Universität Ulm
Aus der Abteilung Innere Medizin II
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Association Between Plasma Levels of the Soluble CD14 Receptor of Lipopolysaccharide and the C(–260)®T Polymorphism in the Promoter of the CD14 Gene and Coronary Artery Disease: Investigations in a Large Case-Control Study.

Dissertation zur Erlangung des Doktorgrades der Medizin
der Medizinischen Fakultät der Universität Ulm

vorgelegt von
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2002

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Tag der Promotion: 26.04.2002

To my family with deep gratitude for its persistent support of my academic endeavours

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Abbreviations and Acronyms

AMI	acute myocardial infarction
AP	angina pectoris
apo B-100	apolipoprotein B-100
BMI	body mass index
bp	base pair
CAD	coronary artery disease
CAM	cellular adhesion molecule
CD14	cluster of differentiation antigen 14
CD36	cluster of differentiation antigen 36
cDNA	complementary DNA
CI	confidence interval
CMV	Cytomegalovirus
C. pneumoniae	*Chlamydia pneumoniae*
CRP	C-reactive protein
dNTP	deoxynucleoside triphosphate
EC	endothelial cell
ECG	electrocardiogram
e.g.	for example
ELAM	endothelial leukocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
ERA	Estrogen Replacement and Atherosclerosis
ESR	erythrocyte sedimentation rate
et al	et alii
FDP	fibrin degradation product
FGF	fibroblast growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidilinositol
Hb A_{1c}	hemoglobin A_{1c}
HERS	Heart and Estrogen/Progestin Replacement Study
HLD	high density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPETE	hydroperoxyeicosatetraenoic acid

H. pylori	*Helicobacter pylori*
HRT	hormone replacement therapy
HSP	heat shock protein
HSV	Herpes simplex virus
ICAM-1	intercellular adhesion molecule-1
i.e.	id est
IgG	immunoglobulin G
INF-γ	interferon-γ
IL	interleukin
IRMA	immunoradiometric assay
kDa	kilo Dalton
LBP	lipopolysaccharide-bind protein
LDL	low density lipoprotein
Lp (a)	lipoprotein (a)
LPS	lipopolysaccharide
12-LO	12/15 lipoxygenase
mAb	monoclonal antobody
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
mm-LDL	minimally oxidized low density lipoprotein
MRFIT	Multiple Risk Factor Intervention Trial
NO	nitric oxide
NF B	nuclear factor-kappa B
O_2^-	superoxid anion
OD	optical density
OR	odds ratio
ox-LDL	highly oxidized low density lipoprotein
PAI-1	plasminogen activator inhibitor-1
PAOD	peripheral arterial occlusive disease
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PHS	Physicians‘ Health Study
PLC	phospholipase C

PNH	paroxysmal nocturnal hemoglobinuria
PROCAM	Prospective Cardiovascular Münster Study
RAS	renin-angiotensin system
RH	relative hazard
ROS	reactive oxygen species
RR	risk ratio
SAA	serum amyloid A
SAS	statistical analysis system
SMC	smooth muscle cell
SR-A	scavenger receptor-A
TIA	transient ischemic attack
TF	tissue factor
TGF-β	transforming growth factor-β
TLR-4	Toll like receptors-4
TNF-α	tumor necrosis factor-α
VCAM-1	vascular adhesion molecule-1
VLA-4	very late antigen-4
VLDL	very low density lipoprotein
vs	versus
vWF	von-Willebrand factor
UKPDS	United Kingdom Prospective Diabetes Study

1 Introduction

1.1 Coronary artery disease (CAD)

1.1.1 Pathogenesis of CAD: cellular and molecular interactions

In the “response to injury” hypothesis of atherosclerosis, endothelial dysfunction represents an initial step for the early local inflammation which precedes plaque development (Ross 1999). The endothelium, with its intercellular tight junctional complexes, functions as a selective permeable barrier between the vessel wall and circulating blood. As one of the important physical forces acting on endothelial cells (EC), fluid shear stress has an effect on EC morphology and therefore can determine, in part, the site of an atherosclerotic lesion (Koenig & Hombach 1995).

It has been well established that atherosclerotic lesions typically develop in the vicinity of branch points and areas of major curvature, where blood flow is non-laminar. ECs in those areas have polygonal shapes and no particular orientation that would increase permeability of the endothelium to macromolecules like low density lipoproteins (LDL). In contrast, ECs in the tubular regions of arteries with a laminar blood flow are ellipsoid in shape and aligned in the direction of flow, that result in relative atherosclerotic resistance, at least in the early phases of the disease (Gimbrone 1999).

The primary events in the pathogenesis of atherosclerosis are accumulation and subsequent modification of LDL in the subendothelial matrix. The LDL particles are spherical, and consist of a polar surface and an apolar core. The surface is composed of unesterified cholesterol, the phospholipids phosphatidilcholine and sphingomyelin and a single polypeptide, the apolipoprotein B-100 (apo B-100). The physiological function of LDL particles is to provide cells with the cholesterol they need to form cellular membranes. LDL either crosses the endothelium in transcytotic vesicles or diffuses passively through EC junctions. Accumulation of LDL in the subendothelial space is greater, when levels of circulating LDL are raised and when both the transport and retention of LDL are increased in lesion-prone sites. LDL remains in the vessel wall as the result of an interaction between positively charged so-called “heparin-binding domains” on apo B-100 particles of LDL and negatively charged sulphate groups of the glycosaminoglycan chains of the matrix proteoglycans (Boren et al. 1998). To become atherogenic, trapped native LDL particles have to undergo modification, including oxidation (Bhakdi 2000), lypolysis, proteolysis, glycation or aggregation. However, the most

significant finding in early lesion formation is lipid oxidation, which occurs as a result of interaction with reactive oxygen species (ROS) including products of 12/15 lipoxygenase (12-LO) like hydroperoxyeicosatetraenoic acid (HPETE) (Cyrus et al. 1999).

The specific properties of oxidized LDL depend on the extent of the modification, which can range from “minimal” modification (or minimally oxidized LDL (mm-LDL) to extensive oxidation (or highly oxidized LDL (ox-LDL). Minimally oxidized LDL stimulates the endothelial cells to produce cellular adhesion molecules (CAM) like intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin, and growth factors, like macrophages colony-stimulating factor (M-CSF), resulting the adhesion of monocytes to the endothelium and subsequent recruitment into the vessel wall. Recent data suggest that certain chemokines (monocyte chemotactic protein-1, MCP-1 and interleukin-8, IL-8) may directly interfere with monocyte adhesion by increasing the interaction with adhesion molecules (Gerszten et al. 1999).

The first step in the adhesion, the monocyte “rolling” along the endothelial surface, is mediated, in part, by P- and E-selectins (Dong et al. 1998). The adhesion of monocytes to the endothelium can be mediated by the integrin very late antigen-4 (VLA-4), which interacts with VCAM (Shih et al. 1999).

Once adhered, the monocytes migrate across the endothelial surface into the intima, where they, on stimulation with MCP-1, M-CSF and transforming growth factor-β (TGF-β), proliferate and differentiate into macrophages. Macrophages in the subendothelial space take up LDL via scavenger receptors (scavenger receptor - A (SR-A) or CD36). However in order to be recognized by these receptors, LDL must be “highly oxidized”. It has been suggested that macrophages may be responsible for converting minimally oxidized LDL into intensively modified LDL. This modification involves several enzymes such as myeloperoxidase, sphingomyelinase and secretory phospholipase.

After uptake of ox-LDL, macrophages convert into foam cells. The formation of foam cells and their continued accumulation in the intima together with T-cells lead to the first ubiquitous lesion of atherosclerosis, the “fatty streak”. With time, foam cells die, contributing their lipid-filled contents to the necrotic core. They also liberate a large number of products that can, in turn, damage the endothelium and thus participate in the further evolution of the fatty streak. Molecules such as

interleukins and tumor necrosis factor-α (TNF-α) may enhance monocyte adhesiveness and chemotaxis and thereby recruit circulating monocytes into the lesion. Moreover, growth factors (platelet-derived growth factor (PDGF) and TGF-β) and some cytokines (IL-1 and TNF-α), secreted by T-cells and macrophages, are important for the production of extracellular matrix and for the proliferation and migration of the smooth muscle cells (SMC) from the medial layer of the arterial wall through the internal elastic lamina. This condition can in turn lead to an expanded and more complex lesion, called a fibrous plaque. Clinically this advanced atherosclerotic lesion can exhibit the ischemic symptoms as a result of the progressive narrowing of the vessel lumen. Changes in the surface characteristics of the fibrous cap, such as ulceration or rupture, can result in the formation of a thrombus which can cause acute cardiovascular events or which can lead to further lesion progression and compromise of local blood flow.

1.1.2 Classical risk factors for CAD

The atherosclerotic lesion is a protective inflammatory-fibroproliferative response against the different agents which can cause the disease.

Epidemiological studies over the last 50 years have established numerous “classical” risk factors for atherosclerosis. They can be grouped into factors with a strong genetic component and those that are largely environmental. More recently, several emerging risk factors such as hyperhomocysteinemia, procoagulatory factors, inhibition of fibrinolysis, (Kullo et al. 2000, Illingworth 1999, Cullen et al. 1998, Koenig 1998) as well as various genetic markers such as polymorphism of the fibrinogen gene (Simmonds et al. 2001, Fowkes et al. 1992) have been identified.

1.1.2.1 Lipoproteins

The relative abundance of the different plasma lipoproteins appears to be of primary importance. It has been clearly shown that elevated LDL cholesterol and triglyceride levels and low level of high density lipoprotein cholesterol (HDL), independently or combined, are risk factors for atherosclerosis and its complications, as reported for example (e.g.), in the Framingham Study, the Helsinki Heart Study and the Prospective Cardiovascular Münster (PROCAM) Study (Andersson et al. 1987, Manninen et al. 1992, Assmann & Schulte 1992).

Recently lipoprotein (a) - Lp(a), a member of lipoprotein family, has been revealed as a potential additional risk factor for CAD, particularly in persons with familial hypercholesterolemia or with a family history of premature coronary disease (Scanu 1992, Genest et al. 1992).

1.1.2.2 Hypertension

Hypertension is also a well-known risk factor, and probably acts via injury of vessel wall, predisposing to atheroma. In the Multiple Risk Factor Intervention Trial (MRFIT), a prospective study with 11.6 years of follow-up, which enrolled over 316.099 subjects, baseline blood pressure was shown to be strongly and independently related to the risk of CAD mortality (Neaton & Wentworth 1992). Elevated blood pressure is associated with an increased risk of reinfarction (Flack et al. 1995) as well as an increased risk of stroke (Thijs et al. 1992). Several observations have linked the renin-angiotensin system (RAS) to an increased risk of thrombosis (Ridker et al. 1993, Van Leeuwen et al. 1994). Clinical trials have consistently demonstrated the benefits of blood pressure reduction, with particularly strong effects in the prevention of stroke (MacMahon et al. 1990, Luft 1998, Staessen et al. 2000). Moreover, the effects of hypertension on CAD are considerably amplified if cholesterol levels are high (Lusis et al. 1998).

1.1.2.3 Diabetes mellitus

It has also long been recognized that diabetic patients have earlier and more severe CAD and that they are prone to “silent” myocardial ischemia, which presumably reflects the impaired perception of ischemic cardiac pain caused by autonomic neuropathy. Diabetes mellitus (both insulin-dependent and non-insulin-dependent) is associated with a markedly increased risk of CAD, especially among women (Harris et al. 1998, Timmis 2001). Diabetic dyslipidemia, which is characterized by elevated levels of triglyceride and small, dense LDL (LDL subclass pattern B), as well as decreased HDL cholesterol levels (Haffner 1998), seems to be a major source of this increased risk. In the United Kingdom Prospective Diabetes Study (UKPDS) it has been shown that each increment of 1 mmol/L in LDL cholesterol is associated with a 1.57-fold increased risk of CAD, and a 0.1 mmol/L increment in HDL cholesterol is associated with a 15% decrease in the risk of CAD (Turner et al. 1998). Improved glycemic control, i.e. the

reduction of glycated hemoglobin A_{1c} (Hb A_{1c}) from 7.9% to 7%, is also associated with a 16% reduction in MI (p=0.0052) (UKPDS group 1998). Recent data have revealed that in patients with type II diabetes mellitus without previous myocardial infarction (MI), the risk of a first MI is equivalent to that observed in non-diabetic patients who have had a previous infarction (Haffner et al. 1998). Diabetic patients also have a high mortality rate from a first MI (Löwel et al. 2000).

1.1.2.4 Gender

CAD is more common in men than in women. The greater severity and longer exposure of men to the major risk factors, particularly dyslipidemia and smoking, may explain part of the gender difference in CAD. Other explanations have focused on hormonal differences between both sexes. Meta-analysis of more than 30 case-control and prospective studies revealed that the summary relative risk for CAD was 0.70 (30% protection) in women who only used estrogen (presumed to be primarily unopposed estrogen) and 0.66 (34% protection) in women who used estrogen plus progestin (Barrett-Connor & Grady 1998). However, the results of the Heart and Estrogen/Progestin Replacement Study (HERS) randomized trial, published in 1998, showed no beneficial effect of long-term hormone replacement therapy (HRT) on the risk of major cardiovascular events among women with established CAD (relative hazard (RH), 0.99; 95% confidential interval (CI), 0.81-1.22) and, in fact, showed an increased risk of CAD events in hormone recipients during the first year of the trial (RH, 1.52; 95% CI, 1.01-2.29) (Hulley et al. 1998). More recently, Estrogen Replacement and Atherosclerosis (ERA) study investigators announced no apparent benefit associated with hormone use during 3.5 years of treatment too (Herrington et al. 2000). Thus, recent evidence from HERS and subsequent trials and clinical studies raises serious concerns about the role of HRT in the prevention of CAD in postmenopausal women.

1.1.2.5 Body mass index

Numerous cross-sectional and prospective studies have shown a close relationship between excessive accumulation of body fat and all-cause mortality and cardiovascular mortality in particular (Troiano et al. 1996). Obesity (body mass index (BMI) $\geq$ 30 kg/m^2) appears to increase the risk for cardiovascular disease

both directly and by promoting the occurrence of comorbid conditions, such as blood pressure, plasma LDL-, HDL-cholesterol levels, and glucose tolerance, which in turn predispose patients to the development of CAD.

1.1.2.6 Physical activity

A sedentary life style is also recognized as an independent risk factor for CAD, and even moderate exercise and physical fitness may provide a reduction of this risk (Berlin & Colditz 1990, Kokkinos et al. 1995, Fischer & Koenig 1998). Meta-analyses of epidemiological studies have shown a 1.8-fold increased risk for the development of CAD and for coronary death among sedentary people (Berlin & Colditz 1990). Furthermore, in a recent study which involved 3.331 Japanese men, those engaged in regular physical activity for ≥ 3 days per week, had the lowest coronary risk (Hsieh et al. 1998). Leisure time physical activity helps to prevent or reduce overweight, lowers high blood pressure, and has beneficial effects on triglyceride and HDL levels. Glucose intolerance is also controlled by moderate and low-level exercise intensity (Fletcher 1999). Furthermore, an improvement of any disturbed hemostatic balance is seen (Imhof & Koenig 2001).

1.1.2.7 Smoking

The favourable effect of smoking cessation on prognosis of CAD is well documented (Van Berkel et al. 1999). Smoking accounts for 2% of male and 4% of female deaths from cardiovascular diseases in Europe (French & White 2000). Several effects of smoking, including endothelial damage, decreased levels of HDL cholesterol, the prothrombotic effects like increased thromboxane production, raised plasma fibrinogen levels, and platelet-dependent thrombin generation, and decreased oxygen-carrying capacity of the blood, may contribute to the increased cardiovascular risk in smokers. Several mediators, i.e. nicotine, carbon monoxide and various toxic components of cigarette smoke, may be also involved (Hioki et al. 2001, Kimura et al. 1994, Wilson & Culleton 1998).

1.1.2.8 Family history of disease

Both genetic and environmental risk factors contribute to the major predisposing factor for the development of CAD, a family history of disease (Burke et al. 1991). CAD is associated with pathological conditions that are now known to have a

genetic component, such as dyslipoproteinemias, hypertension, diabetes mellitus and obesity, as well as with lifestyle behaviours, such as dietary practices, smoking and lack of physical activity.

Thus, it is logical to assume that a disease as variable as atherosclerosis may develop from different combinations of etiological factors and that each of the independent genetic risk factors involves multiple genes.

1.1.3 CAD – an inflammatory process

Apart from the local inflammatory process in the vessel wall, systemic signs of an inflammatory reaction are associated with future cardiovascular end-points. Several studies have established that plasma levels of leukocyte count and various inflammatory proteins such as fibrinogen, plasminogen activator inhibitor (PAI-1), von Willebrand factor (vWF) (Koenig 1998, Ridker 1997 b), and more recently C-reactive protein (CRP) (Danesh et al.1998 a, Koenig et al. 1999 a), as well as several cytokines (Ridker et al. 2000 a, Ridker et al. 2000 b) and adhesion molecules (Ridker et al. 2001 a, Ridker 2001 b) are positively correlated with a risk of future cardiovascular disease.

