one plausible pathological mechanism between CMV infection and increased blood pressure. An association between CMV infection and increased arterial pressure has also been shown in mice [18]. Cheng *et al.* showed that CMV infection induced renin expression in a dose-dependent manner in mouse and human cells and that increased angiotensin-II, interleukin (IL)-6, tumour necrosis factor (TNF)-α and monocyte chemotactic protein-1 (MCP-1) levels were identified in mouse serum. Both IL-6 and TNF-α levels have been shown to correlate with increased blood pressure values [27,28]. The association shown in this study between high CMV antibody titres and with blood pressure values in young men supports the possible relationship between CMV infection and blood pressure. Because renin, angiotensin-II, IL-6, TNF-α and MCP-1 levels were not measured in this study, we were not able to evaluate whether this association depends on these factors. However, CRP concentrations,

Table 2. Determinants of systolic and diastolic blood pressure in a multivariate linear regression model in men ($n = 730$).

Blood pressure	Risk variable	β ± s.e.	*P*-value
Systolic	Age (years)	0·026 ± 0·101	0·800
	Body mass index (kg/m^2)	0·868 ± 0·161	<0·001
	HDL cholesterol (mmol/l)	3·215 ± 2·027	0·113
	Triglycerides (mmol/l)	7·840 ± 2·955	0·008
	Insulin (mU/l)	6·898 ± 2·553	0·007
	C-reactive protein (mg/l)	1·452 ± 1·092	0·184
	Smoking (daily)	−3·147 ± 1·098	0·004
	Non-manual occupation	−2·438 ± 0·991	0·014
	Alcohol (no. drinks per week)	0·087 ± 0·049	0·075
	High CMV antibody (titre)	2·367 ± 1·084	0·029
		$R^2 = 0·181$	
Diastolic	Age (years)	0·385 ± 0·063	<0·001
	Body mass index (kg/m^2)	0·431 ± 0·099	<0·001
	HDL cholesterol (mmol/l)	3·895 ± 1·252	0·002
	Triglycerides (mmol/l)	8·742 ± 1·825	<0·001
	Insulin (mU/l)	5·387 ± 1·577	0·001
	C-reactive protein (mg/l)	0·928 ± 0·674	0·169
	Smoking (daily)	−2·114 ± 0·678	0·002
	Non-manual occupation	−2·151 ± 0·612	<0·001
	Alcohol (no. drinks per week)	0·095 ± 0·030	0·002
	High CMV antibody (titre)	1·927 ± 0·669	0·004
		$R^2 = 0·275$	

High CMV antibody (titre); CMV antibody titres were parameterized with a dummy variable comparing the highest quartile with the bottom three quartiles combined. HDL: high-density lipoprotein; CMV: cytomegalovirus.

which are considered to be indicators of systemic low-grade inflammation, were available from the present cohort. We found that CRP concentrations did not differ between CMV antibody titre quartiles and that the association between CMV antibody titres and blood pressure values was not attenuated after adjustment with CRP. However, it is possible that CMV may mediate other inflammatory pathways than CRP. Based on the works of Li *et al.* and Cheng *et al.*, it can be speculated that the activation of the renin–angiotensin system (RAS) is possibly an underlying mediator behind these findings. It is possible that high CMV antibody titres are indicators of frequent reactivation of CMV or reinfection with new strains of CMV, leading to stronger immunity in these individuals. CMV activity may lead to an increased RAS activation, leading to arterial constriction via the influence of angiotensin-II, a mechanism that possibly explains the observed increased blood pressure values in this study.

Table 3. Determinants of flow-mediated dilation in a multivariate linear regression model in men ($n = 657$).

Risk variable	β ± s.e.	*P*-value
Age (years)	0·004 ± 0·032	0·929
Baseline brachial diameter (mm)	−2·828 ± 0·361	<0·001
Body mass index (kg/m^2)	0·236 ± 0·054	<0·001
Systolic blood pressure (mmHg)	−0·025 ± 0·013	0·050
HDL cholesterol (mmol/l)	0·355 ± 0·641	0·580
Triglycerides (mmol/l)	−0·098 ± 0·945	0·917
Insulin (mU/l)	−0·209 ± 0·819	0·799
C-reactive protein (mg/l)	−0·093 ± 0·346	0·787
Smoking (daily)	−0·273 ± 0·351	0·436
Non-manual occupation	−0·386 ± 0·313	0·219
Alcohol (no. drinks per week)	−0·019 ± 0·016	0·231
High CMV antibody (titre)	−0·844 ± 0·343	0·014
	$R^2 = 0·118$	

High CMV antibody (titre); CMV antibody titres were parameterized with a dummy variable comparing the highest quartile with the bottom three quartiles combined. HDL: high-density lipoprotein; s.e.: standard error.

