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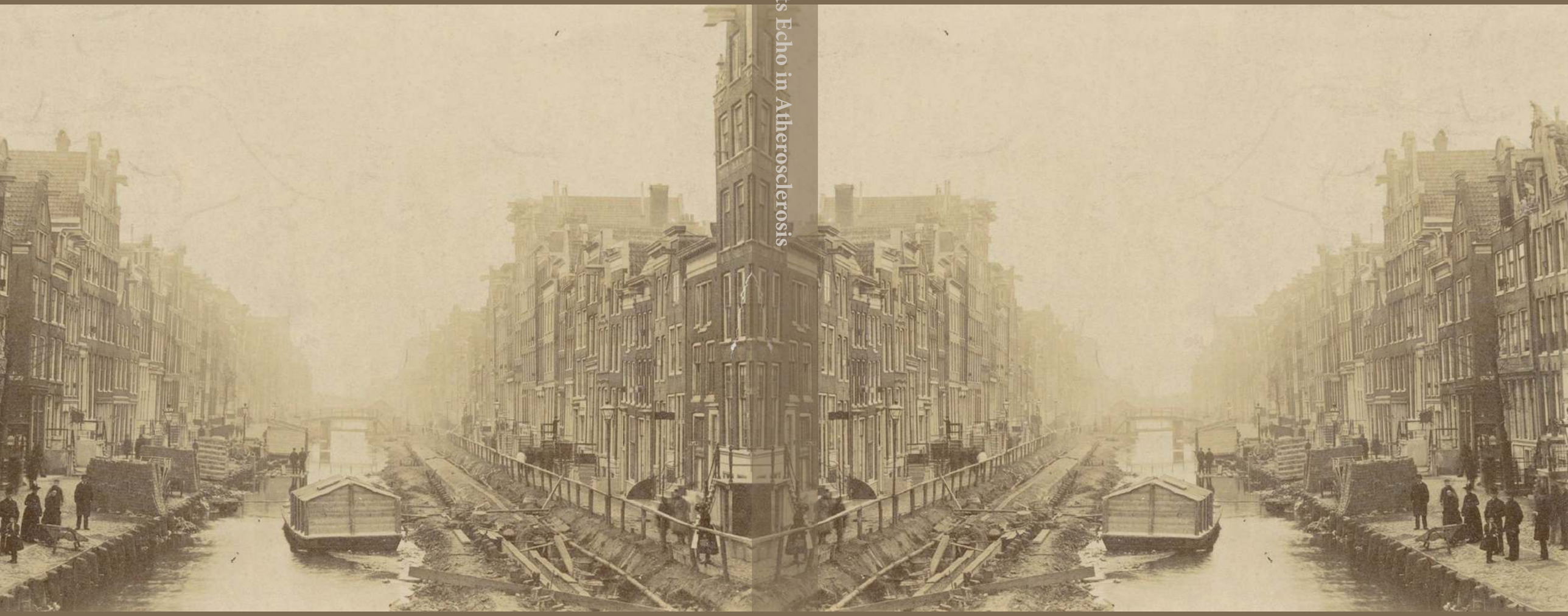

Sander van Leuven

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Dissertation, University of Amsterdam, the Netherlands

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Inflammation and its Echo in Atherosclerosis

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Chapter 1

Introduction and Outline of this Thesis

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Introduction

I Inflammation and Atherosclerosis

Oxidative Modification and the Endothelium

Atherosclerosis is a chronic disorder of the arterial wall which may initiate even before birth.[1] Over the last few decades of the previous millenium it has become clear that the atherosclerotic process can be regarded as an excessive and rampant inflammatory response to amongst others the deposition of lipids in the vessel wall. The key event which has been suggested to initiate atherosclerosis is the subendothelial retention of apolipoprotein (apo)B-containing lipoproteins. Low-density lipoprotein cholesterol (LDL) particles penetrate the endothelial barrier and become trapped by virtue of a charge-mediated interaction with proteoglycans, components of the extracellular matrix that are produced, for instance, by smooth-muscle cells (SMC).[2] Indeed, the increased retention of small LDL particles in the vessel wall is illustrated by the observation that the concentration of LDL in the subendothelium of arteries is twice that in the circulation.[3] Furthermore it facilitates for LDL particles, which are now subjected to a metabolic milieu that differs from that of the systemic circulation, to be chemically modified as a result of which they can acquire altered functional characteristics. In fact, a variety of (subtle) modifications of native LDL such as carbamylation, acetylation, and even methylation of lysine residues of apoB have been described and were shown to render the modified LDL immunogenic.[4] Moreover, the oxidative-modification hypothesis of atherosclerosis dictates that once LDL particles become stranded in the subendothelium, they are subjected to oxidization by resident vascular cells (e.g. smooth-muscle cells, endothelial cells and macrophages).[5] Among the active factors involved in these processes are reactive oxygen species such as superoxide anion, myeloperoxidase, sphingomyelinase and secretory phospholipases.[6] The oxidation of LDL substantially enhances its atherogenicity and depending on the degree of oxidation, minimally-modified LDL (mmLDL) can be differentiated from fully oxidized LDL (oxLDL). In contrast to native LDL, mmLDL is bound and

internalized not only by the LDL receptor but also by a number of scavenger receptors, whereas oxLDL exclusively binds to scavenger receptors.[7] These modified lipids exert various atherogenic effects for instance on the endothelium on which two receptors have been identified via which ox-LDL can promote atherosclerosis, CD36[8] and lectin-like ox-LDL receptor-1 (LOX-1).[9] Indeed, ox-LDL mediated ligation of CD36 and LOX-1 results in the activation of nuclear factor-κ B (NFκB).[10,11] Moreover, stimulation of endothelial cells with ox-LDL in vitro results in the expression of various leukocyte adhesion molecules such as vascular cell adhesion molecule (VCAM)-1,[12] intercellular adhesion molecule (ICAM)-1,[12,13] P-selectin[14] and E-selectin.[15] Under normal circumstances, there is only minimal interaction between the endothelium and circulating leukocytes. However, once the endothelium becomes activated, various leukocytes can adhere to the endothelial monolayer.

Involvement of leukocytes

Endothelial activation and the subsequent expression of leukocyte adhesion molecules enables the recruitment of leukocytes to the arterial wall which occurs in several phases. Contact between leukocytes and the endothelium is initiated by selectins (L-, P-, and E-selectin) which mediate the capture and the subsequent rolling of leukocytes along the endothelium. L-selectin is expressed constitutively on almost all leukocytes, whereas E-selectin and P-selectin are expressed on the surface of activated endothelium. P-selectin is also expressed by activated platelets.[16] Selectins interact with P-selectin glycoprotein ligand 1 (PSGL-1) as well as several other glycosylated ligands.[17] The significance of selectins in atherogenesis is highlighted by the observation that L-selectin deficient lymphocytes are characterized by a reduced capacity to invade the aorta compared with wild-type lymphocytes.[18] Furthermore, genetic deficiency of either P-selectin[19] or E-selectin[20] in apoE$^{-/-}$ mice resulted in reduced atherosclerotic lesion size. The interaction of selectins with their ligands however, does not support the firm adhesion that is required for the subsequent extravasation of leukocytes unless the initial attachment is followed by the engagement of integrins. Integrins are cell surface receptors formed by the noncovalent association of α- and β-subunits

and interact with a class of ligands that belong to the immunoglobin superfamily, e.g. ICAM-1 and VCAM-1.[21] The most relevant integrins for leukocyte migration are members of the β2 integrin family, especially αLβ2 (LFA-1) and αMβ2 (Mac-1), and the two α4 integrins, α4β1 (VLA-4) and α4β7.[22] Indeed, antibodies directed against LFA-1[23], Mac-1[24] or VLA-4[25] significantly attenuate atherosclerosis in various animal models. The role of α4β7 in the atherosclerotic process remains unknown. Interestingly, under physiological circumstances integrins are expressed on leukocytes in a state that has a low affinity for its ligands and they must undergo activation by a chemokine signal to mediate firm adhesion.[21] Indeed, chemokines (chemotactic cytokines) direct the movement of circulating leukocytes to sites of inflammation such as the arterial wall and play a crucial role in atherogenesis. Approximately 50 human chemokines have been identified thus far and can be classified into four subfamilies (C, CC, CXC, and CXXXC) depending on the relative position of the first two cysteines. Virtually all cellular constituents of the vascular wall produce chemokines in response to pro-inflammatory stimuli. The interaction with specific chemokine receptors first causes leukocyte rolling along the endothelium to come to a halt via the activation of adhesion receptors as well as their subsequent extravasation.[26] In particular, CCL2 (monocyte migration protein-1/MCP-1) appears to play a critical role in the initiation and development of atherosclerotic lesions by facilitating the recruitment of monocytes into the arterial wall. Indeed, contrary to normal arteries, high levels of CCL2 expression are observed in atherosclerotic lesions as CCL2 mRNA is expressed by endothelial cells, macrophages and smooth muscle cells.[27] Furthermore, genetic deficiecy of CCL2[28] or its receptor CCR2[29] resulted in significantly reduced atherosclerosis in murine studies.

Thus, activation of endothelial cells underlies an increased expression of leukocyte adhesion molecules. Leukocytes adhere to the endothelium and their subsequent extravasation is facilitated by chemokines. Various leukocytes play an important role in the atherosclerotic process.

Monocytes/Macrophages

Upon entering the subendothelial area monocytes differentiate into macrophages, a process driven by interactions with the extracellular matrix and cytokines, including macrophage colony-stimulating factor (M-CSF) and members of the tumor necrosis factor family.[30] Monocyte-to macrophage differentiation is associated with upregulation of numerous pattern recognition receptors for innate immunity, such as various several toll-like-receptors (TLR) and scavenger receptors (see figure 1).[31] In particular SR-AI and CD36 are considered to play a crucial role in the uptake of ox-LDL by macrophages. When the excess of cholesteryl esters cannot be adequatly processed this will lead to the accumulation of cytosolic lipid droplets and ultimately to the formation of foam cells, which are considered the hallmark of atherosclerosis. Although the uptake of ox-LDL by scavenger receptors does not directly stimulate inflammation it can lead to MHC-class-II-restricted antigen presentation of internalized material, thereby linking innate and adaptive immunity.[32] Contrary to scavenger receptors however, TLR activation leads to the activation of subendothelial macrophages and thereby the production of proinflammatory cytokines, chemokines, matrix metalloproteinases and antimicrobial molecules such as nitric oxide (NO) (see figure 1).[33] Various pathogen-associated molecular patterns can ligate the different TLRs such as ox-LDL, heat-shock proteins (HSPs), microbial components and unmethylated CpG DNA.