Recent reports have suggested a link between the blood concentration of CRP and the risk of cardiovascular disease. CRP is the clinical acute phase protein and represents a sensitive marker of inflammation. In healthy, young subjects the serum concentrations of this protein are below 1.5 mg/L (De Maat & Kluft 2001). During an acute inflammatory stimulus, the CRP concentration can increase up to several thousand-fold. Various studies have established a solid association between CRP and clinical manifestations of atherosclerosis in several vascular beds (Ridker et al. 1998 c, Danesh et al. 2000 b). In the Physicians‘ Health Study (PHS), a prospective study of apparently healthy male physicians, baseline levels of CRP were significantly and independently predictive of future MI and stroke (Ridker et al. 1997 a), whereas the rise in CRP after AMI or during unstable angina pectoris correlates with outcome (Liuzzo et al. 1994, Pietilä et al. 1996, Haverkate et al. 1997). CRP may be an even stronger predictor of cardiovascular events in low-risk populations (women) (Ridker et al. 1998 b).

Concentration of CRP is usually strongly correlated with IL-6 concentration. IL-6 is a local and circulating marker of coronary plaque inflammation. Raised circulating

concentrations are found in patients with unstable angina (Biasucci et al. 1996) and in stable CAD patients at risk of future recurrent events (Ridker et al. 2000 b).
Increased levels of CAMs may serve as markers of early atherosclerosis and thereby may indicate an enhanced risk of CAD. This has been shown in the PHS, where sICAM-1 was associated with an increased risk of MI (Ridker et al. 1998 a) and in the Women's Health Study, where levels of soluble P-selectin were significantly associated with an increased future risk (Ridker et al. 2001 a). Another group of investigators found higher levels of circulating ICAM-1 in patients with CAD, with the highest levels in patients with acute ischemic syndromes (Haught et al. 1996). Moreover, levels of all four soluble CAMs (ICAM-1, VCAM, E-selectin and P-selectin) were siginifically elevated in patients with unstable angina and non-Q wave MI (Mulvihill et al. 2000). However, a recent meta-analysis (Malik et al. 2001) leaves doubt about the additional predictive ability of these molecules.
Recently it has been revealed that relatively inexpensive and simple laboratory test like erythrocyte sedimentation rate (ESR) may carry prognostic information on the risk of sustaining CAD events. Danesh et al. published an analysis of pooled data from four population-based prospective studies (1703 cases with fatal or non-fatal CAD) on the association of ESR and the development of CAD, comparing ESR in individuals in the top third with those in the bottom third at baseline, and found a risk ratio (RR) of 1.33 (95% CI, 1.15 to 1.54; $p \leq 0.0001$) (Danesh et al. 2000 a). Furthermore, in the study by Erikssen et al. ESR turned out to be a strong predictor of subsequent CAD mortality among 403 patients who had developed angina pectoris and/or who had a positive exercise ECG test at the second survey study 7 years later (Erikssen et al. 2000).
Thus, these observations taken together suggest the possibility that local and systemic inflammation accompanies atherosclerosis from its initiation to the evolution of clinical endpoints.

1.1.4 Possible role of infectious agents in the pathogenesis of CAD

Recently increasing interest has centred on the infectious theory of atherosclerosis. However, such a relationship between infectious diseases and the genesis of atherosclerosis has still to be proven.
The concept of infection as a risk factor in the atherosclerotic process and related clinical cardiovascular disease is approximately 100 years old. In the early 1900s,

Osler wrote of a potential link between “acute infections” and atherosclerosis (Osler 1908). But this did not receive much attention until the 1970s, when Fabricant et al. found that Marek’s disease virus, an avian herpesvirus, caused typical atherosclerotic lesion in chickens (Fabricant et al. 1978). Since then a number of infectious agents such as Herpes simplex virus (HSV), Enterovirus, Cytomegalo-virus (CMV), *Chlamydia pneumoniae* (*C.pneumoniae*) and *Helicobacter pylori (H.pylori)* have been implicated as primary etiologic factors or cofactors in the pathogenesis of atherosclerosis (Danesh et al. 1997). The accumulated evidence in favour of a possible role of these infections in atherogenesis consist mainly of seroepidemiological data (Nieto et al. 1996, Patel et al. 1995, Mendall et al. 1994), but the identification of viruses and bacteria in atherosclerotic plaque material (Hendrix et al. 1990, Shor et al. 1992, Blasi et al. 1996) and some experimental models showing an induction or acceleration of atherosclerosis in animals (Muhlestein et al. 1998) also contribute to the evidence.

The results of numerous prospective studies based on serological data that reported an association between the presence of antibodies to *H.pylori* and atherosclerosis are relatively weak (Pasceri et al. 1998, Gunn et al. 2000, Osawa et al. 2001) and there are many potential confounding factors, particularly low socioeconomic status, which is strongly associated with both CAD and *H.pylori* infection (Koenig et al. 1999 b). Moreover, a meta-analysis of 18 studies failed to show any correlation of seropositivity against *H.pylori* with the presence or extent of coronary disease (Danesh & Peto 1998 b). Recently Malnick et al., using a PCR technique, were also unable to demonstrate *H.pylori* infection in the carotid artery in symptomatic patients (Malnick et al. 1999).

CMV probably also plays some role, at least in the so-called “diffuse coronary heart disease after heart transplantation” (Mattila et al. 1998) and was found be associated with coronary artery restenosis following coronary angioplasty/ atheroctomy (Zhou et al. 1996).

However, among all pathogens which have been associated with atherosclerosis, *C.pneumoniae* appears to have the strongest association. Numerous seroepidemiological data (Libby et al. 1997), isolation from human atherosclerotic plaque (Kuo et al. 1997, Campbell et al. 1995), identification in the atheroma by culture or directly by microscopy (Jackson et al. 1997, Ramirez 1996) and early antibiotic trials directed against *C.pneumoniae* (Grayston 1998) together, suggest

that *C.pneumoniae* infection, especially in its chronic form, may play a role in the development of atherosclerosis and its clinical complications. The mechanisms by which *C.pneumoniae* influences atherogenesis are still poorly understood. It has recently been shown that *C.pneumoniae* induces foam cell formation, which represents the key cell in the initiation of atherosclerotic plaques and that chlamydial lipopolysaccharide (chlamydial LPS) is the main mediator of such formation (Kalayoglu & Byrne 1998). The LPS of Chlamydia is relatively non-toxic, but is a potent inducer of cytokines and adhesins (Saikku 2000).

Chlamydial heat shock protein (HSP)-60 has been demonstrated within plaque macrophages in human atherosclerotic lesions (Kol et al. 1998). This bacterial product stimulates macrophage function with release of proinflammatory cytokines, such as TNF-α, and matrix signalling metalloproteinases (Kol et al. 1998). Moreover, it has recently been shown that antibodies to chlamydial HSPs also induce endothelial cytotoxicity, a key event in the pathogenesis of atherosclerosis (Mayr et al. 1999)

Thus, infectious agents, especially *C.pneumoniae,* can be involved in atherogenesis, and specific microbial products, such as LPS and HSP, seem to play a pivotal role in triggering the atherosclerotic process. In addition, infection is associated with many classic risk factors, such as smoking, lipid abnormalities, hemostatic disorders and diabetes. Under these circumstances, infection may act as a synergistic factor, together with the classical risk factors in the etiopathogenesis of atherosclerosis.

1.2 CD14 – major receptor for bacterial lipopolysaccharide

1.2.1 Chemical structure and biological role of LPS

Almost all groups of Gram-negative bacteria express a biologically active substance on their surface, called lipopolysaccharide (LPS), which together with proteins and phospholipids form the outer membrane of these bacteria.

Chemically, LPS, as the name implies, consists of a polysaccharide portion (subdivided into the O-specific chain (O-antigen) and the core region) and a lipid part, termed lipid A (Bannerman & Goldblum 1999). The O-specific chain, comprising up to 50 repeating oligosaccharide units, is characteristic and unique for each bacterial strain and therefore is the most variable segment of the LPS molecule. It determines the serological specificity of the various bacterial

serotypes, i.e., their O-antigenicity. The core portion displays far less diversity and is composed of an inner core region (the part linked to the hydrophobic lipid A) and the outer core which couples the inner core to the O-specific chain (Rietschel et al. 1994). The lipid A component has been identified as the portion of the LPS molecule responsible for its biological activities and is called the “endotoxic principle” of LPS. Lipid A is also essential for bacterial survival and the interaction of LPS with recognition proteins such as LBP or lipoproteins (Schumann et al. 1994 b).

Endotoxin exerts its profound biological effects on the whole in an indirect manner. It stimulates host cells (monocytes/macrophages, ECs, SMCs and neutrophils) to produce and release different endogenous mediators (Liao 1996). LPS can induce endothelial activation and subsequent endothelial dysfunction. Endothelial cellular response to endotoxin includes increased expression of PAI-1, IL-1, IL-6, IL-8, tissue factor (TF), CAMs (endothelial leukocyte adhesion molecule (ELAM), ICAM and VCAM) (Chakravotty et al. 1999, Bierhaus et al. 2000). Besides inducing a proinflammatory and procoagulatory activity, LPS increases vascular permeability, induces endothelial cell detachment from the underlying matrix and disrupts endothelial barrier function (Bannerman et al. 1998, Bannerman & Goldblum 1999). Monocytes/macrophages, upon activation with endotoxin, release proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (Liuzzo et al. 2001, Tani et al. 2001, Baumgarten et al. 2001). LPS stimulation of macrophages leads to the production of polypeptides that can induce cell growth. These include fibroblast growth factor (FGF), PDGF and TGF-β. Endotoxin also stimulates SMC proliferation and migration, resulting in intimal hyperplasia (Liao 1996). In addition, LPS can provoke disturbance in lipoprotein metabolism, leading to a hyperlipidemic state: an increase of LDL and very low density lipoprotein (VLDL) and a decrease in HDL levels (Hardardottir et al. 1994). LPS can induce oxidative modification of LDL by stimulating the oxidative metabolism of monocytes with the release of superoxid anions (O_2^-) (Lopes-Verella 1993). LPS is also a potent stimulus for inducible nitric oxide (NO) synthase activity, leading to the formation of large amounts of NO (Moncada et al. 1991).

Thus, LPS may play a pivotal role in the development of atherosclerosis by injuring and activating the endothelium, stimulating SMC migration and proliferation,

activating the coagulant cascade, and by inducing hyperlipidemia, which in turn may disrupt the normal repair process.

1.2.2 Mechanism of interaction between LPS and LPS-recognition molecules

1.2.2.1 Structure and role of lipopolysaccharide-binding protein

After entering the blood stream, endotoxin is recognized and bound by a binding protein/receptor system, involving the lipopolysaccharide-binding protein (LBP) and the CD14 molecule, the latter in its membrane-bound and soluble form. Furthermore, LPS can bind to other membranes and serum proteins (Fenton & Golenbock 1998). However, the majority of these components, like the macrophage scavenger receptor for acetylated LDL, members of the CD18/CD11 β-integrin family, or lipoproteins are, most likely, only involved in detoxification of LPS, without taking part in LPS-induced cell activation. So far, the roles of LBP and CD14 are well established in endotoxin signal transduction that leads to a secretory response of LPS-activated cells.

LBP is synthesized in hepatocytes as a 50 kDa precursor and secreted as the mature 60 kDa glycosylated protein (Ramadori et al.1990). LBP is released into the bloodstream upon acute-phase stimulation. Its normal concentration in human serum is 2-20 µg/mL and this level increases during the acute phase response by up to 200 µg/mL (Schumann et al, 1994 a). Biochemical and biophysical studies revealed that LBP binds to the lipid A portion of LPS with high affinity (10^{-9} M) (Mathison et al. 1992) and then delivers LPS to both mCD14 and sCD14, thereby catalyzing the formation of CD14-LPS complexes (Hailman et al. 1994, Yu & Wright 1996). Constitutive LBP levels enhance LPS effects and initiate cellular responses at subthreshhold LPS levels, potentially enabling the host to detect invading Gram-negative bacteria and LPS early. However, recently it has been shown that different LBP concentrations may have distinct modulating activities on LPS dependent effects. While low concentration of LBP enhances LPS activity, the acute-phase rise in LBP concentration inhibits LPS-induced cellular stimulation (Lamping et al. 1998).

Thus, LBP enables monocytes/macrophages to respond to extremely low (sub-nanogram) concentrations of LPS via the CD14 dependent pathway. In addition to

its ability to transfer LPS to CD14, LBP is associated with plasma lipoproteins that may play an important role in the neutralization of LPS in vivo. (Wurfel et al. 1994)

1.2.3 Molecular characteristic and functional properties of CD14

1.2.3.1 Membrane-bound form of CD14

Cluster of differentiation antigen 14 was originally described during the First Leukocyte Typing Workshop in 1982 (Bernard et al. 1984) and was identified as a myeloid differentiation antigen, present on mature cells but absent on myeloid precursors. Recently, CD14 was defined as a central pattern recognition receptor and accordingly was becoming appreciated as a key molecule for recognition of the Gram-negative bacteria and the initiation of the inflammatory response by the host (Pugin et al.1994, Wright et al. 1990, Wright 1995).

Membrane-bound form of CD14 (mCD14) is not a transmembrane molecule, but is attached to the cell surface by a glycosylphosphatidilinositol (GPI) anchor, that leaves it mobile in the plane of the cell membrane (Haziot et al. 1988). The molecular weight of mCD14 is 55 kDa. The mCD14 molecule is preferentially expressed on the surface of monocytes and some tissue macrophages and less frequently on polymorphonuclear granulocytes. Furthermore, mCD14 expression on tissue macrophages can vary depending upon the source: alveolar macrophages, Kupffer cells and macrophages of the intestinal mucosa express low levels of mCD14, whereas peritoneal, pleural and perivascular brain macrophages show much higher levels of mCD14 expression. The synthesis and expression of mCD14 can also be altered and regulated by a variety of factors including in vitro monocyte to macrophage maturation, various cytokines and LPS (Landmann et al. 1991 a, Landmann et al. 1991 b). The effects exerted by LPS on mCD14 expression appear controversial, varying from no effect to either enhancement or reduction in mCD14 expression. This diversity can probably be explained by the following findings: the magnitude of mCD14 expression upon LPS stimulation shows a quick upregulation (50-100%) in 30-180 min, followed by a decrease (50-70%) after 3-6 h, then again a marked increase (200-300%) after 1-6 days (Landmann et al. 1991 a, Landmann et al. 1996, Marchant et al. 1992).

The role of mCD14 in LPS binding was identified in vitro with the use of anti-CD14 monoclonal antibodies (anti-CD14 mAb) that inhibit the binding of LPS and prevent LPS-induced TNF-α, IL-6 and IL-8 release by monocytes (Wright et al. 1990).

Since this inhibition most pronounced at low LPS concentrations and only partly at higher LPS concentration, it has been suggested that at high concentrations of endotoxin cytokines release via both CD14-dependent and independent pathway. It has been also shown that transgenic mice expressing high levels of human mCD14 are more sensitive to endotoxin than their normal littermates (Ferrero et al. 1993). In contrast, CD14 knockout mice were found to be at least 10-fold more resistant to LPS-induced shock than wild-type controls (Haziot et al. 1996).

Thus, much evidence exists to support mCD14 as the only receptor known to be involved in both LPS binding and cellular activation.