The association between CMV infection and endothelial function has been reported previously in relatively small study populations, but the findings have not been consistent. In a population consisting of paediatric heart transplant patients ($n = 50$), Simmond *et al.* demonstrated that decreased FMD is associated with CMV replication after transplantation [12]. In a middle-aged population (mean age 38 years), which consisted of diabetic and non-diabetic subjects ($n = 157$), Grahame-Clarke *et al.* showed that CMV seropositivity was associated with impaired vascular function measured by venous occlusion plethysmography with bradykinin and glyceryl trinitrate [13]. Additionally, CMV infection has been shown to cause arterial dysfunction in a mouse model [29]. However, negative findings have also been reported in small cohorts, which consisted of young Japanese men ($n = 81$) [16] and young Canadian men ($n = 65$) [15]. In our study we demonstrated for the first time, in a large cohort of young men ($n = 657$), that high

CMV antibody titres were associated independently with endothelial function. *In vitro*, CMV infection has been shown to activate the P38-mitogen-activated protein kinase (MAPK) signalling pathway and up-regulate phosphatase and tensin homologue [30,31]. Via this pathway, endothelial nitric oxide synthase is inhibited and reduced nitric oxide (NO) production leads to endothelial dysfunction [32,33]. The association between CMV and endothelial dysfunction has been hypothesized further to be one possible pathological mechanism of hypertension [33]. This mechanism is a possible explanation for the demonstrated association between CMV and reduced FMD in our study, and it is also a possible alternative explanation for the demonstrated association between CMV and increased blood pressure. In this study, high CMV antibody titres were not associated with IMT or Cdist. Previously, in a cohort consisting of middle-aged subjects, high CMV antibody titres were shown to be a risk factor for increased IMT [14]. One possible explanation for this discrepancy between the results could be the age difference between the cohorts. Possible deleterious effects of CMV immune response on IMT may not be currently detectable in young adult ages.

In this study, CMV antibody titres were significantly higher among women, an observation that has also been seen in other populations [2]. Interestingly, high CMV antibody titres were not associated with decreased FMD values or with increased blood pressure values in women. Thus, it might be possible that CMV infection leads to dissimilar immune responses and subclinical manifestations in women and men. Zhu *et al.* found that the association of an immune response to CMV and the risk of coronary artery disease differed between the sexes [34]. In their study, coronary artery disease risk was increased among women who had humoral antibodies against CMV but not in those women who had only a cell-mediated immune response to CMV. In men, there was no difference between different immune responses and coronary artery disease risk. Our work also supports the hypothesis that immune responses to CMV may differ between the sexes. Zhu *et al.* proposed that the differences in the findings between the sexes may be due to the association found between CMV and CRP in men, but not in women. In our study, CMV antibody titres were not associated with CRP. All in all, we did not find any significant implications for high CMV antibody titres in young women. Based on our current knowledge, we do not have an explanation for the observed sex-related differences in our results.

The role of CMV as a risk factor for CVD is controversial. A great deal of previous studies investigated the role of CMV seropositivity as a risk factor for CVD indices [6,7,10]. Few studies have investigated the role of CMV antibody titres [8,9,11]. Regardless of the approach, both negative and positive associations have been reported. Numerous mechanisms have been reported on how CMV infection may lead to the development and exacerbation of atherosclerosis. CMV has been shown to infect human endothelial cells [35] and leucocytes [36]. Infection of these cells leads to neutrophil transendothelial migration [37], smooth muscle cell migration [38], intracellular adhesion molecule expression and leucocyte adhesion [39]. Conversely, it has been demonstrated that CMV-independent TNF-α production can induce CMV reactivation [40]. Thus, the alternative explanation could also be the reverse causality. High CMV antibody titres might be a result of immunoresponses stimulated by pathological changes independent of CMV.

In this study, the subjects with high CMV antibody titres were compared to those with moderate, low or seronegative CMV antibody titres, whereas the traditional set-up has been to compare seropositive and seronegative subjects. The latter set-up may be problematic due to the high prevalence of the virus in humans. More than half of humans have serological evidence of CMV infection and it is also possible that there are some seronegative cases that have had prior CMV infection without leaving detectable serological evidence. This methodology raises the question of how an almost ubiquitous infection could be a sole risk factor. Along these lines, we used a set-up where subjects with high CMV antibody titres were compared to other subjects. This strong antibody-specific immunity against CMV could be the result of various factors: severe primary infection, recent infection, reinfections of CMV with a different strain, frequent reactivations of the virus in the body or non-viral-related individual differences in immunity. The advantage of this set-up is that these subjects certainly have a strong CMV-specific humoral immune response, but conversely, we do not know what mechanism may have caused it. Further studies are needed to differentiate the aetiology of strong CMV-specific immune response. Because our study cohort was ethnically homogeneous, the generalizability of our results is limited to white European subjects. It is also important to remember that observational studies cannot establish causality.

In conclusion, this study showed that high CMV antibody titres associate directly with blood pressure and inversely with FMD in young men. These associations were not found in women. Our results support the idea that this common virus could be a risk factor for unfavourable changes in the cardiovascular system at an early age.

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Disclosure

The authors declare that there is no conflict of interest.

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