T-cells

In addition to macrophages, T-cells play a pivotal role in the atherosclerotic process which is illustrated by the observation that activated T-cells are present in all phases of atherogenesis. The majority of T-cells in human atherosclerotic lesions are $CD4^+$ T-helper cells that express the αβ T-cell antigen receptor (TCR).[34] There is a significant presence of $CD8^+$ T-cells in human lesions, but little data exist regarding the precise role of $CD8^+$ T-cells in atherogenesis. In addition to $TCR\alpha\beta^+$ T-cells there are smaller numbers of T-cells expressing invariant TCRs, including $TCR\gamma\delta^+$T-cells and iNKT cells.[34]

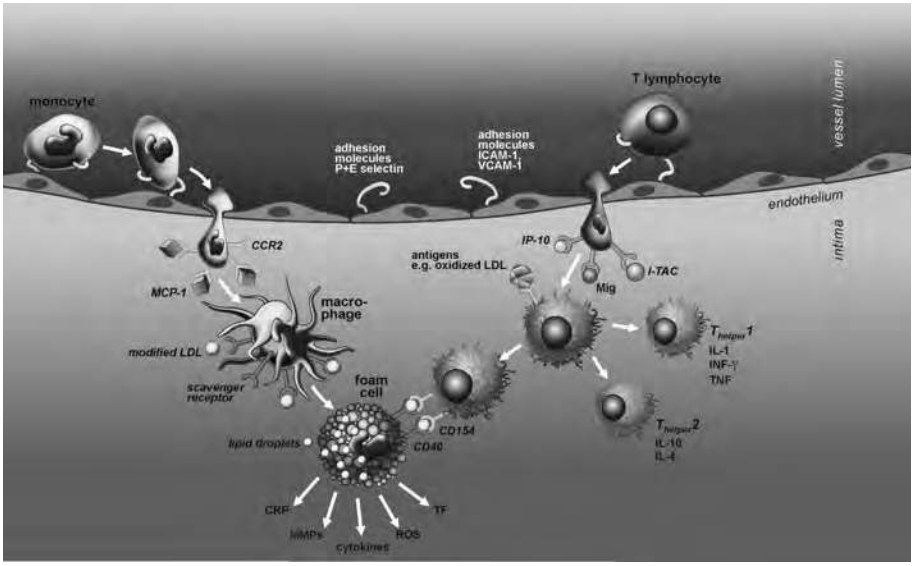

Figure 1. Activation of endothelial cells underlies an increased expression of leukocyte adhesion molecules such as ICAM-1, VCAM-1, P-selectin and E-selectin. Monocytes adhere to the endothelium and their subsequent extravasation is facilitated by chemokines such as MCP-1. In the subendothelium macrophages express scavenger receptors facilitating ox-LDL uptake and the formation of foam cells. Activated macrophages produce proinflammatory cytokines, chemokines, matrix metalloproteinases and express tissue factor thereby contributing to an inflammatory and thrombogenic micro-environment while compromising plaque stability. Chemokines such as inducible protein-10 (IP-10), monokine induced by IFN-γ (Mig) and IFN-inducible T-cell α chemoattractant (I-TAC) orchestrate T-cell transmigration to the subendothelial area. T cells can subsequently polarize into T helper-1 (Th1) cells secreting various pro-inflammatory cytokines and T helper-2 (Th2) cells which primarily secrete anti-inflammatory cytokines. Chronic immune activation characterized by subendothelial crosstalk between the different leukocyte subpopulations drives the development of atherosclerosis and ultimately compromises plaque stability.

Naïve T-cells, as part of immune surveillance, traffic from the systemic circulation, through peripheral tissues. Upon encountering antigen(s), they become activated and may subsequently differentiate and proliferate. Although the exact location is not

known, it is thought that the initial presentation of antigens to T-cells takes place in regional lymph nodes, presumably by dendritic cells (DC). When effector or memory T-cells subsequently home to the (inflamed) arterial wall, they can be reactivated by different antigen-presenting cell (APCs) such as DCs or macrophages.[31] Several potential antigens have been hypothesized to underlie the initiation and propagation of atherosclerotic vascular disease such as oxidized LDL, heat shock protein 60 (HSP 60) and Chlamydia antigens. In addition to ligation of the antigen receptor directly by an antigen or via MHC, ligation of the costimulatory molecules on T-cells is an essential step in the activation of naive T-cells. Two major families of costimulatory molecules include the B7 and the tumor necrosis factor (TNF) families which bind to receptors on T-cells belonging to the CD28 or TNF receptor families, respectively.[34]

Upon activation, T-cells can polarize into various subtypes including effector T-cells such as T helper-1 (Th1) cells or T helper-2 (Th2) cells (see figure 1), of which the first subtype predominates in the atherosclerotic plaque. Th1 cytokines, such as interferon-γ and IL-2, contribute to a pro-inflammatory environment in the subendothelium whereas Th2-biased responses (characterized by secretion of IL-4, IL-5, IL-10 and IL-13) were proposed to antagonize Th1 driven immune activation. The role of the Th2 driven responses in the development of atherosclerosis however remains controversial. Whereas IL-5 deficiency for instance has been shown to reduce the production of atheroprotective ox-LDL antibodies and accelerate atherosclerosis in $ApoE^{-/-}$ mice[35] the prototypic Th2 cytokine IL-4 exhibits inflammatory (e.g., induction of chemokines) and oxidative (activation of NADPH oxidase) properties and has been suggested to contribute to vascular inflammation.[36] Indeed, in $apoE^{-/-}$ mice genetic IL-4 deficiency was shown to attenuate atherosclerotic lesion formation, particularly at the advanced stages of lesion progression.[37]

Interestingly, it has been postulated that an imbalance between pathogenic (Th1 and/or Th2) and regulatory T-cells (Tregs) in response to "altered" self antigens facilitates reciprocal and mutual amplification of the innate and adaptive immune responses, responsible for plaque development and progression.[38] Tregs are a diverse population of lymphocytes that suppress pathogenic and autoreactive immune responses. Indeed,

in different mouse models, such regulatory T-cells have been shown to significantly reduce the development of atherosclerosis.[39,40]

Additional leukocyte subsets

The cellular composition of a human atherosclerotic plaque is generally comprised of approximately 40% macrophages, 10% $CD3^+$ T-cells while the majority of the remaining cells posses characteristics of SMC.[32] As such, the focus of leukocyte involvement in atherothrombotic disease has been directed at monocytes/macrophages and T-cells. Various additional leukocyte subsets however appear to play a pivotal role in the atherosclerotic process.

B-cells

Although B-cells are encountered in relatively low numbers in atherosclerotic lesions, their role in atherogenesis is underlined by the observation that substantial amounts of B-cells and plasma cells can be found in periadventitial lymphoid infiltrates surrounding advanced plaques.[31] Several lines of evidence support an atheroprotective role of B-cells in atherogenesis. Indeed, splenectomy in $apoE^{-/-}$ mice led to acceleration of the atherosclerotic process whereas administration of B-cells from atherosclerotic to splenectomized $apoE^{-/-}$ mice attenuated atherogenesis.[41] Moreover, transplantation of bone marrow cells from B-cell-deficient mice into $LDLR^{-/-}$ mice results in a 30% to 40% increase in the atherosclerotic lesion area in the proximal and distal aorta of these mice.[42] As such, the currently available data point towards B-cell mediated protective immunity during atherogenesis which may at least partly depend on antibody production against potential atherogenic antigens (e.g. oxLDL). On the other hand, B-cells may nonetheless play an important role in modulating the atherosclerotic immune response, namely through antigen presentation and cytokine secretion.[43]

Mast cells

Several observations suggest that mast cells play a significant role in atherothrombosis. A robust increase (200-fold) in the number of activated mast cells at sites of

atheromatous erosion or rupture is observed in coronary arteries of subjects who had died of myocardial infarction.[44] In line with this, mast cell–deficient mice showed a reduction of atherosclerotic lesion size of >50%.[45] Various mechanisms via which mast cells may promote atherothrombotic disease have been suggested. Activated mast cells release a variety of mediators such as proteases (tryptase, chymase and cathepsin G) and pro-inflammatory cytokines (e.g. IL-6, IFN-γ) with can exert atherogenic effects.[46] Indeed, protease-dependent activation of the inactive proforms of matrix metalloproteinases (proMMPs) generates activated MMPs which degrade various extracellular components of the atherosclerotic plaque thereby compromising plaque stability. Even more so considering proteolytic degradation of the fibronectin component of the pericellular matrix of subendothelial smooth muscle cells results in their apoptotic death.[47] As a result of diminished plaque SMC, local production of collagen is reduced further attributing to plaque instability. Finally, chymase and tryptase can actively degrade HDL particles compromising their anti-atherothrombotic functions (see below).[46]

Leukocyte crosstalk and stability of the atherosclerotic plaque

In human atherosclerotic plaques, MHC class II–expressing macrophages and dendritic cells can be detected close to activated T cells which suggests that there is an ongoing immune activation in atheromas.[31] Indeed, chronic inflammation characterized by subendothelial crosstalk between the different leukocyte subpopulations drives the development of atherosclerosis and ultimately compromises plaque stability.[48] Activated plaque cells secrete mediators that thin and weaken the fibrous cap overlying an atherosclerotic lesion by reducing synthesis and increasing degradation of collagen. SMC apoptosis also contributes to depletion of collagen in the fibrous cap.[49] Ultimately, the phenotype of the plaque evolves into an atherosclerotic lesion which is characterized by a thin fibrous cap, a large lipid pool, an abundance of inflammatory cells and few SMC and is thereby susceptible to rupture. Disruption of the thin fibrous cap of such vulnerable plaques causes the direct contact of blood coagulation factors to tissue factor and can trigger occlusive thrombus formation.[49] Indeed, the most

frequent patho-anatomical substrate for sudden coronary thrombosis is rupture of the fibrous cap that overlies the lipid core of the plaque.[48]

II The Echo of Inflammation: A Heartbreaking Sound

Although immune responses within the arterial wall thus facilitate plaque initiation, growth and eventually rupture, activation of the immune system outside of the arterial wall (extravascular, systemic inflammation) may also accelerate the atherosclerotic process. This has been illustrated by enhanced atherogenesis associated with various chronic inflammatory disorders. The incidence of cardiovascular disease is for instance increased 5-8 fold in systemic lupus erythematosus (SLE)[50] and 2-4 fold in rheumatoid arthritis (RA).[51] The association between these disorders and atherosclerotic vascular disease can not be fully explained by classical risk factors such as an (secondary) atherogenic lipid profile, hypertension or hyperglycemia[51,52] and is considered to result from the atherogenic effects of a systemic inflammatory state.[53-55]