Because mCD14 lacks a traditional transmembrane domain (Ulevitch & Tobias 1994, Viriyakosol & Kirkland 1995), it is incapable to transduce an intracellular signal alone. Thus additional cell membrane proteins are required for the initiation of transmembrane signalling. The principal signalling component of the LPS receptor complex appears to be the TLR-4 protein. Recent studies indicated that the CD14-LPS complex on the cell surface binds directly to TLR-4 and eventually results in the activation of monocytes through nuclear factor-kappa B (NFκB) translocation (Aderem & Ulevitch 2000)

1.2.3.2 Soluble form of CD14

The soluble form of CD14 (sCD14) lacks the GPI anchor and is present in normal plasma at concentrations of 1-6 µg/mL (Bazil et al. 1989). Two forms of sCD14, differing in molecular weight, have been described: an alpha form (sCD14α) with a lower (48-49 kDa) and a beta form (sCD14β) with a higher (50-53 kDa) molecular weight. Some reports have elucidated the mechanisms of the variability in the release of sCD14. Firstly, various stimuli, like interferon-γ (INF-γ) or LPS, induce shedding of the GPI-anchored mCD14, resulting in a sCD14 with a slightly smaller molecular mass (48-49 kDa) than mCD14. This type of shedding is mediated by the activation of a membrane-associated serine protease (Bazil & Strominger 1991, Bufler et al. 1995). The sCD14β form (55-56 kDa) can originate from enzymatic cleavage of mCD14 via the cell-associated pospholipase C (PLC) (Bazil & Strominger 1991, Bufler et al. 1995). Some investigators describe LPS-triggered activation of PLC in murine macrophages. sCD14 may be also released directly without first passing through an mCD14 stage, e.g. by generating the low-

molecular mass form of sCD14 in a process involving endocytosis of mCD14 and subsequent releasing of sCD14α form by exocytosis; by direct secretion of the sCD14, which escaped GPI-anchor attachment, that result in molecular weight of 55-56 kDa (Durieux et al. 1994). Moreover, the minor forms of a lower molecular weight can be not completely glycosylated. The biological differences between these two forms of sCD14 are not clear yet. It has been established that patients suffering from paroxysmal nocturnal hemoglobinuria (PNH), whose monocytes do not express mCD14 as result of defect in GPI synthesis, have elevated levels of the beta form of sCD14 (56-kDa), as has been also shown in septic patients. However, purified 48-kDa sCD14 enhances the LPS-induced TNF-α, and IL-6 release by monocytic cells. Some investigators have also demonstrated that CD14 shedding reduces the expression of this receptor on monocytes (decrease of mCD14 on blood monocytes and the concomitant increase of sCD14 was found in patients after cardiopulmonary bypass operation and in the plasma of trauma patients) (Fingerle-Rowson et al. 1998, Carrillo et al. 2001). It has also been shown that treatment with LPS or TNF-α increases sCD14 levels, while treatment with IFN-γ or IL-4 leads to a decrease (Schutt et al. 1992).

sCD14 plays an important role in activation of cells that are normally deficient in mCD14, so-called “CD14-negative cells” (endothelial, epithelial and SMCs). Activation of HUVEC by low concentration of LPS is serum dependent and immunodepletion of sCD14 abrogate cell activation. Surprisingly, sCD14 also appears to at least enhance the LPS-mediated activation of CD14 bearing cell such as macrophages and neutrophils (Hailman et al. 1996). However, sCD14 may also inhibit LPS-induced activation of monocytes by competition with the membrane-bound molecule: Schutt et al. found that excess of sCD14 (>70-100μg/mL) blocks LPS binding to monocytes and the oxidative burst response of mononuclear cells (Schutt et al. 1992). Thus, sCD14 seems to play a dual role as either an activator or inhibitor of LPS-mediated activation.

1.2.4 Polymorphism of the CD14 gene

The human CD14-cDNA was cloned in 1988 by Ferrero and Goyert and the gene has been mapped to chromosome 5 into a region (5q23-5q31), which codes also for growth factors like granulocyte/macrophage colony-stimulating factor (GMCSF),

IL-3, IL-4, IL-13, macrophage-colony-stimulating factor (M-CSF), endothelial cell groth factor (ECGF) as well as receptors like M-CSF-r, PDGF-r, β_2-adrenergic receptor (ADR-β_2), glucocorticoid receptor and human *c-fsm* protooncogene (Ferrero & Goyert 1988). The gene encoding CD14 is organized in an unusual simple fashion: it is a single gene spannig 1.5 kb and consists of only 2 exons, starting with an ATG sequence directly followed by an 88-bp intron. The first exon only codes for the first amino acid methionine. Recently, the Sp1 transcription factor binding site was identified as critical for CD14 expression (Zhang et al. 1994).

In several publications, a polymorphism in the upstream, untranslated region of the CD14 gene has been identified in patients AMI, alcoholic liver disease (ALD), bronchial hyperresponsiveness, and atopic disease (Hubacek et al. 1999, Unkelbach et al. 1999, Shimada et al. 2000, Jarvelainen et al. 2001, Koppelman et al. 2001, Baldini et al. 1999). This polymorphism (i.e. the existence of two or more alleles at significant frequencies in the population) consists in the single base exchange (C→T) at position -260 (corresponding to position -159 in the studies of Unkelbach et al., Baldini et al., Jarvelainen et al. and Koppelman et al.). Moreover, this polymorphism is located near the Sp 1 transcription factor binding site and it was also suggested that the C→T change in the promoter region affects the level of CD14 gene expression. More recently, *in vitro* studies have also shown that within an 80-kb human genomic fragment (including the CD14 coding region as well), a 0.7-kb enhancer located ~6-kb 5' of the CD14 transcription initiation site, appers to be essential for CD14 expression in hepatocytes, while additional upstream elements are required to direct transcription in monocytes (Pan et al. 2000).

1.3 Aim of the study

We conducted a case-control study with a large sample of apparently healthy subjects and a well defined group of patients to investigate the possible association between an "infectious state" measured by plasma levels of the soluble lipopolysaccharide receptor CD14 and the presence of angiographically documented, clinically stable coronary artery disease. A further aim of this study was to determine whether the C(-260)→T polymorphism of the promoter of CD14

gene is associated with a risk of CAD, after carefully controlling for other potentially confounding factors in multivariable analysis. In addition, we tested the hypotheses as to whether or not CD14 genotype influences sCD14 plasma levels, and whether or not seropositivity to *Chlamydia pneumoniae (C.pneumoniae),* or *Helicobacter pylori (H.pylori),* or both, is associated with increased sCD14 levels. Finally, a variety of sensitive systemic markers of inflammation and hemostasis as well as lipid parameters, all reflecting established predictors of coronary risk, were also measured to estimate their relationship with CD14 polymorphism and plasma levels of sCD14.

2 Material and methods

2.1 Study design and study population

For the present study a case-control design had been chosen. Case-control studies, often called “retrospective” studies, provide a research method to investigate factors that may prevent or cause disease. In the case-control study, a group of individuals with a particular disease (termed as “cases”) are identified for comparison with an unaffected group (“controls”) that consists of persons who are free of the disease under study. The aim of this comparison is to determine risk factors that may differ in the two groups and may be relevant to the development of the disease.

This project was carried out in the Department of Cardiology and the Department of Epidemiology at the University of Ulm Medical Centre in collaboration with the Red Cross blood bank serving the University hospitals.

All participants were enrolled between October 1996 and November 1997. The case group consisted of 312 patients aged 40-68 years with clinically stable CAD who underwent elective coronary angiography in the Department of Cardiology at the University of Ulm Medical Centre during this period and who had ≥1 coronary stenosis of ≥50% of luminal diameter of at least one major coronary artery. All patients, with a stenosis of ≥50% at catheterization during the last 6 months before October 1996, were also asked to participate. In order to reduce the likelihood of survival bias (“selective survival” effect), patients with diagnosis of CAD, which had been established for the first time only within the previous two years, were included in the study.

Patients with acute coronary syndromes (unstable angina pectoris or AMI) and patients on anticoagulant therapy (i.e., full-dose heparin (at least 20000 U per day) or coumarin) within the past four weeks were excluded from the study.

The control group was sampled during the same period of time and consisted of 476 voluntary blood donors from the local blood donor centre at the University of Ulm. Subjects, who had neither suspicion on nor manifestation of CAD as well as no history of angina pectoris according to the Rose angina questionnaire (Rose et al. 1977) were eligible for participation. Exclusion criteria for the control group were: insulin dependent diabetes mellitus (type I), chronic heart failure and arrhythmias. Individuals, who had elevated blood pressure (≥ 180 mmHg systolic

and ≥ 100 mmHg diastolic) or who had been treated with oral hypoglycemic agents, were accepted as blood donors only after careful examination of each individual case, in accordance with the Red Cross blood bank requirements.

None of the study participants had any of the following disorders, associated with an acute phase reaction: febrile acute infection or acute state of a chronic infection or an inflammatory disease, underlying hematologic or malignant diseases, severe liver and renal disorders, surgery within the previous four weeks, or tooth extraction during the last seven days.

Cases and controls were “frequency matched” by age and gender, and a sampling ratio of about 1:1.5 was intended to ensure adequate power of the study.

The study was approved by the ethics committee of the University of Ulm. All participants signed informed consent forms upon entry into the study.

2.2 Data collection

2.2.1 Cases

Venous blood was drawn between 8:00 a.m. and 2:30 p.m. (93% between 8:00 and noon) directly before diagnostic coronary angiography. Blood samples were obtained from the antecubital fossa with only short-term venous occlusion and minimal suction, using a large (19-21 gauge) needle. Total volume of obtained blood was about 30 mL and blood was collected into four different tubes. Nine mL of blood were taken into a tube with 0.109 M trisodium citrat as anticoagulant; both 9 mL and 2.7 mL in a potassium ethylenediaminetetraacetic acid (EDTA) tubes and 9 mL into a tube without anticoagulants to obtain serum. The nominal volume ratio of anticoagulant (citrat) to blood was 1:9 and molar concentration of EDTA was in the range of 4.55 ± 0.85 mmol/L. In order to avoid premature activation of the coagulation system and possible changes in sensitive rheological parameters, particular attention was paid to thorough mixing of the blood specimen with the anticoagulant as quickly as possible after blood collection (not more than 2 minutes from the beginning of sampling).

After the proper mixing of blood samples by gentle inverting the tube approximately 10 times, 2.7 mL EDTA samples were processed immediately for total leukocyte count. The remaining 9 mL EDTA- and 9 mL citrat blood for obtaining plasma were centrifuged at 3000 g for 10 min within 15 min after

venipuncture. Plasma was aliquoted with an Eppendorf pipette into polystyrene tubes from 200 µL up to 1500 µL, sealed and pre-stored in the freezer at –18° C.
To avoid so-called “post-coagulation”, which can cause volume errors in dispensing, as well as blockage in the analyzed system, serum samples were stored undisturbed for 30-45 min at room temperature after drowning. Clotted blood (serum-tube) was centrifuged at 3000 g for 10 min and immediately divided into aliquots under the same conditions as described above.
Finally, all 24 obtained blood samples from each patient were labelled with a sub-number system, which is linked to the identification number of patient and code letter. Serum and plasma specimen were deep frozen and stored at –70° C until analysis. No specimen inadvertently thawed during storage.
In case that CAD in a patient was confirmed by coronary angiography, further data were collected from the patient’s medical records and by a standardized interview. If CAD had been excluded, no further data were collected.

2.2.2 Controls

Occasional blood donors, who came to the local blood donor centre at the University of Ulm, and appeared eligible for the study, were invited to participate. Participation was voluntary. All individuals, who agreed to take part, gave written informed consent in a form acceptable to the local ethics committee after a full explanation of the study and guarantee of total privacy.
Controls were matched to cases on the basis of age and gender, and were evaluated by a standardized questionnaire.
Venous blood for the study was obtained on the day of the interview after 500 ml blood donation. Sampling and proceeding of the blood were done in the same way, as described above for cases.
Recruitment of both groups (cases and controls) was carried out at the same period of time.

2.3 Standardized questionnaire

All study participants underwent a standardized interview carried out by a specially trained team of interviewers and a brief standardized questionnaire on personal and lifestyle factors was filled out in order to control for potential confounders in

multivariable analysis subsequently. Cases and controls were of German nationality to avoid misunderstanding during the interview.

Participants answered on sociodemographic questions, questions on personal medical history (including questions related to history of CAD and particular symptoms of angina pectoris according to the Rose questionnaire, physician diagnosed hypertension, diabetes mellitus, gastroduodenal and pulmonary diseases), previous antibiotic therapy and current medication.

Interviewers obtained information on participant's occupation and education as well. Education was determined on the basis of school years completed (more or less than 10 years).

Data on food and lifestyle habits (in relation to smoking and alcohol consumption, diet, including weight reduction) and physical exercise were also collected.

Smoking status subjects were classified as current-, non- or former-smokers and cigarette consumption was given as pack years (1 pack year: e.g. 20 cigarettes per day for one year). With regard to alcohol intake, each participant was asked about the average number of units of beer, wine and spirits they had consumed per week during the previous 12 months. Leisure-time physical activity was assessed by a four-level graded scale during summer and winter time (0, <1, to 2 and >2 hours/week) as well as work activity (none, light, medium and heavy).

BMI was calculated as body weight in kilograms divided by height in meters squared.

2.4 Coronary angiography

Coronary angiography was carried out among cases according to the Judkins technique (Judkins 1967). Three different scores were used to evaluate the angiographic severity and extension of CAD: first, all patients were classified according to the number of stenosis (>50% of luminal diameter) or occluded vessels (1- to 3-vessel disease); second, the quantitative extension score (1 to 15 segments) according to the guidelines of the American Heart Association; and third, the qualitative and quantitative evaluation by the Gensini score (Gensini 1975). All angiographic images were reviewed by an experienced observer who was unaware of the clinical and laboratory data.

The intra-class correlation coefficient for intra-rater-reliability was 1.0 (1-3 vessel disease score), 0.79 (tertiles of the extension score), and 0.85 (tertiles of the Gensini score).

2.5 Review of patient medical records

Further information about CAD patients was obtained by reviewing the hospital medical records of each case. An important point of interest was the first manifestation of CAD (stable angina pectoris, angina at rest, myocardial infarction, dyspnoe or arrhythmias). Furthermore, the location of the infarct zone as well as the month and year of the first diagnosis were also registered in MI patients. In addition, information about medical history of prior diseases, medications taken at study entry (generic or brand name and total daily dose), findings from coronary angiography (one-, two- and three-vessel disease), and ejection fractions were also recorded (see appendix).

2.6 Infectious state

2.6.1 sCD14 ELISA

Plasma levels of sCD14 were determined with a commercial sCD14-ELISA kit supplied by IBL (Hamburg). The sCD14-ELISA is a sandwich enzyme immunoassay (enzyme-linked immunosorbent assay) for the quantitative determination of sCD14 in human plasma (standard range, 0.55-9 µg/mL). This system measured only free sCD14 and not sCD14 bound in any complex with LPS. The two forms described for sCD14 were equally recognized. Samples had to be diluted with diluent buffer depending on their sCD14 content. For plasma samples, a dilution of 1:100 was recommended. Standards, controls and samples were incubated in microtitre wells previously precoated with an anti-sCD14 oligoclonal antibody. Any sCD14 present in the sample was bound to the wells. After that, any excess material was removed by washing and aspiration. The assay employed also second monoclonal antibody, coupled to biotin, which revealed sCD14, bound to microtitre wells. Enzyme conjugate, consisting of streptavidin conjugated with horseradish peroxidase, detected the binding of second anti-sCD14 monoclonal antibodies to the well. “Ready to use” TMB (tetramethylbensidin) was used as a substrate to determine the amount of peroxidase, which results in a colour development. The enzyme-substrate reaction

was stopped by quickly pipetting of an acid solution (H_2SO_4) into each well to completely and uniformly inactivate the enzyme. The obtained coloration (or observed optical density, OD) was measured at a wavelength of 450 nm in a microtitre plate spectrophotometer (an ELISA-Reader) and was directly proportional to the concentration of sCD14, which was determined using interpolation from a standard curve.

Assay procedure:

- Pipet 50 µL of standard, control or diluted sample into the microtiter wells
- Add 50 µL anti-sCD14 conjugated with biotin
- Incubate for 2 h at room temperature on a shaker
- After washing add 150 µL of streptavidin conjugated with peroxidase
- Incubate for 1 h at room temperature on a shaker
- After washing add 200 µL substrate solution
- Incubate for 10-20 min at room temperature
- Add 100 µL stopping solution (H2SO4)
- Measure OD at 450 nm with an ELISA-Reader

The inter-assay coefficient of variation was 15.7%.

2.6.2 Polymorphism of the CD14 gene

Genomic DNA was isolated from white blood cells by a standard method (Miller et al. 1988). Polymerase chain reaction (PCR) was performed at a total volume of 20 µL (100 ng of genomic DNA, 10 pmol of each primer, 250 µmol/L of each dNTPs and 1 U *Taq*DNA polymerase) in the provided reaction buffer (Boehringer Mannheim). The promoter of CD14 receptor gene was amplified by the primers 5'-ATCATCCTTTTCCCACACC-3', and 5'-AACTCTTCGGCTGCCTCT-3' under the following conditions: an initial denaturation at 95°C for 3 minutes, followed by 35 cycles at 95°C for 30 seconds, 1 minute of annealing at 60°C, 30 seconds of extension at 72°C, and a final extension time of 5 minutes at 72°C. The PCR product was digested 3 hours at 37°C with 10 U *Hae*III restriction enzyme (New England Biolabs). The DNA fragments were separated by electrophoresis through a 2% NuSieve agarose gel containing 0.5 µg/mL of ethidium bromide and were visualized under UV light. Results were scored blinded as to case-control status.

2.6.3 Specific anti-*H pylori* IgG

H.pylori infection was evaluate by the measurement of *H.pylori*-specific Immunoglobulin G titres with a commercially available ELISA (*H pylori*-IgG-ELISA; Medac, Wedel, Germany) in duplicate. This is a quantitative assay, using plates coated with *H.pylori* antigens. IgG-specific *H.pylori*–antibodies were quantified in units/mL and the recommended cut-off value of 8 U/mL was considered as seropositive.