Similar to the atherosclerotic plaque, sites of inflammation in RA and SLE (i.e. arthritic joint or lupus nephritis) are infiltrated by (activated) leukocytes. Activated leukocytes, cytokines and other inflammatory mediators are subsequently released into the systemic circulation. These circulating cytokines alter function of distant tissues, resulting in various proatherogenic changes that include insulin resistance, pro-oxidative stress, endothelial dysfunction and (secundary) dyslipidemia.[53] In addition, circulating inflammatory mediators may also stimulate leukocytes and smooth muscle cells within the atherosclerotic plaque thereby promoting plaque growth or rupture. The notion that a systemic inflammatory state is a universal risk factor for atherosclerotic vascular disease has been refered to as the echo of inflammation[56] and has been corroborated by several studies demonstrating that inflammatory mediators, derived from another source than the atherosclerotic plaque itself, can stimulate plaque growth. Indeed, administration of either IL-2,[57] IL-6,[58] IL-12,[59] IL-18,[60] IFN-g[61] or CRP[62] to the systemic circulation, was shown to exacerbate atherosclerosis in vivo. Moreover, introduction of leukocytes overexpressing atherogenic mediators such as MCP-1[63] or sPLA2[64]

significantly accelerates atherosclerosis in murine studies. Of note, the same holds true for anti-atherogenic mediators as it has been shown that administration of IL-10[65] to the systemic circulation can counterbalance atherothrombotic changes. Thus, considering administration of exogenous inflammatory mediators or oversecreting macrophages to the systemic circulation accelerates atherosclerosis, it is likely the same can occur when cytokines or activated leukocytes are released endogenously into the circulation in the case of lupus nephritis or an arthritic joint.

SLE and RA however, are not the only inflammatory disorders associated with enhanced atherogenesis. Indeed, acceleration of the atherosclerotic process secondary to a systemic inflammatory state has also been suggested in Wegener's granulomatosis,[66] Takayasu arteritis,[67] Sjogren's disease,[68] air polution,[69] chronic sleep apnoea,[70] psoriasis [71] as well as chronic infections that are associated with a chronic inflammatory state such as periodontitis[72] and human immunodeficiency virus (HIV).[73]

III Immunomodulation and Atherosclerosis

The clinical outcome of the immune responses within the arterial wall is determined by the balance between the abovementioned pro-inflammatory processes and endogenous counterregulatory mechanisms aimed at diminishing immune activation and maintaining the integrity and homeostasis of the arterial vasculature. As such, modulation of the immune responses that comprise vascular inflammation, characterized either by inhibition of pro-inflammatory or enhancing anti-inflammatory mechanisms has emerged as a potential means to attenuate atherosclerotic vascular disease.

Inhibition of pro-inflammatory mediators

Different approaches to attenuate activation of the immune system within the (atherosclerotic) arterial wall by inhibiting pro-inflammatory mediators have been undertaken. Various strategies interfering with the signalling of chemokines with their recepors have been shown to attenuate atherosclerosis. In apoE$^{-/-}$ mice for instance,

genetic deficiency of either CCR2,[74] CCR5,[75] CX3CR1,[76] CXCR3[77] or CXCR6[78] resulted in decreased atherosclerotic lesion size. In line, anti-CCL2 gene therapy[79] and blockade of MIF[80] exerted beneficial effects on the atherosclerotic process. In contrast, deletion of CCR1[81] as well as CXCR4[82] aggravated diet-induced atherosclerosis in $LDLr^{-/-}$ and $apoE^{-/-}$ mice respectively. As such, functional interactions between different chemokines in atherogenesis as well as the effects of chemokines on vascular cell homeostasis beyond the recruitment process need to be taken into account.[83] In addition to abrogation of chemokine signalling, T-cell costimulation constitutes another signalling pathway where (pharmacological) interference may attenuate intra-arterial immune activation. The primary costimulatory dyad is B7-1 (CD80) and B7-2 (CD86) expressed on antigen-presenting cells and CD28 expressed on T-cells. Indeed, absence of B7-1 and B7-2 significantly reduced early cholesterol diet–induced atherosclerotic lesion development in $LDLr^{-/-}$ mice compared with B7-1/B7-2–expressing control mice.[84] Similar results have been generated by interruption of signalling of the TNF and TNF receptor family members such as the OX40/OX40L and the CD137/CD137L pathway. Treatment of $LDLr^{-/-}$ mice with an anti-OX40L antibody resulted in a a 53% decrease in atherosclerotic lesion formation[85] whereas treatment of $apoE^{-/-}$ mice with a CD137 agonist resulted in a prominent increase in $CD3^+$ T cells in atherosclerotic plaques as well as larger atherosclerotic lesions.[86]

Enhancing anti-inflammatory mediators

T-cell coinhibition

Similar to inhibition of T-cell costimulation, stimulation of co-inhibitory pathways has recently emerged as a potential anti-atherothrombotic strategy. Thus far, four co-inhibitory pathways that involve binding of B7 family molecules to CD28 family receptors on T-cells have been identified.[34] The first pathway shown to play an significant role in atherogenesis involves programmed death–1 (PD-1, CD279).[87] PD-1 expressed on T-cells binds programmed death–ligand 1 (PD-L1) and PD-L2 (also known as B7-H1 (CD274) and B7-DC (CD273), respectively) on APCs and transduces signals that inhibit T-cell immune-mediated responses. In $LDLr^{-/-}$ mice PD-L1/2 deficiency led

to significantly increased atherosclerotic burden. Moreover, atherosclerotic lesions were characterized by increased infiltration of $CD4^+$ T-cells and $CD8^+$ T-cells.[87] The significance of other the co-inhibitory pathways in the atherosclerotic process remains to be elucidated

HDL and atherosclerosis

Over the course of the last decades, high-density lipoprotein cholesterol (HDL) HDL has gained wide acceptance as a potent antiatherogenic mediator. The most widely acknowledged mechanism by virtue of which HDL has been considered to protect the arterial vasculature is its role in reverse cholesterol transport (RCT). RCT constitutes the transport of excess cholesterol from lipid-laden macrophages in peripheral tissues to the liver via HDL (see figure 2). Beyond this crucial role, progressive insights also place HDL at the crossroads of the "inflammation" and "oxidative modification" hypotheses of atherosclerosis. In addition to its role in RCT, one of the most prominent protective aspects of HDL is its anti-inflammatory potential. Indeed, HDL has the ability to impede leukocytes invading the arterial wall via several modes of action. HDL not only diminishes expression of adhesion molecules by the endothelium, but also inhibits the production of chemokines such as monocyte chemoattractant protein-1 (MCP-1). As a consequence, HDL has been shown to attenuate leukocyte migration across the endothelial barrier. Administration of reconstituted HDL to rabbits with a periarterial collar significantly reduced endothelial expression of adhesion molecules such as VCAM-1 and ICAM-1 and leukocyte infiltration of the arterial wall.[88] Adding to its anti-inflammatory repertoire, HDL has also been suggested to inhibit subendothelial activation and crosstalk of leukocytes as it has been shown that apoAI can inhibit activation of monocytes by activated T-cells in vitro.[89] Another pillar supporting HDL's biological role in preserving the physiological structure and function of the arterial wall lies in its ability to inhibit oxidation. HDL is enriched with several anti-oxidant proteins such as paraoxonase (PON) and platelet activating factor acetylhydrolase (PAF-AH) which help prevent the oxidation of LDL. Furthermore, anti-oxidant properties have also been ascribed to apoAI itself. In addition to inhibiting inflammatory and

oxidative processes, HDL protects the arterial vasculature by exerting beneficial effects on the endothelium and by inhibiting coagulation. Indeed, HDL has been shown to promote endothelial generation of the atheroprotective signaling molecule nitric oxide (NO) in vitro and improve endothelial function and arterial vasoreactivity in vivo.[90] HDL exerts additional atheroprotective effects on endothelial cells by promoting their proliferation and migration and inhibiting their apoptosis.[91] Finally, HDL protects against atherothrombosis by exerting various antithrombotic actions which relate to Virchow's triad i.e. alterations in normal blood flow (e.g. increasing NO and prostacyclin production), injuries to the vascular endothelium (e.g. inhibition of apoptosis and expression of adhesion molecules and tissue factor) and alterations in the constitution of blood (e.g. enhanced activity of activated protein C and protein S, downregulation thromboxane A2 (TxA2) and PAF-AH) (see figure 2).[92]

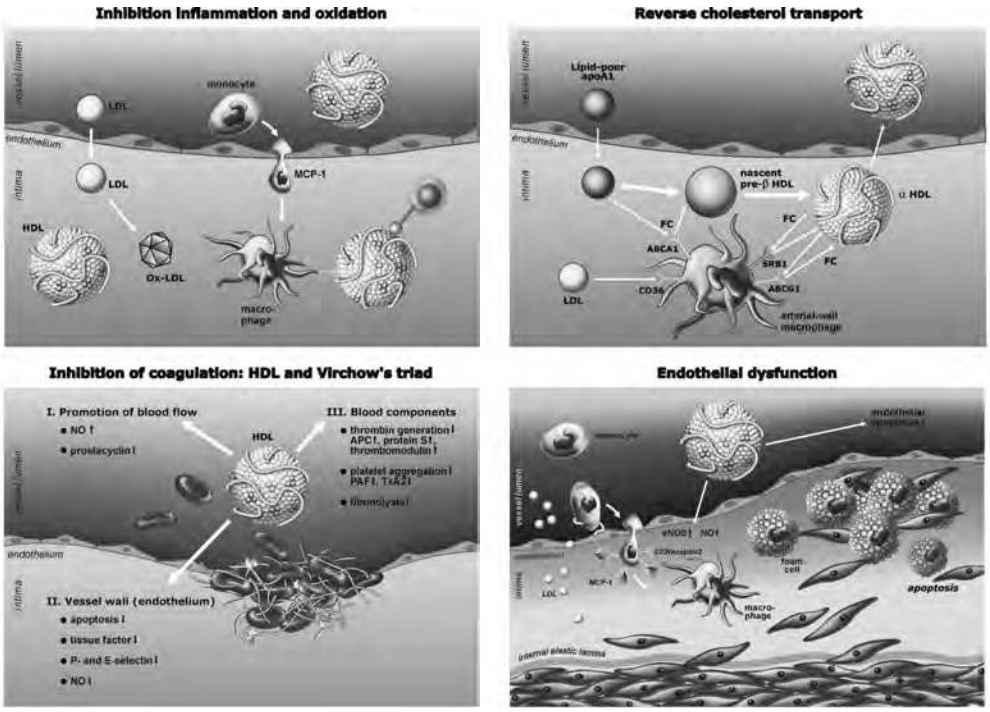

Figure 2. HDL exerts various anti-atherthrombotic functions. In reverse cholesterol transport, newly synthesized lipid-poor apolipoprotein A-I interacts with ABCA1, removing excess cellular cholesterol and forming pre-β-HDL. Pre-β-HDL is converted into mature α-HDL which is returned to systemic circulation and thereby the liver (left upper panel). HDL inhibits the oxidative modification of LDL, expression of adhesion molecules and chemokines, as well as leukocyte extravasation. Furtermore, it has been suggested to inhibit subendothelial activation and crosstalk of leukocytes (right upper panel). HDL protects against dysfunctioning of the endothelial monolayer by attenuating endothelial cell apoptosis and promoting NO production by eNOS (left lower panel). HDL posseses various anti-thrombotic actions relating to Virchow's triad: I Alterations in normal blood flow (e.g. increasing NO and prostacyclin production); II Injuries to the vascular endothelium (e.g. inhibition of apoptosis and expression of adhesion molecules and tissue factor, and the promotion of NO production); III Alterations in the constitution of blood (e.g. enhanced activity of activated protein C and protein S as well as upregulation of endothelial cell thrombomodulin results in decreased thrombin generation. HDL attenuates platelet activation mediated by downregulation of thromboxane A2 (TxA2) synthesis and platelet activating factor (PAF) release (right lower panel).

Raising HDL levels

The undisputed significance of HDL in atherogenesis has generated considerable interest in interventions aimed at elevating HDL levels. This has resulted in a continuously expanding armature of strategies targeting HDL at various points of its metabolism. One potential approach to manipulate HDL levels is to inhibit enzymes involved in the remodeling of HDL of which CETP has drawn most attention. Considering CETP mediates the transfer of cholesteryl ester from HDL to apoB–containing lipoproteins and the simultaneous transfer of triglycerides in the opposite direction, blockings its action is hypothesized to decelerate atherosclerosis secondary to increased levels of HDL.[93] Indeed, genetic CETP deficiency leads to elevated HDL levels[94] whereas elevated CETP levels were shown to be associated with an increased risk of future coronary artery disease in apparently healthy subjects.[95] It does however appear that the role of CETP is determined by the metabolic milieu in which it operates. For instance, it has been shown that CETP has an incremental impact at higher levels of triglycerides.[95] Unfortunately, the use of animal models has failed to further clarify the role of CETP in atherogenesis. Considering rodents are naturally deficient in CETP, transgenic models were created and expression of CETP in transgenic mice and rats was shown to increase atherosclerosis in most[96-98] but not all models.[99] Rabbits however, naturally express CETP and inhibition of CETP by means of small molecule inhibitors,[100] a vaccine[101] or antisense oligonucleotides[102] has consistently led to attenuated atherosclerosis in rabbits. Ín humans, a randomized placebo-controlled trial in almost 200 healthy subjects, showed that CETP inhibitor JTT-705 dose-dependently lowers plasma CETP activity and increases HDL concentration. Indeed, treatment with the maximal dose of 900 mg JTT-705 for 4 weeks led to a 37% decrease in CETP activity and a 34% increase in HDL cholesterol without any signals concerning safety or tolerability.[103]

Although a continuously expanding plethora of evidence consisting in vitro data as well as studies in experimental animal models supports the notion that immmunomodulation is a promising anti-atherothrombotic stategy, studies in humans evaluating the effect of immunomodulatory strategies on atherosclerotic

plaque phenotype, remain scarce. Encouraging results were recently observed in a study evaluating the effect of infusion of reconstitued HDL (rHDL) on atherosclerotic plaque morphology.[104] Patients undergoing percutaneous superficial femoral artery revascularization received either a single infusion of rHDL or placebo, five to days prior to endatherectomy. Excised specimens were collected for histological and immunohistochemical analyses. Interstingly, a single infusion of rHDL significantly attenuated expression of VCAM-1 as well as macrophage size. In addition, there was a reduction in lipid content in the plaque of HDL-treated patients compared to placebo.[104]

Various strategies targeting immune and inflammatory pathways have been shown to exert potent anti-atherothrombotic actions thus far predominantly in experimental animal models. Clearly, pilot studies in human are needed to further test this hypothesis.

Outline of this thesis

In this thesis the role of inflammation in the pathophysiology of atherosclerosis will be examined from different angles. In part I the effect of various pro-inflammatory mediators is evaluated in the context of atherosclerosis. Chapter 2 and 3 describe the relation between components of the immune system, mannose-binding lectin (MBL) and monocyte migration protein-1 (MCP-1) respectively, and the risk of coronary heart disease in apparently healthy individuals. The remainder of part I focuses on the atherosclerotic effects of acute inflammatory stimuli. Chapter 4 assesses the effects of C-reactive protein (CRP) on glucose metabolism whereas in chapter 5 the ability of high-density lipoprotein cholesterol (HDL) to counterbalance CRP-mediated atherothrombotic effects is evaluated. In order to gain more insight in the atheroprotective effects of HDL by means of evaluating its ability to neutralize pro-inflammatory stimuli, the effect of endotoxin in individuals with isolated low and high HDL levels is studied in chapter 6.

In part II the association of atherosclerosis with chronic inflammatory disorders is explored. Chapter 7 reviews the available literature with regard to the role of carotid intima-media thickness (IMT) as a marker for progression and regression of atherosclerosis. By employing IMT measurements, chapter 8 and 9 assess whether atherogenesis is enhanced in two distinct pro-inflammatory conditions, Crohn's disease and early onset rheumatoid arthritis respectively. Finally, chapter 10 provides an overview of systemic inflammation as a risk factor for atherothrombotic disease.

Part III focuses on the anti-atherosclerotic effects of various immunomodulatory interventions characterized either by inhibition of pro-inflammatory or enhancing anti-inflammatory mechanisms. With regard to the latter, chapter 11 describes the results of the RADIANCE 1 (Rating Atherosclerotic Disease change by Imaging with a new CETP Inhibitor) study, designed to evaluate the effects of torcetrapib, a novel drug with the ability to increase HDL levels by virtue of its ability to inhibit of cholesteryl ester transfer protein (CETP). A pooled analysis of the RADIANCE 1 and 2 trials is described in chapter 12. The remainder of part I focuses on strategies aimed at diminishing pro-inflammatory mechanisms. Indeed, chapter 13 asseses the anti-atherosclerotic effects of anti-TNFα therapy in patients with rheumatoid arthritis on lipoproteins and macrophage migration inhibitory factor. Chapter 14 provides an overview of atheroprotective effects of mycophenolate mofetil (MMF) on various components of the atherosclerotic plaque such as T-lymphocytes, monocytes/ macrophages and the endothelium. These potential beneficial effects are studied in chapter 15 which describes the results of a study in which patients with carotid artery stenosis were treated with either MMFor placebo for at least 2 weeks prior to undergoing carotid endarterectomy. Endarterectomy specimens were collected to verify whether treatment with MMF was associated with altered cellular infiltration and/or changes in plaque inflammatory activity, as assessed with mRNA expression profiling as compared to placebo.

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PART 1

Inflammation and Atherosclerosis

Chapter 2

Serum levels of mannose-binding lectin and the risk of future coronary artery disease in apparently healthy men and women.

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Abstract

Objective: To determine the association between serum levels of mannose-binding lectin (MBL) and the risk of future coronary artery disease (CAD) in apparently healthy men and women.

Methods: We performed a prospective case-control study among apparently healthy men and women nested in the EPIC-Norfolk cohort. Baseline concentrations of MBL were measured in serum samples of 946 patients who experienced a myocardial infarction or died of CAD during follow-up, and 1799 matched controls who remained free of CAD.

Results: Among men, median MBL levels were 1.63 ng/mL (IQR: 0.59 - 3.80) in cases and 1.20 ng/mL (IQR: 0.48 - 3.37) in controls. Among women, median MBL levels were 1.02 ng/mL (IQR: 0.43 - 2.95) in cases and 1.01 ng/mL (IQR: 0.43 - 2.94) in controls. After adjustment, the odds ratio in men for future CAD was 1.59 (95% CI: 1.09-2.32; p for linearity = 0.01) for those in the highest quartile compared to those in the lowest quartile. In women no such relation was observed.

Conclusions: Elevated levels of MBL are associated with an increased risk of future CAD in apparently healthy men but not in women. The sex difference merits further exploration.

Introduction

Inflammation plays a major role in all phases of atherogenesis from plaque initiation to plaque rupture. Several inflammatory markers have been associated with an increased risk of atherosclerotic vascular disease, including CRP,[1] SPLA2, [2] and IL-6.[3] In addition, markers of innate immunity have been shown to predict the development of CAD.[4-7] However, prospective evidence for a causative role of inflammatory factors is still limited.[8] Recently, evidence has emerged concerning the role of mannose-binding lectin (MBL) in the development of atherosclerosis. MBL is a part of the complement cascade and plays an important role in the first line of defense of the innate immune system against pathogenic micro-organisms.[9, 10] MBL recognizes sugar patterns on the surface of many pathogens[11], phospholipids,[12] immune complexes[13] and apoptotic cells.[14] In the circulation, MBL forms a complex with MBL-associated serine proteases (MASPs). This complex becomes enzymatically active and activates the classical complement route. This facilitates complement-dependent opsonization and subsequent uptake and clearance by phagocytes.[11] In addition, there are indications that MBL binds directly to granulocytes, monocytes and macrophages, which may stimulate the production of pro-inflammatory cytokines.[15] Since innate immunity has been implicated in atherogenesis, MBL has been suggested to play a role in the formation of atherosclerotic plaque. However, studies examining the relations between either serum levels of MBL or MBL genetic variants associated with low serum levels of MBL and CAD risk have reported equivocal results.[7, 16-21 22-24] At present, no conclusive data are available about the relationship between serum MBL levels and future CAD risk in healthy individuals.

We hypothesized that MBL levels are associated with an increased risk of future CAD in healthy individuals. We tested this hypothesis in a large prospective case-control study nested in the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) prospective population study. We measured serum levels of MBL in apparently healthy men and women at baseline and assessed the development of CAD during a follow-up period of 6 years.