2.6.4 IgG antibodies against *Chlamydia*

IgG antibodies to *Chlamydia* species (chlamydial lipopolysaccharide) were measured by recombinant antigen-based ELISA (Medac, Wedel, Germany) This ELISA used a cLPS antigen produced by recombinant DNA technology. This antigen contains a common epitope shared by all *Chlamydia* species (Brade et al. 1990). An antibody titre ≥ 100 was defined as seropositive according to manufacturer's instructions.

2.7 Sensitive systemic markers of inflammation and hemostasis and lipids parameters

2.7.1 C-reactive proteine

Measurement of CRP, the classical acute phase protein, was obtained by means of a high-sensitive immunoradiometric assay – IRMA with a range of 0.05-10 mg/L. This assay used monospecific polyclonal and monoclonal antibodies produced by immunization with highly purified CRP. The amount of radioactivity found in the precipitate is directly proportional to the amount of CRP in the test samples which can be quantitated by reference to a 5-point standard curve calibrated with the WHO international reference standard 85/506 (Hutchinson et al. 2000).

All samples were measured in triplicate and values were averaged for analysis. Samples with concentrations of more than 10 mg/L were remeasured at higher dilutions and adjusted for the final results using the dilution factor.

CRP monoclonal and polyclonal antibodies were kindly provided by Professor Mark B.Pepys (FRS), Royal Free and University College Medical School, London, United Kingdom. Inter-assay coefficient of variation for CRP over all ranges was 12%.

2.7.2 Fibrinogen

Fibrinogen is both an acute phase reactant and the precursor of fibrin to the thrombus. It was assayed by immunonephelometry in EDTA plasma using a fibrinogen antiserum kit (Dade Behring Co, Marburg, Germany) on a Behring Nephelometer II analyser (BNA II), according to the manufacturer‘s instructions. In this assay, fibrinogen forms an immune complex with specific antibodies and these complexes scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the fibrinogen concentration.

Fibrinogen was also measured according to the semiautomatic Clauss method (Clauss 1957), where various dilutions of standard plasma with known fibrinogen concentration and test citrate plasma were clotted by the addition of relatively large amounts of thrombin (0.1-0.5 g/L). The relationship between fibrinogen level and clotting time was linear over a certain range of concentrations. The normal analytic range for fibrinogen is 1.8-3.5 g/L and the inter-assay coefficient of variation was 5%.

2.7.3 Leucocytes

Leucocytes and neutrophils from 2,7mL EDTA blood were measured automatically by a Coulter STKS hematology analyser (Coulter, Krefeld, Germany) within 30 minutes after blood collection. The recognition and calculation of various white blood cells by using this standard impedance counting technique is based on different physical properties of these cells.

2.7.4 Plasma viscosity

Measurement of plasma viscosity (millipascal-seconds, mPa•s), a test of the resistance of plasma to flow deformation, was done in a 37°C water bath in a Coulter Harkness capillary viscometer (Coulter Electronics Co, Luton, United Kingdom) at high shear rate (>300s^{-1}) (Harkness 1963). Each EDTA blood sample was measured in duplicate. For quality control, daily comparisons with a water control were made. At irregular intervals duplicates were remeasured in a single-blinded fashion. The inter-assay coefficient of variation was 2%.

2.7.5 Serum amyloid A

SAA levels were also analysed by immunonephelometry using Berhring equipment and reagents (Dade Behring Co, Marburg, Germany). This assay used lyophilized polystyrene particles, coated with sheep antibodies to human SAA, which agglutinated after mixing with SAA-containing samples. The intensity of the scattered light in the nephelometer depended on the concentration of the analyte in the sample and consecutively its concentration was determined by comparison with dilutions of a standard of known concentration. The results of measurement were calculated automatically using a logit-log function. Inter-assay coefficient of variation was 7.4%

2.7.6 HDL-Cholesterol

Routine enzymatic method (CHOD-PAP) was used for HDL cholesterol measurement. HDL cholesterol was measured in the supernate after precipitation of apo B-containing lipoproteins with phosphtungstate acid and $MgCl_2$ obtained from Roche Diagnostics (Mannheim, Germany).

2.7.7 Lipoprotein (a)

Lp(a) was determined with antisera from Greiner Biochemicals (Flacht, Germany) by immunoturbidimetry on a WAKO R-30 automated analyzer in a laboratory setting certified according to ISO 9001.

2.7.8 Plasminogen activator inhibitor-1

PAI-1, the major inhibitor of fibrinolysis, was determined by a commercially available ELISA (Immuno, Heidelberg, Germany), which is based on a sandwich enzyme immunoassay technique with anti-t-PA specific capture antibodies, adsorbed on microplate and second, detection anti-PAI-1-POD monoclonal antibodies, labelled with peroxidase. Inter-assay coefficient of variation for PAI -1 was 11%. Standard range: 1.5-32.5 U/mL.

2.7.9 Von Willebrand factor

This adhesive protein, which mediates the adhesion and spreading of platelets to the subendothelium, was assayed using vWF-ELISA kit supplied by Haemochrom,

Essen, Germany, according to manufacturer's instruction.
vWF was measured by capturing on to a microtitre plate coated with an affinity adsorbed polyclonal antibody to vWF. After washing, the captured vWF was detected by goat anti-vWF IgG. Inter-assay coefficient of variation was 15.8%. Normal analytic range is 50-100% activity.

2.7.10 D-Dimers

For determinbation of D-Dimer levels, the Dimertest Gold EIA (commercially available ELISA) was used (American Diagnostica Inc, Greenwich, USA). Fibrin degradation products (FDP) - D-Dimers were recognized by the Dimertest monoclonal antibodies DD-3B6, which were covalently bound to microtiter wells. These antibodies recognize aspecific epitope on the cross-linked γ-polypeptide chains in the D domain of fibrine. Second polyclonal *Tag* antibody (DD-1D2), conjugated to horseradish peroxidase, bound to immobilized FDP fragments. The test procedure was carried out according to the manufacturer‘s instruction with a standard range 32-2000 ng/mL. Inter-assay coefficient of variation was 7.2%.

2.7.11 Soluble Inter Cellular Adhesion Molecule-1

sICAM-1 was determined by quantative sandwich enzyme immunoassay technique by using biotinylated monoclonal antibody specific for ICAM-1 and microtitre plate method. sICAM-1 ELISA kit was provided by Diaclone, Besancon, France. The inter-assay coefficient of variation was 14.2% and analytic range is 219-1042 ng/mL.

2.7.12 Tumor necrosis factor α

TNF-α was measured by high sensitive human TNF-α ELISA kit provided by R&D Systems, Wiesbaden, Germany according to manufacturer's instruction. This assay was carried out using two different antibodies: a murine monoclonal antibody precoated onto microplate and conjugated polyclonal antibody against TNF-α. The inter-assay coefficient of variation for this measurement was 17.9. Normal analytic range: 0.5-32 pg/mL.

2.7.13 Interleukin 6

IL-6 was determined by immonochemical ELISA method on plates of microtitre by using reagents from HS-Quantikine, R&D Systems, Wiesbaden, Germany. In this ELISA an alkolinephosphatase-conjugated polyclonal antibody against IL-6 was used for colour development. Normal analytic range is 0.156-10 pg/mL. Inter-assay coefficient of variation for IL-6 was 7%.

All laboratory analyses were done in a blinded fashion.

In order to ensure reliability and precision, all evaluation procedures were standardized.

2.8 Data management and statistical analysis:

All data were manually reviewed for completeness and plausibility. Data from the patient medical records and completed questionnaires were recorded in standardized form using “Epi-info” software. For quality control purposes, a double entry was performed by two independent persons. At audit yielded data errors were subsequently corrected. All data were stored electronically using a unique identification number for each study participant.

Baseline characteristics of cases and controls were compared in a descriptive way. Differences between the expected and observed allele frequencies and possible genotype distortion from Hardy-Weinberg equilibrium were assessed among cases and controls by Chi-square (χ^2) analysis. Furthermore, age and gender adjusted mean values for sCD14 in patients and controls, and according to CD14 genotypes were computed using a linear regression model in order to detect statistically significant relationships between a number of independent variables and of dependent ones. The same method was used to assess the relationship of age and gender adjusted plasma levels of sCD14 according to serostatus of *C.pneumoniae* and serostatus of *H.pylori*, and the combination of both, in patients and controls. The association of plasma levels of sCD14 or CD14 genotype with the presence of CAD was compared by Chi-square (χ^2) test. sCD14 levels were measured and divided in quintiles defined by the distribution of the control values and unconditional logistic regression analysis was carried out to estimate the independent association of sCD14 distribution (quintiles) and CD14 genotypes with CAD, while simultaneously controlling for BMI, duration of school education,

cigarette smoking (pack-years), alcohol consumption, history of hypertension and history of diabetes mellitus and HDL-cholesterol values (Hosmer & Lemeshow 1989).

Blood, plasma, and serum parameters are reported as means (arithmetic, or if skewed geometric). To assess the association of sCD14 (upper quintile) and the CD14 genotype with markers of inflammation and hemostasis and lipid parameters age and gender-adjusted mean values were calculated for these markers for patients and controls separately. All *p* values were two-tailed and values lower than 0.05 were considered statistically significant. Odds ratios (OR) were calculated and expressed with their 95% CI.

Data processing and all statistical analyses were performed using the Statistical Analysis System (SAS) software package (version 6.12 for Windows, SAS institute) (SAS Institute 1996).

3 Results

3.1 Study population

Overall, 312 patients and 476 age and gender-matched controls were enrolled in the study. The response rate among cases was 78% and among controls it was 84%.

Table 1 shows baseline characteristics of the study participants. Cases were slightly older than controls (57.7 and 55.8 years, respectively). A total of 267 out of 312 cases (85.6%) were male. Also, cases had lower school education (<10 years of education: 69.2 % versus 58.5 %, respectively), whereas there was little difference between both groups in family status (85.9% in cases vs 83.8% in controls were married) and daily alcohol consumption habits (29.5% vs 28.8%, respectively).

With regard to smoking status, patients on average had smoked more cigarettes (mean number of smoked park-years in cases was 20.3 vs. 10.9 pack-years in controls), but current smoking was more common in the control group than in the cases (14.3 % vs 9.6%, respectively). However, the number of former smokers was somewhat higher among patients, compared to controls (66.3% vs 42%, respectively). This difference is, probably, due to fact that most patients have stopped smoking completely after the first manifestation of CAD.

As expected, cases more frequently reported to have a physician diagnosed history of hypertension (57.7%) whereas only 20.4% of controls did so. A total of 13.5% and 2.7% of cases and controls, respectively, reported also a history of diabetes mellitus, and 67.3% of cases had a history of hyperlipidemia versus 20.9% of controls. Cases showed a higher BMI than controls (27.3 vs 26.3 kg/m², respectively).

Myocardial infarction had occurred in approximately two thirds of the patients (62%) within the previous 2 years. Based on results of coronary angiography, 48% of patients had single-vessel disease, 34% had double-vessel disease, and 18% had triple-vessel disease (data not shown).

Mean HDL cholesterol was considerably lower in CAD patients (42.4 mg/dL) compared with healthy subjects (51.6 mg/dL).

When compared with men, women on average had a lower school education, and consumed less amounts of alcohol and cigarettes. There were no significant sex differences in BMI.

Table 1: Characteristics of patients with coronary artery disease and controls

	Men		Women		All	
	CAD patients	Controls	CAD patients	Controls	CAD patients	Controls
n	267	359	45	117	312	476
Age (y,SD)	57.6 (7.5)	55.8 (7.3)	58.6 (6.7)	55.9 (7.1)	57.7 (7.4)	55.8 (7.2)
Family status Married [%]	87.3	86.4	77.8	76.1	5.9	83.8
School education < 10 yrs [%]	67.0	58.6	82.2	58.8	69.2	59.5
Daily alcohol consumption [%]	33.7	34.7	4.4	0.3	29.5	28.8
Smoking status [%]						
current	9.4	14.2	11.1	14.5	6	14.3
ex-smoker	70.4	47.1	42.2	26.5	66.3	42.0
never-smoker	20.2	38.7	46.7	59.0	24.0	43.7
Smoked pack-years (µ)	21.8	12.6	11.4	5.5	20.3	10.9
Body mass index (kg/m^2), µ (SD)	27.3 (3.5)	26.7 (3.1)	27.5 (4.1)	25.2 (3.3)	27.3 (3.6)	26.3 (3.2)
History of high blood pressure [%]	56.2	21.2	66.7	18.0	57.7	20.4
History of diabetes mellitus [%]	12.0	3.3	22.2	0.9	13.5	2.7
History of hyperlipidemia [%]	65.9	22.3	75.6	17.1	67.3	20.9
HDL-Cholesterol (mg/dL), µ (SD)	41.9 (9.6)	48.5 (11.7)	50.4 (10.7)	61.4 (12.9)	42.4 (10.3)	51.6 (13.2)

3.2 Association between the C(-260) ®T polymorphism, sCD14 plasma levels, and presence of CAD

The distribution of genotypes and the allele frequencies of the C(-260)→T polymorphism in the promoter of the CD14 gene among both groups are shown in **Table 2**. Allele and genotype distribution in cases and controls were consistent with that predicted by the Hardy-Weinberg equilibrium (p=0.81 and p=0.94, respectively). Among the CAD patients, 24% were CC; 52.6% were CT; and 23.4% were TT. This genotype distribution was not significantly different from the distribution in the control group (CC were 26.5%; CT were 51.1%; TT were 22.5%), (p= 0.74, adjusted for age and gender).

Moreover, no difference with respect to the frequencies of the C and T alleles was found among cases and controls. The prevalence for the C allele was 50.3% versus 52%, respectively, and for the T it allele was 49.7% versus 48%.

Table 2: Genotype and allele distribution for CD14 polymorphism in patients and controls

	CAD patients		Controls		p-value
CD 14 genotypes	*N*	%	*N*	%	
CC	75	(24.0)	126	(26.5)	
CT	164	(52.6)	243	(51.1)	0.74
TT	73	(23.4)	107	(22.5)	
All	312	(100)	476	(100)	
Allele	*N*	%	*N*	%	
C	157	(50.3)	248	(52.0)	
T	155	(49.7)	228	(48.0)	

Mean values and distribution of sCD14 plasma levels among cases and controls are shown in **Figure1**. SCD14 plasma levels in cases were found to be 4.2 ± 1.3 μg/mL (median (Q1-Q3), 4.07 μg/mL (3.36-4.81)) and were not different from those of healthy individuals (4.3 ± 1.3 μg/mL; median 4.06 μg/mL (3.38-4.84) (p= 0.51, after adjustment for age and gender) without any significant differences between males and females.

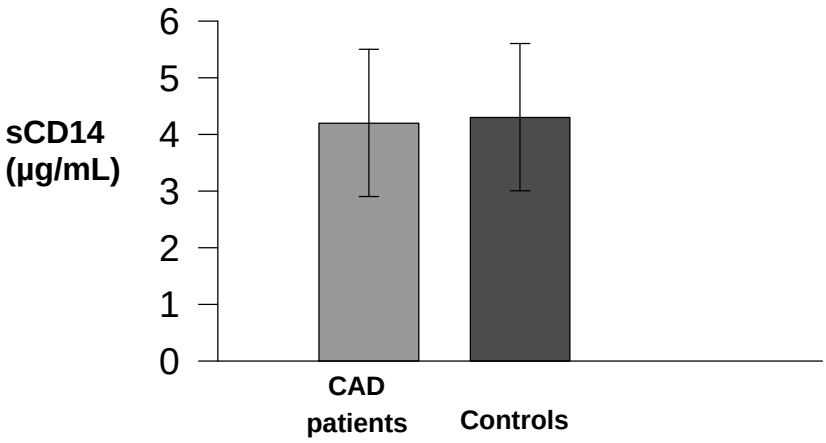

Figure 1 Plasma levels of sCD14 (μg/mL) in CAD patients and controls
sCD14 plasma levels were found to be 4.2 ± 1.3 μg/mL in CAD patients and to be 4.3 ± 1.3 μg/mL in control group (p=0.51)

In this study, no association was found between sCD14 plasma levels, C(-260)→T polymorphism of the CD14 gene and gender, BMI, cigarette smoking and alcohol consumption as well as the prevalence of hypertension, diabetes mellitus and hyperlipidemia (data not shown).

3.3 Association between sCD14 plasma levels and C(-260)®T polymorphism of the CD14 gene

When age and gender-adjusted mean sCD14 plasma levels were analyzed with respect to CD14 genotypes, as demonstrated in **Table 3**, homozygous carriers of the T-allele had significantly increased plasma levels of sCD14. In TT-genotype bearing cases, sCD14 plasma levels were found to be 4.48 µg/mL compared to 4.08 µg/mL in those homozygous for the CC genotype, and 4.02 µg/mL in heterozygous (p=0.03).