Methods

The EPIC study (EPIC, European Prospective Investigation into Cancer and Nutrition), a collaborative study of nine countries in Europe, was designed to assess the determinants of cancer and other diseases. The EPIC-Norfolk cohort which is a part of the EPIC study has been described in detail previously.[25] In brief, investigators recruited 25,663 men and women, all residents in Norfolk (United Kingdom), from general practices and performed a baseline survey between 1993 and 1997. Trained nosologists obtained vital status of the entire cohort based on death certificates of the United Kingdom Office of National Statistics and linkage of the unique National Health Service number linkage with the East Norfolk Health Authority (ENCORE) database, which identifies all hospital contacts throughout England and Wales for Norfolk residents. Follow-up information was obtained up to January 2003, an average of about 6 years. The study was approved by the Norwich District Health Authority Ethics Committee and all participants gave informed consent. We performed a nested case-control study of coronary artery disease in the EPIC-Norfolk cohort. The design, methods and validation of this CAD case control study have been described in detail elsewhere.[2] In brief, we identified apparently healthy participants from the cohort who developed a myocardial infarction or died of coronary heart disease. Two controls were matched to these cases based on sex, age (within 5 years), data of visit (within 3 months) and duration of follow-up time in the study. Controls were those who remained alive and did not had a myocardial infarction during follow-up. For this nested study we excluded participants from the EPIC-Norfolk cohort who reported a history of myocardial infarction or stroke at the baseline visit.

Biochemical analyses

Qualified staff collected blood from all participants at the baseline visit. The Department of Clinical Biochemistry, University of Cambridge processed the samples for assay. Serum levels of total cholesterol, high-density lipoprotein cholesterol (HDL-c), and triglycerides were measured on fresh samples with the RA 1000 (Bayer

Diagnostics, Basingstoke, UK), and low-density lipoprotein cholesterol (LDL-c) levels were calculated with the Friedewald formula.[26] In addition, serum samples were stored at –80˚C for future analyses. We measured serum concentrations of C-reactive protein (CRP) with a sandwich-type enzyme linked immunosorbent assay (ELISA) as previously described,[27] and related the results of this ELISA to a standard consisting of commercially available CRP (Behringwerke AG, Marburg, Germany). We measured serum MBL levels with a commercially available ELISA kit from Sanquin Research (Amsterdam, The Netherlands). We incubated the samples in mannan coated plates. After washing, we visualised the binding of MBL by incubation with biotinylated monoclonal antibody against MBL (CLB anti-MBL-1). Samples were analyzed in random order and researchers and laboratory personnel were blinded as to case status of the samples.

Statistical analysis

MBL levels had a skewed distribution and were therefore log-transformed before being used in statistical analyses, but in tables we present untransformed medians and corresponding interquartile ranges (IQR). MBL quartiles were based on the sex-specific distribution among the controls. Sex-specific analyses were performed using sex-specific quartile cut-offs. In order to estimate the relative risk of future CAD, we calculated odds ratios (OR) and corresponding 95% confidence intervals (95%CI) per MBL quartile and a p-value for linearity across quartiles. ORs were calculated using conditional logistic regression, taking into account the matching for sex and age and enrollment time. ORs were adjusted for cardiovascular risk factors which were significantly related to MBL levels. In a second regression model, ORs were additionally adjusted for serum levels of CRP. The lowest quartile was used as the reference category.

Results

MBL levels in the study population

Table 1 shows the sex specific distribution of risk factors at baseline for cases and controls. As expected, levels of risk factors including blood pressure, total cholesterol, LDL-c, triglycerides, CRP, diabetes, cigarette smoking habit and BMI were higher and HDL-c lower in those who developed CAD during follow-up compared to those who remained free from CAD. Among men, median MBL levels were higher in cases when compared to controls. Among women, median MBL levels were similar in cases and controls.

MBL levels and other risk factors

Table 2 shows the relationship between cardiovascular risk factors and MBL quartiles. MBL levels were correlated with sex, HDL-c and CRP levels. These correlations were consistent over the MBL quartile. Among men, MBL levels were correlated with CRP levels. Among women, no such correlation with CRP was noted, but a significant correlation was seen with BMI. There was no significant relationship between MBL levels and other cardiovascular risk factor, including age, history of diabetes, smoking, blood pressure, LDL-c, and triglycerides.

MBL levels and the risk of future CAD

Table 3 presents the odds ratios for future CAD according to quartile of MBL among men and women (Table 3). In men, after adjustment for determinants of cardiovascular risk and MBL levels, the risk of future CAD was 1.55 (95% CI: 1.08 - 2.22) for men in the highest MBL quartile compared to those in the lowest (p for linearity = 0.01). This remained significant after additional adjustment for CRP. Figure 1 shows odds ratios for future CAD in men stratified by quartiles of CRP and MBL. The relationship between MBL levels and future CAD was solely accounted for by men, whereas in women no such a relation was found (p for linearity = 0.2).

Table 1: Study population

Men	Controls	Cases
	(n = 958)	(n = 486)
Age, year	64 ± 8	65 ± 8
Smoking – Current	8.3 (78)	16.6 (80)
– Former	61.7 (583)	58.0 (279)
– Never	30.1 (284)	25.4 (122)
BMI, kg/m^2	26.4 ± 3.2	27.1 ± 3.4
Diabetes	2.3 (22)	7.2 (35)
SBP, mmHg	140 ± 17	144 ± 18
DBP, mmHg	85 ± 11	86 ± 12
TC, mmol/l	6.0 ± 1.0	6.3 ± 1.2
LDL, mmol/l	3.9 ± 0.9	4.1 ± 1.0
HDL, mmol/l	1.25 ± 0.34	1.16 ± 0.30
TG, mmol/l	1.7 (1.2 - 2.4)	2.0 (1.4 - 2.9)
CRP, mg/l	1.4 (0.7 – 2.7)	2.2 (1.0 - 4.5)
MBL, ng/ml	1.20 (0.48 - 3.37)	1.63 (0.59 - 3.80)
Women		
	(n = 547)	(n = 294)
Age, year	67 ± 7	67 ± 7
Smoking – current	7.0 (38)	15.1 (44)
– Former	36.3 (197)	37.1 (108)
– Never	56.6 (307)	47.8 (139)
BMI, kg/m^2	26.3 ± 4.1	27.3 ± 4.5
Diabetes	1.3 (7)	5.4 (16)
SBP, mmHg	139 ± 19	144 ± 20
DBP, mmHg	82 ± 11	85 ± 12
TC, mmol/l	6.7 ± 1.2	6.9 ± 1.3
LDL, mmol/l	4.3 ± 1.1	4.5 ± 1.1
HDL, mmol/l	1.58 ± 0.41	1.44 ± 0.39
TG, mmol/l	1.5 (1.1 - 2.2)	1.9 (1.5 - 2.6)
CRP, mg/l	1.6 (0.7 - 3.7)	2.6 (1.1 - 5.1)
MBL, ng/ml	1.01 (0.43 - 2.94)	1.02 (0.43 - 2.95)

Data are presented as mean ± SD, % (n), or median (interquartile range). LDL indicates low-density lipoprotein; SBP indicates systolic blood pressure; DBP indicates diastolic blood pressure; HDL indicates high-density lipoprotein; MBL indicates mannose binding lectin. Means, percentages and medians may be based on fewer observations than the indicated number of subjects.

Table 2: Correlation between risk factors and MBL levels

Quartile	1	2	3	4	P*	R	P†
Men							
MBL range, ng/ml	< 0.48	0.49- 1.19	1.20 - 3.37	≥ 3.38			
Case / control	103 / 240	103 / 238	137 / 242	143 / 238			
Age, year	64 ± 8	65 ± 8	64 ± 8	64 ± 8	0.7	0.04	0.9
Smoking - Current	12.9 (44)	7.5 (25)	10.2 (38)	13.5 (51)			
- Former	60.7 (207)	65.3 (218)	57.2 (214)	59.2 (223)	0.9		
- Never	26.4 (90)	27.2 (91)	32.6 (122)	27.3 (103)			
BMI, kg/m^2	26.7± 3.2	26.8± 3.2	26.7±3.2	26.6 ± 3.5	0.7	-0.01	0.8
Diabetes	2.9 (10)	3.2 (11)	5.8 (22)	3.7 (14)	0.2		
SBP, mmHg	141 ± 17	143 ± 18	141 ± 18	141 ± 18	0.8	-0.01	0.7
DBP, mmHg	85 ± 11	86 ± 12	85 ± 11	85 ± 11	0.3	-0.02	0.6
TC, mmol/l	6.2 ± 1.1	6.2 ± 1.1	6.1 ± 1.1	6.1 ± 1.1	0.3	-0.03	0.2
LDL, mmol/l	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 0.9	4.0 ± 0.9	0.6	-0.02	0.5
HDL, mmol/l	1.2 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	0.2	-0.03	0.2
TG, mmol/l	2.1 ± 1.0	2.2 ± 1.3	2.1 ± 1.2	2.0 ± 1.1	0.7	-0.01	0.6
CRP, mg/l	3.1 ± 5.1	2.7 ± 3.7	3.9 ± 5.9	3.9 ± 6.4	0.01	0.07	0.005
Women							
MBL range, ng/ml	< 0.42	0.43 - 1.01	1.02 – 2.92	≥ 2.94			
Case / control	72 / 134	74 / 141	74 / 135	74 / 137			
Age, year	66 ± 8	67 ± 7	67 ± 7	66 ± 7	0.8	0.03	0.4
Smoking - Current	11.2 (23)	8.0 (17)	11.7 (24)	8.5 (18)			
- Former	37.1 (76)	39.2 (83)	38.5 (79)	31.8 (67)	0.3		
- Never	51.7 (106)	52.8 (112)	49.8 (102)	59.7 (126)			
BMI, kg/m^2	27.1 ± 4.6	27.0 ± 4.1	26.4 ± 4.0	26.2 ± 4.3	0.03	-0.07	0.04
Diabetes	1.9 (4)	2.3 (5)	2.9 (6)	3.8 (8)	0.5		
SBP, mmHg	140 ± 19	140 ± 19	142 ± 19	140 ± 20	0.9	-0.01	0.8
DBP, mmHg	83 ± 11	83 ± 11	84 ± 12	83 ± 12	0.6	0.00	1.0
TC, mmol/l	6.8 ± 1.3	6.8 ± 1.2	6.7 ± 1.1	6.8 ± 1.2	0.6	0.01	0.8
LDL, mmol/l	4.4 ± 1.2	4.3 ± 1.1	4.3 ± 1.0	4.4 ± 1.0	0.9	0.02	0.7
HDL, mmol/l	1.6 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	1.5± 0.4	0.6	-0.01	0.7
TG, mmol/l	1.8 ± 0.7	1.8 ± 0.7	1.7 ± 0.6	1.7 ± 0.7	0.04	-0.06	0.07
CRP, mg/l	3.5 ± 5.0	4.1 ± 6.5	4.4 ± 9.2	4.2 ± 6.5	0.4	0.03	0.4

Data are presented as mean ± SD per MBL quartile. MBL indicates mannose binding lectin; SBP indicates systolic blood pressure; DBP indicates diastolic blood pressure; LDL indicates low-density lipoprotein; HDL indicates high-density lipoprotein; P* indicates p-value for linearity between the MBL quartile and risk factor levels; R indicates Pearsons' correlation between log-transformed MBL levels and risk factor levels; P† indicates the corresponding p-value. Because of their skewed distribution, triglycerides, CPR, and MBL levels were log-transformed before analysis as continuous variables.

Table 3: Risk of CAD and MBL quartiles

MBL quartile	1	2	3	4	P linearity
Men					
MBL range, ng/ml	< 0.48	0.49- 1.19	1.20 - 3.37	≥ 3.38	
Cases / controls	103 / 240	103 / 238	137 / 242	143 / 238	
OR (95% CI) (1)	1.00	1.17 (0.80-1.70)	1.43 (1.01-2.04)	1.55 (1.08-2.22)	0.01
OR (95% CI) (2)	1.00	1.16 (0.79-1.71)	1.38 (0.95-2.00)	1.59 (1.09-2.32)	0.01
Women					
MBL range, ng/ml	< 0.42	0.43 – 1.00	1.01 – 2.83	> 2.84	
Cases / controls	71 / 134	74 / 141	74 / 135	74 / 137	
OR (95% CI) (1)	1.00	0.91 (0.56-1.46)	1.08 (0.69-1.70)	0.98 (0.61 -1.56)	0.9
OR (95% CI) (2)	1.00	0.70 (0.40-1.21)	1.06 (0.62-1.79)	0.82 (0.47 -1.43)	0.8

Odds ratios for future CAD were calculated by conditional logistic regression with corresponding 95% confidence intervals per quartile. MBL quartiles were based on the sex-specific distribution among the controls. Because of their skewed distribution, CRP levels were log-transformed before analysis as continuous variables. In model 1 adjustment was done for determinant of cardiovascular risk and MBL levels, excluding CRP (1). Model 2 was additionally adjusted for CRP (2).

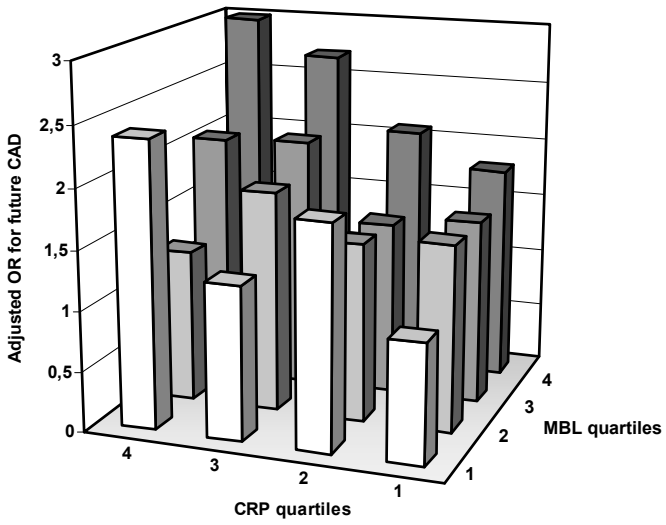

Figure 1: Risk of CAD in apparently healthy men stratified by quartiles of CRPand MBL

Bars represent adjusted odds ratios for future CAD in apparently healthy men stratified by quartiles of CRP and MBL. Odds ratios were calculated by conditional logistic regression analysis. Adjustment was done for determinants of cardiovascular risk and MBL levels. Men in the lowest quartile for both CRP and MBL were used as the reference group.

Discussion

In this large prospective study among apparently healthy people, we showed that in men high serum levels of MBL are associated with an increased risk of future coronary artery disease. This relationship was independent of established cardiovascular risk factors. This observation suggests that MBL levels may reflect or contribute to a pathophysiological process relevant in the development of atherosclerosis.

Recently, evidence emerged about a role of MBL in CAD. In experimental models it was shown that the MBL-pathway is involved in ischemia-induced complement activation in mice.[28] Consequently, the administration of anti-MBL antibodies protects the heart from ischemia-reperfusion injury by reducing neutrophil infiltration and attenuating pro-inflammatory gene expression.[29] In humans, it was recently shown that high MBL levels are associated with a high incidence of re-stenosis in patients with atherosclerotic disease of the carotid artery.[18] Moreover, type I diabetics with a history of cardiovascular disease had significantly elevated MBL levels when compared to type I diabetics without vascular complication.[21] In our study, we were able to show an increased risk for CAD in apparently healthy men with high MBL levels. In women however, no such an association was seen. Sex differences with regard to MBL levels and cardiovascular risk have been reported before but the causes are presently unknown.[18, 24] Endocrine status plays an important role in the regulation of MBL levels.[30, 31] Hormonal differences and cardiovascular disease are an ongoing topic of research. Anti-inflammatory properties of estrogen have sparked much interest and may comprise complement activation. Interestingly, endothelial tissue from males may be more susceptible than that from females to the acute effects of complement activation.[32] Differences in the expression of complement components in adipose tissue of men and women have also been observed.[33] Furthermore, we observed significant differences in CRP levels among the MBL quartiles with highest CRP levels in the highest MBL quartile. High CRP and MBL levels could both be a sign of an inflammatory state. MBL concentrations are normally stable within one person, but can rise threefold during the acute phase response. Therefore, the differences in MBL levels between male cases and controls could be partially attributable to the differences

in the inflammatory state of these individuals. However, the relationship between MBL levels and future CAD risk persisted upon additional adjustment for CRP.

Earlier studies regarding the role of MBL in atherogenesis in humans not conclusive. In one population-based study, DNA analysis in 434 apparently healthy American Indians revealed that genotypes coding for diminished levels of MBL are predictive of CAD.[7] However, measuring MBL protein levels may be more reliable than genotyping. MBL protein levels vary widely between individuals. This is partly influenced by variant MBL genotypes, but serum levels vary markedly between persons with an identical genotype.[24] Depending on the antibody used however, different findings can be seen which may also contribute to the lack of consensus regarding the role of MBL in atherosclerotic vascular disease. In a nested case-control study in the Reykjavik cohort, Saevarsdottir showed that MBL levels are not associated with an increased risk of CAD, but in a sub-analysis high MBL levels were associated with a lower risk of CAD in diabetics.[24] We observed several differences with our own study. Firstly, there is a difference in power, since we included twice as many patients in the study. Secondly, contrary to the former study we matched all cases for both age and sex and enrollment-time and subsequently we performed sex-specific analysis. And finally, we measured also CRP levels which made it possible to correct for CRP levels during the statistical analysis.

There are several mechanisms by which MBL can function atherogenic. MBL activates the complement system which has been implicated in atherogenesis and was recently shown to be associated with increased cardiovascular risk in patients with advanced atherosclerosis.[4] Indeed, increased deposition of complement iC3b in ruptured and vulnerable plaques suggests a role for complement activation in acute coronary syndromes.[34] Furthermore, endothelial oxidative stress, which plays a major role in atherogenesis, activates complement via the lectin complement pathway in human cell cultures. In addition, in this experimental model, anti-MBL monoclonal antibodies inhibited MBL and C3 deposition after endothelial oxidative stress.[35] MBL however, is not only involved in complement activation but also is a potent regulator of inflammatory pathways.[36] Indeed, MBL was shown to enhance the production of chemokines by macrophages.[37] and may thereby enhance phagocyte recruitment to

the subendothelium. Moreover, it was recently shown that MBL binds to leukocytes and can thereby modulate inflammation. Of note, this study suggested that such binding only occurred at extravascular sites in individuals possessing MBL genotypes conferring MBL sufficiency.[38] This may help explain why high MBL levels can influence the atherosclerotic process in the subendothelium. These data support a role for MBL in the atherogenesis and possibly in plaque destabilization and the development of coronary events.

A number of issues have to be taken into account when interpreting the results of the present study. Serum levels of MBL were determined in a single sample that was obtained at a nonuniform time of the day. Diurnal variation and variation over time could have affected the MBL concentrations, although such variation has been shown to be limited compared to other cardiovascular risk factors. In addition, we cannot rule out that sample storage at –80°C for 6 to 10 years may have affected the detection of MBL. However, both these limitations would lead to increased random measurement error, which leads to an underestimation of any relationship, and therefore do not negate our findings. Although the current study was not designed to establish whether the relationship between MBL and CAD is causal, it is unlikely that differences in MBL serum levels occurred as a consequence of cardiovascular events because individuals with symptomatic cardiovascular disease were excluded from our analysis. However, we cannot exclude the possibility that MBL concentration is a marker of advanced subclinical atherosclerosis.

We conclude that in this nested case control study elevated serum concentrations of MBL are associated with an increased risk of future CAD in apparently healthy men. This relationship was independent of other cardiovascular risk factors. These prospective data support the hypothesis that the immune system plays an important role in the development of atherosclerosis and its prominent clinical manifestation: coronary artery disease. The lack of association in women warrants further investigation and may provide insights into possible explanations for the sex difference in coronary heart disease susceptibility.

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Chapter 3

CCL2 Polymorhisms are associated with Monocyte Chemoattractant Protein-1 serum levels and the risk of future coronary artery disease

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ABSTRACT

Background: In humans, genetic evidence regarding the role of monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2) is contradictory.

Methods and Results: We performed a case-control study nested in the prospective EPIC-Norfolk cohort investigating CCL2 genotype, MCP-1 serum concentrations and the risk for future CAD. Cases (n = 1138) were apparently healthy men and women aged 45-79 years who developed fatal or nonfatal CAD during a mean follow-up of 6 years. Controls (n=2237) were matched by age, gender, and enrollment time. Linear regression analysis was used to test for correlations between CCL2 genotype and MCP-1 serum concentrations. The MCP-1-2136T alleles and the MCP-1+764G allele are correlated with higher MCP-1 levels (Beta 2.0; 95% CI 0.51-3.51; p=0.009 and Beta 2.10; 95% CI 0.58-3.62; p=0.007). Using Cox regression analysis we found that homozygotes for the MCP-1-3726C allele have an increased risk of CAD (OR 1.31; 95% CI 1.05-1.65; p-value 0.020) and that the presence of the MCP-1-2835A allele in males (OR 1.28; 95% CI 1.05-1.57; p=0.017) as well as the MCP-1-2578G allele in males (OR 1.26; 95% CI 1.03-1.53; p=0.027) is associated with an increased risk of CAD. Furthermore, we found that CCL2 genotype combinations are associated with both MCP-1 serum concentrations and the risk of future CAD.