Although, sCD14 concentration among controls tended to be slightly higher in the TT genotype-bearing subjects as well (4.55 µg/mL, versus 4.30 µg/mL and 4.21 µg/mL for CT and CC subjects, respectively), this difference did not reach statistical significance (p=0.11, after adjustment for age and gender).

When sCD14 plasma levels were compared according to the genotype distribution among all study participants, TT-genotype bearing subjects had significantly higher sCD14 plasma levels (concentration of sCD14 was 4.56 µg/mL for TT-genotypes, 4.21 µg/mL and 4.19 µg/mL for CT and CC genotypes, respectively) (p=0.005).

Table 3: Soluble CD14 plasma levels (µg/mL) and CD14 C(-260)→T polymorphism

	CD14 Genotypes			
	CC	CT	TT	p-value
Patients n=312				
sCD 14 (µg/mL)				
[1]Mean	4.08	4.02	4.48	0.03
Median (Q1-Q3)	3.81 (3.30-4.64)	4.02 (3.35-4.58)	4.30 (3.73-5.21)	
Controls n=476				
sCD 14 (µg/mL)				
[1]Mean	4.21	4.30	4.55	0.11
Median (Q1-Q3)	3.96(3.35-4.67)	4.03 (3.34-4.83)	4.16 (3.56-5.07)	
Total n=788				
sCD 14 (µg/mL)				
[1]Mean	4.19	4.21	4.56	0.005
Median (Q1-Q3)	3.87 (3.32-4.64)	4.03 (3.34-4.75)	4.20 (3.62-5.10)	

[1] after adjustment for age and gender

Table 4 presents the results of multivariable logistic regression analyses in which the association between sCD14 plasma level or the C(-260)→T polymorphism of CD14 gene and CAD was analysed. Furthermore, the association between combination of the genotypes with high (5th quintile) and low (1-4th quintile) sCD14 plasma levels and CAD adjusted for age and gender, and for age, gender and other potential confounders (body mass index, school education, cigarette smoking, alcohol consumption, history of hypertension, history of diabetes) in the fully adjusted model was studied. Additional adjustment for HDL cholesterol was also performed.

The OR for CAD associated with being in the upper quintile of the sCD14 distribution compared to the 1-4th was 0.97 (95% CI, 0.67-1.39) after adjustment for age and gender and increased to 1.12 (95% CI, 0.74-1.68) after adjustment for other covariates. It increased further slightly after additional adjustment for HDL (OR 1.21; 95% CI, 0.79-1.87). Similar results were seen with bearing the TT genotype. The OR for CAD increased from 1.17 (95% CI, 0.77-1.78, after adjustment for age and gender) to 1.34 (95% CI, 0.84-2.16) in the fully adjusted model. However, additional adjustment for HDL did not change the estimate appreciably (OR 1.32; 95% CI, 0.81-2.16).

The combination of both, being in the top quintile of the sCD14 distribution and bearing the TT genotype resulted in an OR of 1.12 (95% CI, 0.59-2.11) compared to a sCD14 value in the 1.-4.th quintile and the CC or CT genotype, and was 1.31 (95% CI, 0.65-2.66) after adjustment for the covariates.

Table 4: Crude and adjusted odds ratios for CAD associated with soluble CD14 receptor plasma levels and CD14 C(-260) →T polymorphism

Factor	[1]Partly-adjusted OR (95% CI)	[2]Multivariable-adjusted OR (95% CI)
sCD 14 (quintiles)		
1- 4th	1 reference	1 reference
5th	0.97 (0.67-1.39)	1.12 (0.74-1.68)
CD14 genotypes		
CC	1 reference	1 reference
CT	1.16 (0.82-1.65)	1.27 (0.86-1.89)
TT	1.17 (0.77-1.78)	1.34 (0.84-2.16)
sCD14 (quintiles) and CD14 genotypes		
1 - 4th and CC or CT	1 reference	1 reference
1 - 4th and TT	1.02 (0.69-1.52)	1.11 (0.71 – 1.74)
5th and CC or CT	0.92 (0.60 – 1.41)	1.08 (0.67 – 1.75)
5th and TT	1.12 (0.59 –2.11)	1.31 (0.65 –2.66)

[1] adjusted for age and gender

[2] adjusted for age, gender, body mass index, school education, cigarette smoking, alcohol consumption, history of hypertension, history of diabetes.

3.4 Association between sCD14 plasma levels and serostatus of *Chlamydia pneumoniae* and serostatus of *Helicobacter pylori*

In **Table 5** mean values of sCD14 in both groups of study participants were evaluated according to serostatus of *C.pneumoniae* and of *H.pylori*. There was no significant difference regarding plasma levels of sCD14 among either chlamydial LPS- or *H.pylori*- seropositive or seronegateve cases and controls. No association was found when compared with seropositivity to both microorganisms as well.

Also, the combination of seropositivity to *C.pneumoniae* and TT genotype was not associated with higher sCD14 plasma levels than with TT genotype alone (4.57 µg/mL) (data not shown).

Table 5: Age and gender adjusted plasma levels of sCD14 (µg/mL) according to serostatus of *Chlamydia pneumoniae* and serostatus of *Helicobacter pylori*

		Mean sCD 14 (µg/mL)				
		CAD patients			Controls	
Chlamydial LPS (IgG)						
- positive	60.9%	4.05		62.4%	4.40	
- negative		4.24	p = 0.19		4.24	p = 0.17
Helicobacter pylori (IgG)						
- positive	44.2%	4.13		31.3%	4.29	
- negative		4.13	p = 0.99		4.34	p = 0.67
Chlamydial LPS and *Helicobacter pylori*						
- both positive	23.4%	4.14		19.0%	4.31	
- one positive	51.6%	4.05		49.5%	4.41	
- both negative	25.0%	4.34	p = 0.26	31.5%	4.22	p = 0.43

[1]after adjustment for age and gender

3.5 Association between sCD14 plasma levels and C(-260)®T polymorphism of the CD14 gene and various markers of inflammation and hemostasis

3.5.1 Association between sCD14 plasma levels and various markers of inflammation and hemostasis

Table 6 shows the age and gender adjusted mean concentrations of different markers of inflammation and hemostasis in relation to plasma sCD14 levels in the lower four quintile compared to the 5th quintile (>5.18 µg/mL) among cases and controls. Although fibrinogen, D-Dimer, plasma viscosity, leukocyte count, PAI-1, Lp(a), SAA, vWF, IL-6, TNF-α, and ICAM-1 were in general slightly higher in the top quintile of the sCD14 distribution than in the combined four lower quintiles, no clear differences in concentration of these sensitive systemic markers of inflammation and haemostasis and sCD14 distribution was found.

The only exception was CRP which was slightly elevated in subjects being in the top quintile compared to the combined 1-4th quintile in patients (2.15 mg/L versus 1.47 mg/L, respectively) and in controls (1.45 mg/L vs 1.08 mg/L, respectively) (p=0.03, after adjustment for age and gender). PAI-1 activity was slightly higher in controls with plasma levels of sCD14 > 5.18 µg/mL compared to those with lower levels; however, this was with the other group, as well.

By contrast, CAD patients in general showed consistently elevated levels of inflammatory markers than did age and gender matched controls.

Table 6: Mean concentrations[1,2] of various markers of inflammation and hemostasis in patients and controls in the top quintile of soluble CD14 receptor distribution compared to the combined lower four quintiles

		sCD14 1-4th quintile	sCD14 5th quintile	p-value
Patients n=312	-CRP (mg/L) [§]	1.47	2.15	0.03
	-fibrinogen (g/L) Clauss method	2.71	2.85	0.14
	nephelometric	2.77	2.94	0.04
	-plasma viscosity (mPa•s)	1.22	1.23	0.74
	-leukocyte count (in 10^3/ µL)	6.96	6.95	0.96
	-PAI-1 activity (U/mL) [§]	11.6	13.67	0.10
	-Lp(a) (mg/dL) [§]	16.7	17.4	0.89
	-SAA (mg/L) [§]	3.41	3.87	0.27
	-vWF activity (%)	139.2	154.1	0.07
	-IL-6 (pg/mL) [§]	2.34	2.92	0.06
	-TNF- α (pg/mL) [§]	2.78	2.83	0.80
	-ICAM-1 (ng/mL)	527.9	559.1	0.19
	-D-Dimer (ng/mL) [§]	6.01	7.69	0.27
Controls n=476	-CRP (mg/L) [§]	1.08	1.45	0.03
	-fibrinogen (g/L) Clauss method	2.58	2.55	0.57
	nephelometric	2.50	2.51	0.90
	-plasma viscosity (mPa•s)	1.19	1.19	0.49
	-leukocyte count (in 10^3/ µL)	5.78	5.69	0.62
	-PAI-1 activity (U/mL) [§]	6.33	7.86	0.04
	-Lp(a) (mg/dL) [§]	8.69	8.50	0.78
	-SAA (mg/L) [§]	2.87	2.906	0.72
	-vWF activity (%)	134.0	134.9	0.87
	-IL-6 (pg/mL) [§]	1.42	1.46	0.72
	-TNF- α (pg/mL) [§]	2.04	2.06	0.89
	-ICAM-1 (ng/mL)	499.3	473.7	0.11
	-D-Dimer (ng/mL) [§]	2.50	3.10	0.14

[1] Adjusted for age and gender by general linear regression

[2] Arithmetic or [§] geometric means

3.5.2 Association between C(-260) ®T polymorphism of the CD14 gene and various markers of inflammation and hemostasis

In **Table 7**, mean values of different markers of systemic inflammation and hemostatic markers were compared according to CD14 genotypes. None of the variables (CRP, Fibrinogen, D-Dimer, plasma viscosity, leukocyte count, PAI-1, Lp(a), SAA, vWF, IL-6, TNF-α, and ICAM-1) showed a statistically significant difference between CC or CT genotypes and TT genotype, neither in cases nor in healthy controls.

Table 7: Mean concentrations[1,2] of various markers of inflammation and hemostasis in patients and controls according to CD14 genotypes

		CC or CT	TT	p-value
Patients	-CRP (mg/L) §	1.64	1.34	0.23
n=312	-fibrinogen (g/L) Clauss method	2.73	2.77	0.68
	nephelometric	2.80	2.78	0.82
	-plasma viscosity (mPa•s)	1.22	1.22	0.63
	-leukocyte count (in 10^3/ µL)	6.99	6.82	0.50
	-PAI-1 activity (U/mL) §	11.73	12.67	0.42
	-Lp(a) (mg/dL) §	15.90	20.76	0.89
	-SAA (mg/L) §	3.57	3.20	0.33
	-vWF activity (%)	143.9	134.4	0.22
	-IL-6 (pg/mL) §	2.47	2.31	0.52
	-TNF- α (pg/mL) §	2.55	2.39	0.29
	-ICAM-1 (ng/mL)	536.8	521.5	0.49
	-D-Dimer (ng/mL) §	6.35	6.22	0.86
Controls	-CRP (mg/L) §	1.21	1.01	0.15
n=476	-fibrinogen (g/L) Clauss method	2.58	2.55	0.97
	nephelometric	2.51	2.45	0.33
	-plasma viscosity (mPa•s)	1.19	1.19	0.59
	-leukocyte count (in 10^3/ µL)	5.82	5.60	0.19
	-PAI-1 activity (U/mL) §	6.71	6.27	0.51
	-Lp(a) (mg/dL) §	8.77	7.90	0.53
	-SAA (mg/L) §	2.87	3.01	0.72
	-vWF activity (%)	133.5	136.7	0.57
	-IL-6 (pg/mL) §	1.41	1.50	0.40
	-TNF- α (pg/mL) §	1.75	1.87	0.27
	-ICAM-1 (ng/mL)	491.6	507.4	0.30
	-D-Dimer (ng/mL) §	2.97	2.78	0.72

[1] Adjusted for age and gender by general linear regression

[2] Arithmetic or § geometric means

3.6 Association between sCD14 plasma levels and C(-260)®T polymorphism of the CD14 gene and severity and extension of CAD

Table 8 shows the prevalence of elevated soluble CD 14 plasma levels (top quintile versus lower 4 quintiles) according to three different angiographic scores, which represent severity and extension of CAD, in 305 out of 312 patients who underwent coronary angiography. For 7 patients the score could not be calculated due to missing angiograms.

There was no difference in the prevalence of elevated sCD14 plasma levels in 5th quintile with respect to 1-, 2- or 3- vessel disease (p=0.21), as well as with respect to the extension score (p=0.97) and Gensini score (p=0.14) (both analysed in tertiles).

Table 8: Prevalence of elevated soluble CD 14 plasma levels (5th quintile) according to various coronary scores*

Score		% in 5th quiantile	p-value for Trend
Clinical Score	1-vessel disease (n=142)	20.8%	
	2-vessel disease (n=106)	22.6%	
	3-vessel disease (n=57)	12.3%	0.21
Extension Score (tertiles)	0-2 segments	18.3%	
	3-4 segments	23.3%	
	5-10 segments	18,1%	0.97
Gensini Score (tertiles)	0-24 points	16.0%	
	25-40 points	20.6%	
	41-135 points	24.3%	0.14

* for 7 patients the score could not be calculated due to missing angiogramms

The same results could be obtained comparing the prevalence of CD14 genotypes by various coronary scores. No association between CD14 TT-genotypes and any of the three angiographic scores applied, was revealed (p=0.39 for clinical score, p=0.76 for extension score and p=0.16 for Gensini score).

Thus, no relation between severity and extension of CAD and sCD14 plasma levels or C(-260)→T polymorphism of the CD14 gene was seen.

Table 9: Prevalence of CD14 genotype (TT) according to various coronary scores*

Score		% with TT	p-value for Trend
Clinical Score	1-vessel disease (n=142)	21.8%	
	2-vessel disease (n=106)	21.7%	
	3-vessel disease (n=57)	29.8%	0.39
Extension Score (tertiles)	0-2 segments	25.0%	
	3-4 segments	21.4%	
	5-10 segments	22.9%	0.76
Gensini Score (tertiles)	0-24 points	25.0%	
	25-40 points	26.5%	
	41-135 points	18.5%	0.16

* for 7 patients the score could not be calculated due to missing angiograms

4 Discussion

In this large case-control study we found no strong evidence for an association between either sCD14 plasma levels, or the CD14 C(-260)→T gene polymorphism with the presence of clinically stable CAD. Moreover, no consistent relationship between both factors and a variety of sensitive, systemic markers of inflammation and hemostasis, or lipid parameters was seen; there was also no relationship between seropositivity to *C.pneumoniae* or *H.pylori* and increased levels of sCD14. Thus, these results do not support recently published data, suggesting an important role for the CD14 C(-260)→T gene polymorphism or sCD14 plasma levels as risk markers for CAD. At least in part, these data also argue against the notion that bacterial LPS may be a crucial determinant of the atherosclerotic process.

4.1 Association between CD14 genotype and CAD

Several retrospective studies in different populations in Europe and in Japan have described a C→T single nucleotide polymorphism in the promoter region of the gene encoding for CD14 as an independent risk factor for AMI or CAD (Hubacek et al. 1999, Unkelbach et al. 1999, Shimada et al. 2000). Our data are in disagreement with results of these studies, as are data from one prospective study by Zee et al, (2001) which employed a nested case-control design within the PHS. In this latter study, no association was found between C(-260)→T polymorphism of the CD14 gene and the risk of future MI neither in the total cohort, nor in any subgroup evaluated.

Several reasons may account for these discrepancies.

Hubacek et al. found a higher prevalence of the T-allele in MI survivors than in controls (p=0.0005) (Hubacek et al. 1999). However, the control group in this study was rather small (n=135) and the frequency of the T-allele was only 32.5%. Moreover, the frequency was considerably lower than the one observed in our study (48.0% among 476 healthy controls) and in three other studies (Unkelbach et al. 1999, Shimada et al. 2000, Zee et al. 2001). In the study of Unkelbach et al. frequency of the T-allele was 46.0% among 1175 individuals who did not have MI and 48.0% in 501 subjects, in which CAD was excluded by coronary angiography. Shimada et al. also found a frequency of 49% for the T-allele in controls without MI

or AP, and a prevalence of 47.2% for the T-allele was detected in 387 controls in the study of Zee et al. However, in the study by Hubacek et al. the frequency for the T-allele found in cases (49.2%) was similar to we observed in our controls and the frequency found in the studies by Unkelbach and Zee among cases too. Thus, these findings suggest, that the positive results reported by the Czech investigators might be due to an underestimation of the true allele frequency among controls, rather than represent a true increased frequency among cases (Ridker et al. 1999). Furthermore, surprisingly, conventional risk factors were not different between cases and controls in this study.