Conclusions: Our data indicate that MCP-1 is involved in the pathogenesis of atherosclerosis and the progression into CAD.

Introduction

Cardiovascular disease (CVD), including myocardial infarction (MI) and stroke, are currently the leading causes of morbidity and premature mortality worldwide and their incidence is expected to increase.[1] Currently, 60-70% of major cardiovascular events cannot be prevented with therapeutic strategies. This has contributed to the continuing research to unravel the pathophysiology of atherosclerosis to indentify and develop new treatment strategies to further improve cardiovascular outcome.

Chemokines (chemotactic cytokines) are small heparin-binding proteins that direct the movement of circulating leukocytes towards sites of inflammation, such as the atherosclerotic plaque or injury. The most thoroughly characterized CC chemokine is monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2). It is a potent agonist for monocytes, dendritic cells, memory T cells, and basophils.[2] MCP-1 is present in macrophage-rich atherosclerotic plaques,[3,4] and minimally oxidized low-density lipoprotein (LDL) cholesterol induces the production of MCP-1 in endothelial and smooth-muscle cells. MCP-1 has thus emerged as a link between oxidized lipoproteins and the recruitment of foam cells to the vessel wall. The relation between CCL2 polymorphisms, MCP-1 serum levels and cardiovascular risk, however, remains unclear. The inconsistency of these results could be due in part to the different study design, inclusion criteria or insufficient power to detect associations between polymorphisms and cardiovascular risk. To clarify the role of MCP-1 in the pathophysiology of atherosclerosis, we have conducted an analysis of CCL2 genetic variation and its relation to serum levels of MCP-1 and cardiovascular risk in the Epic-Norfolk cohort, a large, longitudinal, observational, community-based study.

Materials and methods

Between 1993 and 1997, the EPIC-Norfolk cohort study was performed. This was a prospective population study of 25,663 male and female inhabitants of Norfolk, United Kingdom, between the ages of 45 and 79 years who completed a baseline questionnaire survey and attended a clinic visit. EPIC-Norfolk is part of the 9-country collaborative EPIC study designed to investigate dietary and other determinants of cancer. Additional data were obtained to enable assessment of determinants of other diseases.

The study cohort was similar to UK population samples with regard to many characteristics, including anthropometry, blood pressure, and lipids, but with a lower proportion of smokers. Participants were recruited by mail from age-gender registers of general practices. At the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire, and additional data collection was performed by trained nurses at a clinic visit as described previously. All individuals have been flagged for mortality at the UK Office of National Statistics, with vital status ascertained for the entire cohort. Death certificates for all decedents were coded by trained nosologists according to the International Classification of Diseases (ICD) 9th revision. Death was considered due to CAD if the underlying cause was coded as ICD 410 to 414. In addition, participants admitted to a hospital were identified by their unique National Health Service number by data linkage with ENCORE (East Norfolk Health Authority database), which identifies all hospital contacts throughout England and Wales for Norfolk residents. Participants were identified as having CAD during follow-up if they had a hospital admission and/or died with CAD listed as an underlying cause. We report results with follow-up up to January 2003, an average of 6 years. The Norwich District Health Authority Ethics Committee approved the study, and all participants gave signed informed consent.

Participants

For the present nested case-control study, we identified apparently healthy individuals who developed fatal or nonfatal CAD during follow-up. Apparently healthy individuals were defined as study participants who did not report a history of heart attack or stroke at the baseline clinic visit. Control subjects were apparently healthy study participants who remained free of CAD during follow-up. Two control subjects were matched to each case subject by gender, age (within 5 years), and date of visit (within 3 months).

Biochemical Analyses

Non-fasting blood samples were taken by vein puncture into serum tubes. Blood samples were processed for assay at the Department of Vascular Medicine, Academic Medical Center, Amsterdam, Netherlands, and stored at minus 80° Celsius before analysis. Serum levels of total cholesterol, HDL cholesterol, and triglycerides were measured on fresh samples with the RA 1000 autoanalyzer (Bayer Diagnostics, Basingstoke, United Kingdom). LDL cholesterol levels were calculated with the Friedewald formula.[5] Serum levels of apoA-I and apoB were measured by rate immunonephelometry (Behring Nephelometer BNII, Marburg, Germany) with calibration traceable to the International Federation of Clinical Chemistry primary standards.[6] The interassay coefficients of variation of the apoA-I and apoB measurements were 5% and 3%, respectively. C-reactive protein (CRP) levels were measured as described previously.[7] Serum MCP-1 levels were determined by a luminex biorad assay. All samples above the 95th percentile were repeated. Samples were analyzed in random order to avoid systematic bias. Researchers and laboratory personnel had no access to identifiable information and could identify samples by number only.

CCL2 genotyping

CCL2 lies on the long arm of chromosome 17. It has 3 exons extending over ≈2000bp. It has both distal and proximal regulatory elements important for cytokine and constitutive activity, respectively. We selected 7 common CCL2 SNPs (RS no: 2857654, 1024611, 1024610, 3760399, 3760396, 2857657, 2530797) spanning the gene based on

previously published selection criteria.[8] MCP-1-2835, MCP-1-2578, MCP-1-2136, MCP-1-1811 are located on the distal regulatory region, wheras MCP-1-927, MCP-1+764 and MCP-1+3726 are located on a promoter, firt intron and 3 ´flanking region respectively. CCL2 genotyping was performed on coded DNA samples by laboratory personnel blinded to clinical information. For the EPIC-Norfolk study genotyping was conducted by KBioscience (http://www.kbioscience.co.uk) using KASPar technology. Genotyping was carried out on an ABI 7900 system, using Assay by Design™ assays (Applied Biosystems, Foster City, CA, USA). Allelic discrimination was performed using FAM and VIC as fluorophore. PCR conditions were denaturation for 10 min at 95°C, followed by 40 cycles (30 sec 92°C, 45 sec 60°C). PCR assay mix was obtained from Applied Biosystems.

Statistical Analysis

Baseline characteristics were compared between case and control subjects with a mixed-effect model for continuous variables or conditional logistic regression for categorical variables. Because MCP-1, triglycerides and CRP levels had a skewed distribution, values were log-transformed before statistical analysis. Our primary objective was to determine the relationships between CCL2-genotype, MCP-1 serum levels, cardiovascular risk factors, and the risk of CAD. Therefore, the relation between MCP-1 genotype and MCP-1 levels were determined by a linear regression model. Multivariable-adjusted Cox regression analyses were conducted to examine the partial contribution of CCL2 genotype to risk of CAD. Data was analyzed with SPSS version 15.0.

From the unphased SNP genotype data, haplotype frequencies and their effect on MCP1 concentrations and MI status were estimated using weighted linear or logistic regression, respectively.[9,10] In short, haplotype effects and haplotype frequencies were jointly estimated using an expectation-maximization (EM) algorithm in which individual haplotypes were handled as missing data. In the first expectation (E) step, the initial probabilities were calculated using Bayes' theorem and estimated haplotype frequencies. In the following E steps, the posterior probabilities of haplotype pairs

compatible with an individual's genotype were calculated based on the phenotype of the individual subject. In the maximization (M) steps, the haplotype effects were estimated using a weighted linear or logistic regression model, where the posterior probabilities functioned as weights. The E and M steps were alternated until convergence. A probability value <0.05 was considered to be statistically significant.

Results

Serum samples from 985 case subjects and 1778 matched control subjects were available. From these individuals, 797 case subjects were matched to 2 control subjects each, whereas 188 case subjects could only be matched to 1 control. Matching ensured that age and gender were comparable between case subjects and control subjects. As expected, individuals who developed CAD during follow-up were more likely than control subjects to smoke and to have diabetes mellitus. Likewise, levels of total cholesterol, LDL cholesterol, triglycerides, apoB/apo-AI ratio, systolic and diastolic blood pressure, BMI, waist circumference and CRP were significantly higher in case subjects than in control subjects, whereas HDL cholesterol was significantly lower in case subjects than in control subjects (Table 1). Table 2 shows the distribution of cardiovascular risk factors as stratified by serum MCP-1 quartiles. Serum MCP-1 levels correlated best with waist circumference, triglycerides and apo-B/apo-AI ratio. There was a weak but significant linear relationship between waist circumference, diastolic and systolic blood pressure and serum MCP-1 levels.

Table 1. Baseline Characteristics of Study Participants

	Controls	Cases	P
Total no. of participants, n	**1778**	**985**	**-**
Age, years	65.3 ± 7.7	65.3 ± 7.8	Matched
Women, n (%)	37.7	35.8	Matched
Body mass index, kg/m^2	26 ± 3.5	27 ± 3.9	<0.001
Waist circumference, cm	91 ± 11	94 ± 12	<0.001
Smoker, (%)	8.5	15.0	<0.001
Diabetes, n (%)	1.6	6.3	<0.001
SBP, mmHg	139 ± 18	144 ± 19	<0.001
DBP, mmHg	83 ± 11	86 ± 12	<0.001
Total cholesterol, mmol/l	6.3 ± 1.1	6.4 ± 1.2	<0.001
LDL-cholesterol, mmol/l	4.1 ± 1.0	4.3 ± 1.0	<0.001
HDL-cholesterol, mmol/l	1.37 ± 0.40	1.26 ± 0.37	<0.001
Triglycerides, mmol/l	1.60 (1.1 - 2.2)	1.80 (1.3 - 2.6)	<0.001
C-reactive protein, mg/l	1.50 (0.7 – 3.1)	2.25 (1.0 – 5.0)	<0.001
Apo-B / Apo- AI ratio	0.81 ± 0.24	0.91 ± 0.26	<0.001

Data are presented as mean (±SD) or number for variables with a normal distribution. Skewed variables are presented as median with the 25th to 75th percentile and log transformed for the p-value calculations. The p-values are for mixed effect model on on continuous variables, and for conditional logistic regression on dichotomous variables. SBP= systolic blood pressure, DBP=diastolic blood pressure, LDL= low-density lipoprotein, HDL= high-density lipoprotein, Apo-B= Apolipoprotein B, Apo-AI= Aoplipoprotein AI, MCP-1= Monocyte chemoattractant protein-1.