In the study by Unkelbach et al., in the overall cohort of 2228 men, who underwent coronary angiography for diagnostic purposes, no association of the CD14 gene polymorphism with CAD of MI was seen. This is in agreement with our results and findings of the study performed by Zee et al. However, the authors performed a subgroup analysis and compared several groups with varying degrees of CAD according to two different angiographic scores (Gensini score and clinical score), groups with or without MI, as well as groups with or without impairment of left ventricular function. Again in these subgroups no statistically significant differences were found for CD14 genotype distributions. Moreover, the allele distribution was unrelated to a number of well established risk factors for CAD. The only positive association in further post-hoc analyses was reported in small, low-risk subgroups, consisting of 76 individuals (46 without and 30 with MI), who were normotensive, non-smokers, and older than 62 years. In this subgroup, the OR for MI and T homozygosity was 3.8 (95% CI, 1.6 to 9.0; $p<0.01$ by multiple logistic regression). Taking into account that the only positive association was shown in post-hoc analysis in rather small subgroup of older low-risk patients out of a total population of 2228 men, we strongly believe that over interpretation of subgroup analyses has led to a false positive finding.

The third retrospective study which has reported a positive association of C(-260)→T polymorphism of the CD14 gene with MI had been carried out in a Japanese population by Shimada et al. (2000). An obvious limitation of this study again was the small number of subjects (83 controls and 128 patients with angiographically documented CAD). Moreover, the investigators found an increased prevalence of the T-allele and T/T homozygotes only in MI patients (n=81), whereas no such positive association was seen in patients with stable

CAD (n=47) (65% prevalence of the T-allele and 52% of T/T homozygotes in MI patients vs 50% prevalence of the T-allele and 21% of T/T homozygotes in patients with AP, giving p=0.01 and p=0.0009, respectively). There were also no significant differences in the prevalence of the T-allele or T/T homozygotes between patients with AP and healthy controls (p=0.8). Based on these results, the authors concluded that the CD14 C(-260)→T gene polymorphism may be linked to plaque vulnerability rather, than to the initiation and development of atherosclerosis. It is also interesting to recognize that the frequency of the TT-genotype in the MI group was more than twice as high in this ethnic group, compared to other reports in Caucasians (TT-genotype frequency was 52% in the study of Shimada et al., 27.5% in the study of Hubacek et al., 19.1% in the study of Zee et al. and 22% in the study of Unkelbach et al.). These discrepancies could be explained by the fact that the genetic background of Japanese and European populations is different. However, in spite of the ethnic differences, distributions of the T-allele and the TT-genotype in Japanese controls were in agreement with the data from both Unkelbach et al. and Zee et al.

In contrast to these at least partly positive studies, in our large (n=788), carefully controlled case-control study, no strong, independent association between CD14 C(-260)→T gene polymorphism and risk of CAD could be demonstrated in multivariable analysis, although, a weak relationship cannot be ruled out completely.

As mentioned above, our data are in accordance with the only so far published prospective study by Zee et al. who investigated the role of CD14 polymorphism as a risk marker for MI in the large-scale, prospective cohort of apparently healthy middle-age men within PHS. Out of 14916 predominantly white US physicians, who were followed over a 12-year period for incident MI, only 387 subjects who developed an MI, were enrolled as cases. 387 participants who were matched for age and smoking but who remained free of vascular disease during follow-up, served as controls. In this large nested case-control study, the observed allele and genotype frequencies were similar among cases and controls (46.9% vs 47.2%, respectively for the T-allele and 19.1% vs 22.2% for the TT-genotype; p=0.28). Thus, no association was observed between CD14 polymorphism and the relative risk of future MI in any of the prespecified subgroups evaluated.

4.2 Association between sCD14 plasma levels and CAD

It is well established that plasma levels of the soluble CD14 receptor increase in response to stimulation by LPS. However, in our study mean levels of sCD14 were not found to be increased in patients with stable CAD compared to control subjects. There was also no association between plasma levels of sCD14 and seropositivity to either *C.pneumoniae,* or *H.pylori,* or both. As a consequence, the OR for the presence of CAD in the top quintile of the sCD14 distribution was not significantly increased in multivariable analysis, compared to the bottom quintile. Only one study, so far, has reported on the correlation of sCD14 levels with the varying extents of CAD. In that study, carried out by Zalai et al. (2001), sCD14 levels were evaluated in 31 patients with different severity of CAD manifestations (stable angina, unstable angina and acute MI) and in 17 normal volunteers. The authors found no differences in sCD14 plasma levels in patients with stable angina, compared with controls, although patients with stable angina showed a trend towards increased levels of sCD14, which did not reach statistical significance ($p=0.066$). However, 41% higher levels of circulating sCD14 were demonstrated in plasma obtained from patients with unstable angina in comparison to that of normal individuals ($p<0.01$). The most surprising finding from this study was that sCD14 plasma levels were 48% higher in patients with unstable angina compared to patients with acute MI ($p<0.01$). Moreover, in several publications patients with unstable angina have consistently shown to present with increased levels of inflammatory (IL-6, CRP, SAA) (Manten et al. 1998, Liuzzo et al. 1999) and hemostatic (TF) markers (Jude et al. 1994) compared to those of acute MI patients. In the case of TF it seems of particular interest, because the sCD14-dependent pathway plays a key role in LPS-induced TF expression (Steinemann et al. 1994).

The observation of increased levels of circulating sCD14 in patients with unstable angina as shown in the study by Zalai et al., can be partly supported by a series of previous published studies evaluating the role of the membrane-bound form CD14 (mCD14) in acute coronary syndromes (ACS). Expression of CD14 on monocytes was significantly increased in patients with ACS when compared with control subjects and to the patients with stable angina, as demonstrated in the studies of Lee et al. (2001) and of Choi et al. (2000). In another study (Meisel et al. 1998) a 30% elevated density of monocyte-specific CD14 in patients in the acute phase of

MI has been described. It has also been shown that expression of membrane-bound CD14 is increased in non-insulin dependent diabetes mellitus patients with cardiovascular disease (Patino et al. 2000).

One prospective cohort study (Wiedermann et al. 1999) reported on the association between levels of plasma endotoxin, a potent stimulator of sCD14, and incident carotid atherosclerosis and cardiovascular disease (combined endpoint of AMI, stroke, transient ischemic attack (TIA) and peripheral arterial occlusive disease (PAOD); n=38). However, no graded relationship between plasma endotoxin levels and incident atherosclerosis was observed; rather, the association was restricted to the top 10% of the endotoxin distribution, and a strong effect modification by smoking status and clinical or laboratory evidence of chronic bacterial infection was found. Although similar results for incident cardiovascular disease were reported, numbers were too small to carry out multivariable adjustments and interaction analyseis, and thus, such results should be interpreted cautiously.

Thus, based on the results of our study as well as from Zalai et al. we may conclude that there is no strong association between sCD14 plasma levels and the presence of clinically stable CAD.

4.3 Association between CD14 genotype and sCD14 plasma levels

We found slightly higher plasma levels of sCD14 in homozygous carriers of the T-allele, compared to the CT or CC genotype in patients (P=0.03) and a tendency toward elevated values was seen in controls (P=0.11). Although no such data are available from other studies in patients with CAD, similar results have been reported in patients with ischemic cerebrovascular disease, where sCD14 levels tended to be slightly higher in patients with the TT genotype, but this tendency failed to reach statistical significance (Ito et al. 2000). In another study, Baldini et al., evaluated the correlation between the CD14 gene polymorphism and the level of sCD14 (Baldini et al. 1999). Circulating sCD14 levels were assessed in 67 unselected subjects with CC genotype and in 42 unselected subjects with TT genotype among 481 children, who were recruited from the general population in Tucson, AZ, USA. This study also demonstrated that TT homozygotes had significantly higher levels of sCD14 (median value 4.5 µg/ml (range 4.1 to 5.0 µg/mL) than did carriers of the CC genotype (median value 4.1 µg/mL (range, 3.6

to 4.5 µg/mL), (p=0.01). Such small differences (0.4-0.5 µg/mL), however, are unlikely to be clinically relevant.

4.4 Association between CD14 genotype, sCD14 plasma levels, and sensitive systemic markers of inflammation

Assuming that genetic variation of the CD14 receptor with increased CD14 gene expression might play a role in atherogenesis, a mechanistic link might contribute to the inflammatory response seen in this disease (Ross 1993). Such an effect could result in either increased levels of sCD14 receptor in plasma, or be demonstrated by increased levels of sensitive systemic markers of inflammation which have been shown to be independently related to coronary disease in prospective studies. We have measured a large variety of such markers and were unable to detect any meaningful relationship with CD14 genotype. Most of these markers are known to increase after stimulation of various cell types, including macrophages, SMCs or ECs by LPS. Furthermore, with the exception of CRP, no other inflammatory marker was associated with plasma levels of sCD14 (top quintile versus lower four quintiles combined). Taking into account the large number of markers tested, such findings are most likely to occur by chance alone.

4.5 Association between CD14 genotype, sCD14 plasma levels, and severity of CAD

We observed no association between CD14 genotype, sCD14 plasma levels, and the severity or extent of atherosclerosis of the coronary arteries evaluated by three different scores (clinical score, extension score according to AHA, and Gensini score). Only one study so far, has assessed the relationship between distribution of CD14 genotypes and the extent and severity of CAD (Unkelbach et al. 1999). In this study, severity of CAD was evaluated by the clinical score (no-, 1-, 2-, or 3-vessel disease) and by the Gensini score (<10 or >90 points). CD14 gene distribution did not differ significantly in the subgroups with varying extents of CAD. Our findings are in agreement with the results from the study by Unkelbach et al. The negative findings of these two studies might be partly explained by the fact that the severity and extent of coronary atherosclerosis is not necessarily representative of the atherosclerotic burden in the human organism.

4.6 Strengths and limitations of the present study

To investigate the possible association between plasma levels of the s CD14 or the C(-260)→T polymorphism in the promoter region of the CD14 gene, and the presence of CAD, a well defined, homogenous group of patients with exclusively chronic, stable CAD was compared to a large sample of apparently healthy subjects. In order to minimize potential selection bias by selective survival, only cases with a diagnosis of CAD established within the previous two years, were included in the study. Taking into account that an acute, ongoing inflammatory response could distort the results of our measurements, we carefully selected cases and controls, and excluded patients with acute coronary syndromes as well as subjects with disorders that might be associated with an acute phase reaction. Controls were “frequency matched” for age and gender to the cases and a sampling ratio of about 1:1.5 was intended to ensure adequate power of the study. Moreover, controls came from the same geographic region as the patients in order to avoid any genetic distinctions. Recruitment of both groups (cases and controls) was done at the same period of time during one year, to prevent any distortion of results due to seasonal variations of rheological and hemostatic parameters and acute-phase reactants (Froehlich et al. 1997).

In addition, we used a detailed, standardized questionnaire to carefully record conventional risk factors and to investigate their relation to sCD14 plasma levels and the CD14 C(-260)→T genotype. We were, therefore, able to assess their potential for confounding by performing multivariable adjustments. Furthermore, controls were asked about their history of CAD and symptoms of angina pectoris according to the Rose questionnaire, allowing us to exclude those subjects with suspected CAD from the study.

To the best of our knowledge, the present study is the first, in which the C(-260)→T polymorphism of the CD14 gene as well as sCD14 plasma levels were examined in a relatively large cohort of patients with clinically stable CAD and in apparently healthy controls. The potential relationship between these two variables was also established. Moreover, sCD14 plasma levels were evaluated with regard to serostatus of *C.pneumoniae* and *H.pylori.* Also, in contrast to other studies, we measured a large variety of sensitive, systemic markers of inflammation to asses the potential contribution of the genetic variation of the LPS receptor or sCD14

plasma levels to the inflammatory response, which is known to represent an integral part of the atherosclerotic process.

The present study has also potential limitations that should be considered. The temporal relationship between markers measured in plasma and the disease under study cannot be established by using a case-control design. Moreover, a case-control design does not allow assessment of the causal role of infection in the initiation or the progression of the atherosclerotic process. To answer this question, a prospective design must be employed. However, the primary aim of our study was to investigate the potential association between various indicators of an infectious state and a variety of sensitive markers of a systemic inflammatory response in the human body.

Another limitation of our study may result from the use of blood donors as controls. Although controls came from the same geographic region as patients, they probably tend to be healthier than population-based controls. Furthermore, asymptomatic CAD in these subjects cannot be ruled out completely, since no electrocardiogram or coronary angiogram could be obtained in controls. However, the prevalence of CAD in an asymptomatic middle-age population appears to be low (Parmley 1989), and selection of controls among subjects who undergo coronary angiography for various reasons would possibly introduce an even more severe bias.

The effect of medication on sCD14 plasma levels might also represent a limitation of our study. Although no such data are available with regard to sCD14, membrane expression of CD14 has been shown to be reduced by treatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (Rothe et al. 1999, Pfeiffer et al. 2001). Moreover, aspirin, which is frequently prescribed to CAD patients (as in our study too), may modulate cytokine-dependent CD14 gene expression (Bhagat et al. 1997, Fearns & Ulevitch 1998).

Soluble CD14 levels were determined by ELISA quantification. This system measures only free sCD14 and not sCD14 bound in any complex with LPS. If the concentration of endotoxin was high, this might lead to false low levels of free circulating sCD14. However, high LPS blood levels were unlikely in our study participants owing to enrolment requirements. Although it is very doubtful that sCD14 binding to LPS reduced the free sCD14 plasma levels in this setting of low-grade inflammation, we cannot formally exclude this possibility.

Nevertheless, all these limitations cannot explain the absence of any significant association demonstrated after control for confounding factors.

4.7 Conclusions

Our results do not confirm an independent and clinically relevant relationship between the polymorphism of the CD14 gene and risk of clinically stable CAD.

Although the present study did not have the power to detect a weak association between sCD14 plasma levels, or the CD14 C(– 260)→T polymorphism and CAD, it had a power of 80% to detect an OR of 1.63 (associated with being in the upper quintile of sCD14) and of 1.59 (associated with the TT genotype), respectively at an α=0.05.

The lack of an association between sCD14 plasma levels and risk of CAD or markers of inflammation, and between CD14 genotype and the inflammatory response constitutes novel findings which contribute to the ongoing discussion of a potential role of chronic infection in atherosclerosis. Therefore, it seems unlikely that in the population studied, sCD14 plasma levels or CD14 C(-260)→T genotype are promising tools for risk assessment of CAD.

5 Summary

A novel inflammatory concept of the pathogenesis of atherosclerosis is presented that has prompted the investigators to search for causative infectious agents. They may act either through direct proinflammatory effect on the vessel wall, or through a less specific, systemic proinflammatory effects. Cellular response in infections with Gram-negative bacteria is mediated by bacterial endotoxin (lipopolysaccharide, LPS), which activates monocytes via the LPS receptor CD14. Recently chronic infections as measured by increased levels of endotoxin have been demonstrated in subjects at risk of CAD.

The primary aim of the present study was to determine whether increased soluble CD14 (sCD14) levels in plasma and C(-260)→T polymorphism in the promoter of CD14 gene are associated with risk of clinically stable coronary artery disease (CAD). To address this question, a case-control design was chosen. 312 patients with angiographically proven CAD and stable angina pectoris were included in the study. Voluntary blood donors (n=476) matched for age and sex served as controls. The study population was well characterized and a large number of potentially confounding variables were collected, which were controlled for in multivariable analyses. Plasma levels of sCD14 were determined by a commercial sCD14-ELISA (enzyme-linked immunosorbent assay) kit and the CD14 genotype was assessed by polymerase chain reaction (PCR). Moreover, a large number of different markers of systemic inflammation and hemostasis as well as lipid parameters were determined, which allowed us to assess the possible association between an „infectious state“ measured by sCD14 levels and various sensitive markers of inflammation in patients and in controls, in addition to the above mentioned primary aim of the study.

All observed genotype frequencies were in Hardy-Weinberg equilibrium. CD14 C(-260)→T genotype was not independently associated with increased risk of CAD after multivariable adjustments (Odds ratio, (OR) 1.34; 95% confidence interval, (CI), 0.84-2.16). However, sCD14 plasma levels were higher in subjects with TT genotype (p=0.005) compared to those with CT or CC genotype. Plasma levels were not different between cases and controls (4.2 ± 1.3 µg/mL versus 4.3 ± 1.3 µg/mL, NS). In multivariable logistic regression, the OR for the presence of CAD was 1.11 (95% CI, 0.65-1.91) if the top quintile of the sCD14 distribution was compared to the bottom quintile. There was no consistent association between

seropositivity to either *Chlamydia pneumoniae,* or *Helicobacter pylori,* or both, and sCD14 levels, and between sCD14 levels or CD14 genotype and the various markers of inflammation.

Thus, our results do not confirm an independent and clinically relevant relationship between CD14 gene polymorphism and risk of CAD. The lack of an association between sCD14 plasma levels and risk of CAD or markers of inflammation, and between CD14 genotype and the inflammatory response constitutes novel findings which contribute to the ongoing discussion of a potential role of chronic infection in atherosclerosis. Therefore, it seems unlikely that in the population studied, sCD14 plasma levels or CD14 C(-260)→T genotype are promising tools for risk assessment of CAD.

6 Appendix

Einleitung zum Interview

Guten Tag, mein Name ist:.....

Die Abteilung Epidemiologie der Universität Ulm und die Abteilung Innere I und Innere II zusammen mit DRK-Blutspendezentrale führen diese Studie durch, um die Bedeutung des weitverbreiteten Magenbakteriums *Helicobacter pylori* für die Entstehung von Herz-Kreislauf- Krankheiten zu untersuchen. Durch die Teilnahme leisten Sie einen wichtigen Beitrag hierzu, wofür wir uns sehr herzlich bedanken.
Zuerst fuhren wir den ersten Teil des Atemtests durch. Anschließend möchte ich Sie bitten, mit mir gemeinsam diesen Fragebogen durchzugehen. Ich werde Ihnen dabei sowohl Fragen zu Ihrer gesundheitlichen Situation als auch zu Ihren Lebensumständen stellen.

Ich möchte Sie noch einmal darauf hinweisen, dass alle Ihre Angaben von uns streng vertraulich behandelt werden. Ihre Angaben werden in anonymisierter Form und ausschließlich für wissenschaftliche Zwecke ausgewertet. Alle erhobenen Angaben unterliegen den Bestimmungen des Datenschutzes.

Falls Sie beim Fragebogen eine Frage nicht verstehen oder nicht beantworten wollen, bitte ich Sie, mir dies mitzuteilen.

	FRAGE	CODE	ANTWORT	Weiter mit Frage
1	ID-Nr.		__ __ __ __ __ __ __ __ __	
		1 2	Fall Blutspender	
			___	
2	Datum des Interviews		Datum: ___/___/_____	
	Uhrzeit Beginn		___.___ ___.___	
3	Hat ein Interview stattgefunden?	1 2 3 4 5 6 7	ja nein, Einverständnis verweigert nein, Ausländer mit sprachlichen Problemen nein, vorzeitig entlassen nein, schlechter psychischer oder physischer Zustand nein, mittlerweile verstorben nein, sonstige Grund .....……	
			___	
4	Geschlecht *(INT.: nicht fragen)*	1 2	Männlich Weiblich	
			___	

	FRAGE	CODE	ANTWORT	Weiter mit Frage

Angaben zur Person

INT: Im folgenden möchte ich Ihnen allgemeine Fragen zu Ihrer Person stellen.

5	In welchem Jahr sind Sie geboren?	Jahr angeben	__ __ __ __	
6	Wieviel Kilogramm wiegen Sie zur Zeit (ohne Schuhe)?		kg __ __ __	
7	Haben Sie in den letzten drei Jahren zu- oder abgenommen?	1 2 3	Zugenommen Abgenommen weder noch	 10
8	Wieviel Kilogramm in etwa?		__ kg __ __ __	
9	Haben Sie absichtlich zugenommen bzw. abgenommen?	1 2	ja nein __	
10	Wie groß sind Sie?		cm __ __ __	
11	Welche Staatsangehörigkeit haben Sie?	1 2 siehe Liste	Deutsch Andere ___ Wenn 2, intern. KFZ-Kennz. __ __	
12	Welchen Familienstand haben Sie?	1 2 3 4	ledig verheiratet geschieden verwitwet ___	
13	Wieviele Geschwister haben bzw. hatten Sie?		__ __ Geschwister	
14	In welchem Jahr sind Ihre Geschwister geboren?		19__ __ 19__ __ 19__ __ 19__ __ 19__ __ 19__ __ 19__ __ 19__ __	

	FRAGE	CODE	ANTWORT	Weiter mit Frage

Angaben zu bisherigen Erkrankungen:
INT: Die nächsten Fragen beziehen sich auf vorbestehende Krankheiten und Beschwerden im Magendarmbereich.

15	Hatten Sie innerhalb der letzten zwei Wochen eine Erkältung	1 2	ja nein ___	
16	Haben oder hatten Sie eine der folgenden Erkrankungen? Wenn ja, in welchem Jahr wurde die Erkrankung von einem Arzt erstmals festgestellt? (*INT.: Erkrankungen einzeln nennen.* *Falls Krankheit vorhanden ist, in welchem Jahr wurde sie erstmals diagnostiziert.)* **Herz-Kreislauferkrankngen** **Erkrankungen der Atmungsorgane** **Magenerkrankungen**	1 2 3	ja nein weiß nicht Krankheit? Jahr der Erstdiagnose Zu hoher Blutdruck __19___ ___ Krampfadern Thrombose __19___ ___ Herzschwäche __19___ ___ Herzrhythmus-störungen __19___ ___ **Herzkranzgefäß-verengung** __19___ ___ **Herzinfarkt** __19___ ___ Schlaganfall __19___ ___ Sonstiges ……__19__ __ (Was genau? Arztdiagnose) Lungenentzündung _19___ ___ chronische Bronchitis ___19___ ___ Asthma ___19___ ___ Sonstiges ……___ 19___ ___ (Was genau? Arztdiagnose) Reizmagen ___ 19__ ___ Schleimhaut-Entzündung (Gastritis)__19__ __ Magengeschwür __19__ __ Zwölffinger-darmgeschwür __19__ __ Magenkrebs __19__ __ Sonstiges ……___19__ __ (Was genau? Arztdiagnose)	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
frs 16	Haben oder hatten Sie eine der folgenden Erkrankungen? Wenn ja, in welchem Jahr wurde die Erkrankung von einem Arzt erstmals festgestellt?	1 2 3	ja nein weiß nicht	
	Darmerkrankungen:		Krankheit? Jahr der Erstdiagnose Morbus Crohn ___19___ ___ Colitis ulcerosa ___19___ ___ Darmkrebs ___19___ ___ Sonstiges ……___19___ ___ (Was genau? Arztdiagnose)	
	Leber- und Gallenerkrankungen:		Leberentzündung (Hepatitis) __ 19__ __ Gallensteine __19__ __ Sonstiges …… __19__ __ (Was genau? Arztdiagnose)	
	Stoffwechselerkrankungen:		Zucker __19__ __ erhöhte Blutfette __ 19__ __ Schilddrüsenerkrankung_19___ Gicht __19__ __ Sonstiges ……___19__ __ (Was genau? Arztdiagnose)	
	Nieren- und Blasenerkrankungen:		Nierensteine __19__ __ Blasenentzündung __19__ __ Nierenentzündung __19__ __ Sonstiges ……___19___ ___ (Was genau? Arztdiagnose)	
	Krankheiten des Bewegungsapparates:		Rheuma __19___ ___ Arthrose __19___ ___ Sonstiges ……___19___ ___ (Was genau? Arztdiagnose)	
			Krankheit? Jahr? Was genau? (Arztdiagnose)	
	Allergien:		____ 19___ ___ ……	
	Sonstige Erkrankungen:		____ 19___ ___ ……	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
17	Wurde bei Ihnen schon einmal eine Magenspiegelung durchgeführt?	1 2	ja nein __	20
18	Wann fand die letzte Magenspiegelung statt?		____ . ____ Monat Jahr	
19	Wurde bei einer Magenspiegelung einer der folgenden krankhaften Befunde festgestellt?	1 2 3 4 5 6 8	Schleimhautentzündung (Gastritis) Magengeschwür Zwölffingerdarmgeschwür *Helicobacter pylori*-Infektion Sonstiges: ..... kein Befund Weiß nicht __	
20	Wurden Sie jemals am Magen operiert?	1 2	ja nein __	22
21	Welche Operationen fanden statt?	 1 2 3 4	Was? im Jahr Nervendurchtrennung __19__ __ Übernähung __19__ __ Magenteilentfernung __19__ __ Sonstiges: …… __19__ __	
22	Wurden Sie in den letzten fünf Jahren mit Antibiotika behandelt?	1 2 8	ja nein weiß nicht __	26 26

	FRAGE	CODE	ANTWORT	Weiter mit Frage
23	Wann war die letzte Behandlung?	1 2 3 4	werde zur Zeit behandelt innerhalb der letzten drei Monate innerhalb des letzten Jahres vor mehr als einem Jahr	
24	Wie oft fand die Behandlung statt?	1 2 3 4	___ einmal 2 bis 3 Mal 4 bis 10 Mal mehr als 10 Mal	
25	Was war der Grund der Behandlung		___ ……	
26	Nehmen Sie zur Zeit Schmerzmittel oder entzündungs-hemmende Medikamente ein?	1 2 3	ja nein weiß nicht. __	28
27	Wie häufig?	1 2	gelegentlich regelmäßig	
28	Welche anderen vom Arzt verordneten Medikamente haben Sie in den letzten drei Monaten eingenommen? *(INT.: ausgenommen den oben genannten)*		__ Medikament: *(INT.: z.B. Ismo 20)* 1…….. 2…….. 3…….. 4…….. 5…….. 6…….. 7…….. 8……..	
29	*(INT.: Diese Frage nur Frauen stellen)* Wie alt waren Sie bei Ihrer ersten Regelblutung?		Jahre __ __	
30	Haben Sie derzeit noch Ihre Regelblutung?	1 2	ja nein __	
31	Wie alt waren Sie bei Ihrer letzten Regelblutung (Menopause)?		Jahre __ __	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
32	Nehmen Sie zur Zeit die Pille oder andere Hormonpräparate ein?	1 2	ja nein __	34
33	Welche genau?		Präparat: 1…… 2…… 3……	
34	Haben (*oder hatten*)Sie jemals Schmerzen oder Engegefühl im Brustraum gespürt?	1 2	ja nein __	48
35	Treten (*oder traten*)diese Schmerzen oder dieses Engegefühl auf, wenn Sie in Eile sind (*waren*), bergauf gehen (*gingen*) oder sich sonstwie körperlich anstrengen (*anstrengten*)?	1 2 3	ja nein ich bin nie in Eile und gehe nicht bergauf __	42
36	Treten (*oder traten*) diese Beschwerden auf, wenn Sie in normalem Tempo auf ebener Strecke gehen (*gingen*)?	1 2	ja nein __	
37	Was tun(*oder taten*) Sie, wenn Sie während des Gehens Schmerzen oder Engegefühl im Brustraum bekommen (bekamen)? (*INT: Wenn der Proband erst weitergeht, nachdem er Nitropräparate genommen hat, Code 1 codieren.*)	1 2	ich gehe langsamer oder bleibe stehen ich gehe im gleichen Tempo weiter _	42
38	Verschwinden diese Beschwerden, wenn Sie langsam gehen oder stehen bleiben?	1 2	ja nein _	40
39	Wie schnell verschwinden diese Beschwerden?	1 2	nach weniger als 10 Minuten nach mehr als 10 Minuten _	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
40	Können Sie mir zeigen, wo diese Schmerzen oder dieses Engegefühl aufgetreten ist? (*INT: Mehrfachnennungen möglich! Den Probanden die Stellen selbst zeigen lassen und dann codieren.*)	1 2	ja nein hinter dem Brustbein __ linke vordere Brust __ Hals/Kieferwinkel __ linke Schulter/Arm __ sonstiges: ..... __	
41	Strahlt der Schmerz oder Engegefühl in den linken Arm aus?	1 2	ja nein __	
42	Was hat der Arzt gesagt, als Sie von diesem Schmerz berichtet haben?	1 2 3	es ist Angina pectoris etwas anderes:..... ich war deswegen nicht beim Arzt ___	
43	Haben Sie jemals einen sehr starken Schmerz quer durch den Brustraum gehabt, der eine halbe Stunden oder länger dauerte?	1 2	ja nein __	
44	Hat bisher ein Arzt bei einem Familienangehörigen (Vater, Mutter, Geschwister oder Kinder) einen Herzinfarkt oder einen Schlaganfall festgestellt?	1 2 8	ja nein weiß nicht __	 46 46
45	Welche der Krankheiten wurden festgestellt? (*INT: Wenn eine Krankheit festgestellt wurde, auch das Alter der erstmaligen Feststellung dieser Krankheit erfragen?*)	1 2 3 4 5	Herzklappenfehler Angina pectoris Herzinfarkt Schlaganfall sonstiges:…… In welchem Was? Bei wem? Alter? __ ..... __ __Jahre __ ..... __ __Jahre __ ..... __ __Jahre __ .....__ __Jahre __ .....__ __Jahre __ .....__ __Jahre	
46	Husten Sie im Winter üblicherweise morgens nach dem Aufstehen?	1 2	ja nein __	
47	Husten Sie im Winter üblicherweise während des Tages oder in der Nacht?	1 2	ja nein __	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
48	Husten Sie üblicherweise mehr als 3 Monate im Jahr auf diese Weise?	1 2	ja nein __	
49	Husten Sie im Winter üblicherweise morgens nach dem Aufstehen zähen Schleim ab?	1 2	ja nein __	
50	Husten Sie im Winter üblicherweise während des Tages oder in der Nacht zähen Schleim aus der Lunge ab?	1 2	ja nein __	
51	Husten Sie üblicherweise mehr als 3 Monate im Jahr auf diese Weise Schleim ab?	1 2	ja nein __	
52	Hatten Sie in den letzten drei Jahren eine Pause mit (*zunehmendem*)∗ Husten und Schleimproduktion für drei Wochen oder länger? *(INT: ∗ für Personen mit üblicherweise)*	1 2	ja nein __	
	Ausbildung und Beruf *INT: Abschließend möchte ich Ihnen einige Fragen zu Ihrer Ausbildung und Ihrer letzten ausgeübten beruflichen Tätigkeit stellen.*			
53	Welches ist Ihr höchster Schulabschluß?	1 2 3 4 5 6	Hauptschule/Volksschule Mittlere Reife/Realschule Fachhochschulreife Abitur sonstiges ..... kein Abschluß ___	
54	Haben Sie eine abgeschlossene Berufsausbildung oder eine Hochschulausbildung? Wenn ja, welche? *(INT: Falls mehrere Ausbildungen die Letzte angeben)*	1 2 3 4 5 6 7 8 9	gewerbl. oder landwirt. Lehre Kaufmännische oder sonstige Lehre Berufsfachschule, Handelsschule Fachschule (z.B. Meister, Techniker) Beamtenausbildung Fachhochschule, Ingenieurschule Universität, Hochschule sonstigen Ausbildungsabschluß nein, keinen Ausbildungsabschluß ___	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
55	Sind Sie zur Zeit ..... *(INT: vor diesem Krankenhaus-aufenthalt!)*	1 2 3 4 5 6 7 8	voll berufstätig (jeden Arbeitstag ganz-tägig, Familienbetrieb – nicht Lehrling) teilweise berufstätig arbeitslos Hausfrau/-mann in Ausbildung wegen Erreichen der Altersgrenze in Rente/pensioniert freiwillig vorzeitig in Rente/ pensioniert vorzeitig aus gesundheitlichen Gründen in Rente/pensioniert ___	
56	Welche berufliche Tätigkeit üben Sie zur Zeit aus, bzw. falls nicht mehr berufstätig, haben Sie zuletzt ausgeübt?	genaue Tätig-keit (Berufs-schlüss)	..... __ __ __ __	
57	Welche Stellung haben Sie bzw. hatten Sie in diesem Beruf?	1 2 3 4 5	Arbeiter Angestellter Beamter Selbständiger Noch nie berufstätig gewesen __	
58	Welchen höchsten Schulabschluß haben bzw. hatten Ihre Eltern?	1 2 3 4 5 6	Volksschule/Hauptschule Mittlere Reife/Realschule Fachhochschulreife Abitur sonstiges: ..... kein Abschluß Mutter __ Vater __	

	FRAGE	CODE	ANTWORT	Weiter mit Frage

Ernährungs- und Lebensgewohnheiten:

INT: Jetzt stelle ich einige Fragen zu Ihren Ernährungs- und Lebensgewohnheiten.

59	Trinken Sie täglich Kaffee?	1 2	ja nein __	62
60	Wieviele Tassen trinken Sie täglich?		Tassen 0,2 L __ __	
61	Wie trinken Sie gewöhnlich Ihren Kaffee?	1 2 3 4	schwarz mit Milch mit Zucker mit Milch und Zucker __	
62	Haben Sie in den letzten 12 Monaten Alkohol getrunken?	1 2 3	ja, täglich nein ja, gelegentlich __	64
63	Wieviel haben Sie durchschnittlich innerhalb einer Woche an Wein, Bier oder Schnaps getrunken?	Anzahl eintragen	Gläser Wein, Sekt o. Most 0,25L__ __ Flaschen Bier 0,5 L __ __ Gläser Spirituosen 2 cL __ __	
64	Haben Sie bisher in Ihrem Leben mindestens 100 Zigaretten geraucht?	1 2	ja nein __	69
65	Wie alt waren Sie, als Sie mit dem Zigarettenrauchen begonnen haben?	Alter in Jahren	__ __ Jahre	

	FRAGE	CODE	ANTWORT	Weiter mit Frage
66	Haben Sie mit dem Zigaretten-rauchen aufgehört?	1 2	ja nein __	 68
67	Wann haben Sie mit dem Rauchen aufgehört? *(INT.: falls länger zurück: wie alt waren Sie, als Sie mit dem Rauchen aufgehört haben?*)	Zeit-punkt Alter in Jahren	Monat ___ Jahr_____ __ __ Jahre	
68	Wieviel Zigaretten haben Sie in den letzten 12 Monaten (*INT.: Ex-Raucher: damals*) durchschnittlich pro Tag geraucht? *(INT.: Gelegentlich = 0,5 Stück)*	Anzahl Zigaretten pro Tag	__ __ Stück	
69	Haben Sie jemals Zigarren oder Pfeife geraucht?	1 2 3 4	ja, regelmäßig (mind. eine Zigarre, Zigarillo oder Pfeife pro Tag) nein ja, gelegentlich ja, aber jetzt nicht mehr __	
70	Wieviele Personen leben derzeit ständig in Ihrem Haushalt, sie selbst eingeschlossen?	Anzahl eintra-gen	__ __	

	FRAGE	CODE	ANTWORT	Weiter mit Frage

Körperliche Aktivität:

INT: Im folgenden möchte ich Ihnen noch einige Fragen zu Ihrer körperlichen Aktivität stellen.

71	Wie oft betreiben Sie im Winter Sport? (*INT: es ist nur körperlich anstrengender Sport gemeint.*)	1 2 3 4	regelmäßig mehr als zwei Stunden in der Woche regelmäßig ein bis zwei Stunden in der Woche weniger als eine Stunde in der Woche keine sportliche Betätigung __	
72	Wie oft betreiben Sie im Sommer Sport? (*INT: es ist nur körperlich anstrengender Sport gemeint.*)	1 2 3 4	regelmäßig mehr als zwei Stunden in der Woche regelmäßig ein bis zwei Stunden in der Woche weniger als eine Stunde in der Woche keine sportliche Betätigung __	
73	Wie würden Sie Ihre derzeitige Arbeit bzw. Hauptbeschäftigung einstufen?	1 2 3 4	als schwere körperliche Arbeit als mittelschwere körperliche Arbeit als leichte körperliche Arbeit keine nennenswerte körperliche Arbeit __	
74	Wie lange sind Sie darüber hinaus an Werktagen normalerweise zu Fuß bzw. mit dem Fahrrad unterwegs, wie z.B. Wege zur Arbeit, Einkaufen, Freizeit?	1 2 3 4	mehr als eine Stunde eine halbe bis zu einer Stunde eine Viertel- bis zu einer halben Stunde weniger als eine Viertelstunde __	
75	Ist Ihre körperliche Aktivität wegen eines gesundheitlichen Problems eingeschränkt?	1 2	ja nein __	
76	Wie würden Sie Ihre körperliche Aktivität im Laufe Ihres Lebens einschätzen?	1 2 3 4	sehr aktiv etwas aktiv wenig aktiv kaum aktiv bis zum 20. Lebensjahr __ im Alter von 20-40 Jahren __ im Alter von 40-50 Jahren __ im Alter über 50 Jahre __	

Fragen zum Atemtest:

(Wird vom Untersucher abgefragt)

1.	Haben bzw. hatten Sie eine Lungenerkrankung Wenn **ja: *** Art der Erkrankung:	O Ja O Nein …… ……
2.	Haben Sie heute oder gestern Vitamin C-Tabletten eingenommen?	O Ja O Nein
3.	Möchten Sie über das Ergebnis des Atemtests Informiert werden? Wenn **ja: *** Tragen Sie bitte Ihren Namen und Ihre Anschrift in dem unteren Feld ein.	O Ja O Nein
4.	Sind Sie bisher schon einmal auf Helicobacter pylori untersucht worden? Wenn **ja: *** Wann war das? ***** War as Ergebnis positiv? ***** Fand eine Behandlung statt?	_____/19_____ Monat Jahr O Ja O Nein O Weiß ich nicht O Ja O Nein

5.	Ergebnis des Atemtests	O negativ O positiv DOB: ……

✂ ✂ ✂ ✂ ✂ ✂ ✂ ✂ ✂ ✂ ✂

Sehr geehrte Dame, sehr geehrte Herr

Wir möchten uns noch einmal für Ihre Mitarbeit an der „Fall-Kontroll Studie zum Zusammenhang zwischen Infektion mit Helicobacter pylori und koronarer Herzkrankheit“ bedanken. Sie unterstützen damit die wissenschaftliche Untersuchung über die Auswirkung dieser weitverbreiteten Infektion. Anbei erhalten Sie das Ergebnis des Atemtests. Zur Beurteilung des Ergebnisses lesen Sie bitte das beiliegende Merkblatt. Wir möchten hiermit nochmals darauf hinweisen, daß nach dem Versand dieser Informationen keine personenbezogenen Daten an der Universität Ulm mehr vorliegen.

…….
Vor- und Nachname

…….
Straße/Hausnummer:

…….
PLZ/Wohnort

Ergebnis des Atemtests

……..

UNIVERSITÄT ULM

- KLINIKUM -

Medizinische Universitätsklinik und Poliklinik

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Prof. Dr. V. Hombach
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Sehr geehrte Patienten,

die Abteilung Innere Medizin I und Innere II, die Abteilung Epidemiologie und DRK-Blutspendezentrale der Universität Ulm führen eine **Fall-Kontroll Studie zum Zusammenhang zwischen Infektion mit Helicobacter pylori und koronarer Herzkrankheit** durch. In einer Fall-Kontroll Studie werden bereits Erkrankte (Fälle) mit gesunden Personen verglichen um Hinweise auf den Einfluß eines Risikofaktors zu ermitteln.

Im folgenden möchten wir Sie um ihre Mitarbeit bei der Durchführung der Studie als Patient/-in mit einer Erkrankung der Herzgefäße bitten.

Hintergrundinformation: Das Bakterium Helicobacter pylori wird gegenwärtig als Ursache der Magenschleimhautentzündung, des Magengeschwürs, sowie als Mitursache bei der Entwicklung von Magenkrebs angesehen. **Neuerdings wird ein Zusammenhang zwischen H. pylori und der Entwicklung einer Herzerkrankung vermutet.**

Ziel der Studie: Diese Studie soll nun helfen, die Beziehung der Infektion von Helicobacter pylori und der koronaren Herzkrankheit zu untersuchen.

Untersuchungsablauf:
1. Nach Aufklärung durch einen Arzt wird ein/e Mitarbeiter/in der Studie Sie in einer persönlichen Befragung um Angaben zu Ihrer Person (ausführlicher Fragebogen) bitten.

2. Ihr Arzt wird Sie dann um eine Blutprobe (30 ml) bitten, die wir zur Erforschung von mit der Infektion zusammenhängenden Risikofaktoren benötigen. Die Blutprobe wird vor der Herzkatheteruntersuchung aus der bereits liegen Braunüle abgenommen.

3. Ob bei Ihnen eine Infektion mit dem Magenkeim Helicobacter pylori vorliegt, wird mit Atemtest festgestellt. **Der Test kann aufgrund der fehlenden Belastung vollkommen risikolos eingesetzt werden.** Der Test wird nach er Untersuchung auf der Station durchgeführt.

Zur Durchführung des Tests wird ein Glas Apfelsaft mit einer geringen Menge der Testsubstanz ^{13}C-Harnstoff getrunken. Vor und ca. 30 Minuten nach Einnahme der Testsubstanz werden Atemproben in einem Beutel gesammelt und analysiert. ^{13}C, ein nicht-radioaktives Isotop des Kohlenstoffs, und Harnstoff kommen überall, also auch beispielsweise in der Nahrung, vor. Es sind keinerlei Risiken und Nebenwirkungen des Tests bekannt. Aus dem Testergebnis kann auf eine Magenbesiedlung mit Helicobacter pylori geschlossen werden. Auf Wunsch wird Ihnen das Testergebnis und eine ausführliche Erläuterung gerne mitgeteilt.

Datenverarbeitung und Auswertung: das Testergebnis und die Angaben auf dem Fragebogen sind streng vertraulich. Sie können sicher sein, daß nach der Ergebnismitteilung an Sie kein Personenbezug der Daten mehr herstellbar ist.

Die wissenschaftliche Auswertung und Publikation der Studie erfolgt in vollkommen anonymisierter Form. Fragebogen und Einwilligungserklärung werden nach einem Jahr vernichtet.

Um gültige Ergebnisse zu erhalten sind wir auf die Mitarbeit möglichst aller Teilnehmer angewiesen! Wir möchten Sie deshalb ganz dringend bitten, bei der Durchführung der Studie zu helfen.

Durch die freiwillige Mitarbeit an diesem Projekt leisten Sie einen wichtigen Beitrag zur Untersuchung der Auswirkungen dieser weitverbreiteten Infektion, der letztendlich uns allen zu Gute kommt. Dafür bereits im Voraus vielen Dank!

Mit freundlichen Grüßen

Prof. Dr. med. Vinzenz Hombach
Abt. Innere Medizin II
Universität Ulm

Prof. Dr. med. Hermann Brenner
Abt. Epidemiologie
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Einwilligungserklärung (Patienten)

Name der Studie: **Fall-Kontroll Studie zum Zusammenhang zwischen Infektion mit Helicobacter pylori und koronarer Herzkrankheit**

Ich bin über Inhalt, die Vorgehenswiese und den Zweck des oben genannten Forschungsprojektes ausreichend informiert worden. Ich hatte Gelegenheit, Fragen zu stellen und habe hierauf Antwort erhalten. Ich hatte ausreichend Zeit, mich für oder gegen die Teilnahme am Projekt zu entscheiden.
Ich willige in die Durchführung des Atemtests bei mir und in die Befragung ein. Weiterhin bin ich damit einverstanden, dass 30 ml venöses Blut zu Studienzwecken abgenommen werden.
Hiermit befreie ich die mit meiner Krankheit befassten Ärzte und die Mitarbeiter des Forschungsteams insoweit von der ärztlichen Schweigepflicht, als ich Ihnen erlaube, alle für das Projekt notwendigen Informationen aus meinen ärztlichen Unterlagen in anonymisierter Form zu entnehmen.
Mir ist bekannt, dass dir Teilnahme freiwillig ist und jederzeit widerrufen werden kann. Ich weiß auch, dass mir das Ergebnis des Atemtests auf Wunsch mitgeteilt wird.

……
(Name des Patienten)

…….. ……
(Ort, Datum) (Unterschrift des Patienten)

Mit der edv-mäßigen Verarbeitung der bei mir im Rahmen des oben genannten Forschungsprojektes erhobenen Daten und der Verwendung in anonymisierter Form in wissenschaftlichen Veröffentlichungen bin ich einverstanden.

…….. ……

(Ort, Datum) (Unterschrift des Patienten)

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Sehr geehrte Damen und Herren,

die Abteilung Innere Medizin I und Innere II, die Abteilung Epidemiologie und DRK-Blutspendezentrale der Universität Ulm führen eine **Fall-Kontroll Studie zum Zusammenhang zwischen Infektion mit Helicobacter pylori und koronarer Herzkrankheit** durch. In einer Fall-Kontroll Studie werden bereits Erkrankte (Fälle) mit gesunden Personen verglichen um Hinweise auf den Einfluß eines Risikofaktors zu ermitteln.

Im folgenden möchten wir Sie um ihre Mitarbeit bei der Durchführung der Studie als Kontrollpresonen ohne einer Erkrankung der Herzgefäße bitten.

Hintergrundinformation: Das Bakterium Helicobacter pylori wird gegenwärtig als Ursache der Magenschleimhautentzündung, des Magengeschwürs, sowie als Mitursache bei der Entwicklung von Magenkrebs angesehen. **Neuerdings wird ein Zusammenhang zwischen H. pylori und der Entwicklung einer Herzerkrankung vermutet.**

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2. Ihr Arzt wird Sie dann um eine Blutprobe (27 ml) bitten, die wir zur Erforschung von mit der Infektion zusammenhängenden Risikofaktoren benötigen. Die Blutprobe wird vor der Herzkatheteruntersuchung aus der bereits liegen Braunüle abgenommen.

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Einwilligungserklärung (Kontrollpersonen)

Name der Studie: **Fall-Kontroll Studie zum Zusammenhang zwischen Infektion mit Helicobacter pylori und koronarer Herzkrankheit**

Ich bin über Inhalt, die Vorgehenswiese und den Zweck des oben genannten Forschungsprojektes ausreichend informiert worden. Ich hatte Gelegenheit, Fragen zu stellen und habe hierauf Antwort erhalten. Ich hatte ausreichend Zeit, mich für oder gegen die Teilnahme am Projekt zu entscheiden.
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……
(Name)

…….. ……
(Ort, Datum) (Unterschrift)

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…….. ……
(Ort, Datum) (Unterschrift)

Heli-Cor Stuidie

ANGABE AUS DER PATIENTENAKTE ID……

Nr.	Fragen	Antworten
1.	Diagnose(-n) ischämische Herzkrankheit(-en) (Art, Erstdisgnose (Monat/Jahr))	…… …… …… ……
2.	Bekannte Vorerkrankungen?	Wenn **ja:** Was genau (Diagnose, ICD-9Nr)? Ja Nein Sonst. Herz-Kreislauferkrankungen (1) O O …… Erkrankungen der Atmungsorgane (2) O O …… Magen- oder Darmerkrankungen (3) O O …… Leber- oder Galleerkrankungen (4) O O …… Stoffwechselerkrankungen (5) O O …… Nieren- oder Blasenerkrankungen (6) O O …… Allergien (7) O O …… Erkrankungen des Bewegungsapparates (8) O O …… Sonstiges (9) O O ……
3.a	Befund der Koronarangiographie **bei Erstdiagnose**	O 1 GE O 2 GE O 3 GE O Hauptsammstenose O ..... O .....
3.b	Befund der heutigen Koronarangiographie *(Entfällt bei ED zur Zeit)*	O 1 GE O 2 GE O 3 GE O Hauptsammstenose O ..... O .....

Nr.	Fragen	Antworten
4.	Zur Zeit verordnete Medikamente? Präparatname und Dosierung hier eintragen	o Beta-Rezeptoblocker **27**1 o CA-Antagonisten **27**2 o ACE-Hemmer **27**3 o Nitrate **55** o Diuretika **36** o Thrombozyten-aggregationshemmer o ASS 100 **791** o Tiklyd **792** o Lipidsenker o Fibrate **581** o HMG-CoA-Redukt. **582** o Broncholytika/Antiasthmatika **28** o Antirheumatika Analgetika o- Nichtopoide Analgetika **05**1 o- Opoide Analgetika **05**2 o- Antiphlogistika (NSAIDS) **23** o Antibiotika/Chemotherapeutika **10** o Magen-Darmmittel o- Antacida **60**1 o- H2-Antagonisten **60**2 o- Protonenpumpenhemmer **60**3 o- Pirenzepin **60**4 o- sonst. Ulcustherapeutika **60**5 o- motilitätsteigernde Mittel **60**6 o- Enzympräparate **60**7 o- Carminativa **60**8 o- Med. gg. chronisch entzündl. Darmerkrankungen **60**9 o- Antidiarrhoika **60**10 o Psychopharmaka **71** o Low-dose Heparin **20** o …… o ……

7 References

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8 Acknowledgments

I would like to thank Prof. Dr. Vinzenz Hombach for the opportunity to work in his department.

I am deeply indebted to Prof. Dr. Wolfgang Koenig from the Department of Internal Medicine II, Cardiology, University of Ulm Medical Centre for his critical and helpful discussions and comments during the preparation of this MD thesis. Moreover, I am very obliged to him for his persistent support during all my stay in Germany.

I would like to express my special gratitude to Mrs. Gerlinde Trischler for introducing me to the IRMA, ELISA and PCR techniques, for excellent laboratory collaboration and expert advice as well as for her friendly assistance.

I extend my sincere gratitude to Dr. Albrecht Hoffmeister, Dr. Margit Froehlich and to Dr. Armin Imhof from the Department of Internal Medicine II, Cardiology, University of Ulm Medical Centre for the friendly working atmosphere and their cooperation. I am also indebted to Dr. Margit Froehlich for critical reading of this manuscript.

I wish to thank PD Dr. Dietrich Rothenbacher from the Department of Epidemiology at the University of Ulm Medical Centre for his full cooperation on the statistical analysis.

Sincere thanks are given to all patients and voluntary blood donors for their participation in the study as well as to the University of Ulm blood bank staff and all medical technicians for collecting and carefully handling the blood samples.

This MD thesis was supported by MD scholarship from the Boehringer Ingelheim Foundation, Germany.