Table 2. Clinical determinants of MCP-1 Serum Concentration.

Variable	MCP-1 serum concentration quartiles (pg/ml)				P	R	P†
	1 (<38.3)	2 (38.3-51.2)	3 (51.2-67.8)	4 (>67.8)			
Total no. of participants	695	686	665	717	-	-	-
Age, years	65.2 ± 7.7	65.1 ± 8.0	65.4 ± 7.7	65.6 ± 7.6	0.195	0.027	0.158
Women, n (%)	264 (38)	258 (38)	240 (36)	257 (36)	0.331	-	-
Body mass index, kg/m2	26.4 ± 3.5	26.5 ± 3.6	26.7 ± 3.9	26.8 ± 3.5	0.016	0.053	0.005
Waist circumference, cm	91.1 ± 11.5	91.6 ± 11.2	92.5 ± 11.9	93.2 ± 11.7	<0.001	0.080	<0.001
Cigarette smoking	64 (9)	74 (11)	73 (11)	88 (12)	0.073	-	-
Diabetes, n (%)	19 (3)	26 (4)	27 (4)	19 (3)	0.337	-	-
SBP, mmHg	139.4 ± 19	140 ± 19	141 ± 19	142 ± 17	0.017	0.045	0.018
DBP, mmHg	83 ± 12	84 ± 11	85 ± 11	85 ± 11	0.079	0.041	0.033
Total cholesterol, mmol/l	6.3 ± 1.2	6.3 ± 1.1	6.3 ± 1.1	6.4 ± 1.2	0.151	0.021	0.268
LDL-cholesterol, mmol/l	4.2 ± 1.0	4.1 ± 1.0	4.2 ± 1.0	4.2 ± 1.0	0.550	0.007	0.707
HDL-cholesterol, mmol/l	1.33 ± 0.39	1.35 ± 0.40	1.34 ± 0.38	1.31 ± 0.40	0.267	-0.31	0.105
Triglycerides, mmol/l	1.6 (1.1 -2.2)	1.7 (1.2 -2.3)	1.7 (1.2 -2.4)	1.8 (1.3 -2.4)	<0.001	0.087	<0.001
C-reactive protein, mg/l	1.8 (0.8 – 3.7)	1.5 (0.7 -3.9)	1.7 (0.8 -3.9)	1.7 (0.8 -3.6)	0.994	0.002	0.933
Apo-B/apo-AI ratio	0.83 ± 0.24	0.84 ± 0.25	0.84 ± 0.25	0.88 ± 0.26	<0.001	0.085	<0.001

Values are mean (±SD) or number. Data for C-reactive protein and triglycerides are presented as median (± interquartile range). P = p-value for linearity between MCP-1 serum concentration quartiles and risk factor levels; R = Pearson's or Spearmans correlation between MCP-1 serum concentration and risk factors, and the corresponding p-value. MCP-1= Monocyte chemoattractant protein-1, SBP= systolic blood pressure, DBP=diastolic blood pressure, LDL= low-density lipoprotein, HDL= high-density lipoprotein, Apo-B= Apolipoprotein B, Apo-AI= Aoplipoprotein AI.

CCL2 polymorphisms and Haplotype frequencies

CCL2 polymorphisms nucleotide changes and the function of the region where the polymorphisms are located are shown in Table 3. Slight differences for the minor allele frequencies (MAF) between cases and controls were found for MCP-1-2835, MCP-1-

2578, MCP-1-1811 and MCP-1+764. Chi-squared testing for deviations of the Hardy Weinberg equilibrium in the control group showed a significant p-value for MCP-1-2136. All other polymorphisms in the control population were in complete Hardy Weinberg Equilibrium.

We estimated CCL2 genotype combinations to determine the effect of specific haplotypes on MCP-1 serum concentration and CAD. The six most common haplotype combinations and the corresponding frequencies are shown in table 4.

Table 3. CCL2 genotypes characteristics

Gene position	RS no.	Nucleotide change	Variant type	MAF cohort	MAF control	X^2	HWE P	Genotyping success, %
MCP-1-2835	2857654	C > A	DRR	0.28	0.27	0.01	0.92	93.6
MCP-1-2578	1024611	A > G	DRR	0.28	0.27	0.01	0.92	94.3
MCP-1-2136	1024610	A > T	DRR	0.20	0.20	5.58	0.02	93.5
MCP-1-1811	3760399	A > G	DRR	0.04	0.05	3.95	0.05	94.5
MCP-1-927	3760396	G > C	Promoter	0.21	0.21	0.60	0.44	94.6
MCP-1+764	2857657	C > G	First intron	0.20	0.19	1.59	0.21	94.4
MCP-1+3726	2530797	T > C	3´Flanking	0.38	0.38	0.21	0.65	93.7

MAF cohort = Minor allele frequencies calculated in the cases and controls and of the controls only (MAF control). Chi-squared distribution with 1 degree of freedom (X^2) to test for deviations from Hardy–Weinberg equilibrium with corresponding p-value (HWE P). MAF = minor allele frequency, DDR = distal regulatory region, HWE = Hardy Weinberg equilibrium.

Table 4. CCL2 genotypes and estimated genotype combination frequencies

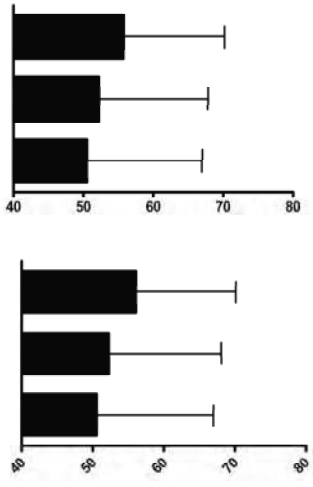
Gene position and RS no. of tested SNPs with nucleotides detected							Haplotype	Freq %
-2835 2857654	-2578 1024611	-2136 1024610	-1811 3760399	-927 3760396	+764 2857657	+3726 2530797		
A	G	A	A	G	C	T	H1	27.8
C	A	A	A	C	C	T	H2	21.0
C	A	T	A	G	G	C	H3	19.6
C	A	A	A	G	C	C	H4	18.5
C	A	A	A	G	C	T	H5	7.9
C	A	A	G	G	C	T	H6	4.4

CCL2 genotype, haplotypes and serum MCP-1 concentration

For the CCL2 polymorphism MCP-1-2136, the TT genotype was associated with serum MCP-1 levels that were 12% higher than those associated with the AA genotype (beta 2.01; p=0.009) (table 5). A significant difference in serum MCP-1 level was also associated with the MCP-1+764 polymorphism (GG vs. CC genotype, 13% increase; beta 2.10; p=0.007). Additional haplotype analysis showed a beta of -1.61 for serum MCP-1 levels (p=0.033) for haplotype 4. MCP-1+764 remained significant after correction for the parameters correlating with serum MCP-1 concentration in table 2 and after correction of the Framingham Risk Score (p=0.0044 and p=0.016 respectively). Full numbers and percentages for individuals according to polymorphism and MCP-1 serum concentration quartiles are given in supplemental table 1.

Table 5. Serum Levels of MCP-1 according to CCL2 genotype and genotype combinations.

CCL2 genotype combination	Participants, No.	Median ± interquartile range	ΔMCP-1, %	Beta (95% CI)	P	P *	P ‡
H4	511			-1.61 (-3.08-0.13)	0.033	0.560	0.046
CCL2 genotype							
MCP-1-2136 A/T	2584			2.01 (0.51-3.51)	0.009	0.066	0.022
MCP-1-2136 TT	120		9				
MCP-1-2136 AT	785		2				
MCP-1-2136 AA	1679		-1				
MCP-1+764 C/G	2609			2.10 (0.58-3.62)	0.007	0.044	0.016
MCP-1+764 GG	106		10				
MCP-1+764 CG	805		2				
MCP-1+764 CC	1698		-1				



Beta for MCP-1 serum concentration according to H4 and CCL2 polymorphisms. * Adjustment for BMI, waist circumference, systolic blood pressure, diastolic blood pressure, triglycerides and apo-B/apo-A1 ratio. ‡ Adjustment for the Framingham Risk Score.

Supplemental Table 1. Numbers of study participants in MCP-1 serum concentration quartiles according to CCL2 genotype.

	MCP-1 serum concentration quartiles (pg/ml)			
	1	2	3	4
	< 38.3	38.3 – 51.2	51.2 – 66.8	66.8 – 1348.0
Total no. of patients, n	695	686	665	717
MCP-1-2835 C/A (RS 2857654)				
-MCP-1-2835 CC, n (%)	331 (48)	332 (48)	339 (51)	347 (48)
-MCP-1-2835 CA, n (%)	270 (39)	264 (39)	252 (38)	259 (36)
-MCP-1-2835 AA, n (%)	51 (7)	49 (7)	33 (5)	58 (8)
-MCP-1-2835 undetermined, n (%)	43 (6)	41 (6)	41 (6)	53 (7)
MCP-1-2578 A/G (RS 1024611)				
-MCP-1-2578 AA, n (%)	330 (48)	335 (49)	343 (52)	352 (49)
-MCP-1-2578 AG, n (%)	271 (39)	266 (39)	256 (39)	259 (36)
-MCP-1-2578 GG, n (%)	53 (8)	51 (7)	32 (5)	58 (8)
-MCP-1-2578 55 undetermined, n (%)	41 (6)	34 (5)	34 (5)	48 (7)
MCP-1-2136 A/T (RS 1024610)				
-MCP-1-2136 AA, n (%)	443 (64)	420 (61)	394 (59)	422 (59)
-MCP-1-2136 AT, n (%)	186 (27)	197(29)	196 (30)	206 (29)
-MCP-1-2136 TT, n (%)	25 (4)	27 (4)	32 (5)	36 (5)
-MCP-1-2136 undetermined, n (%)	41 (6)	42 (6)	43 (7)	53 (7)
MCP-1-1811 A/G (RS 3760399)				
-MCP-1-1811 AG, n (%)	63 (9)	57 (8)	55 (8)	57 (8)
-MCP-1-1811 GG, n (%)	601 (87)	593 (86)	574 (86)	610 (85)
-MCP-1-1811 undetermined, n (%)	31 (5)	36 (5)	36 (5)	50 (7)
MCP-1-927 G/C (RS 3760396)				
-MCP-1-927 GG, n (%)	429 (62)	410 (60)	394 (59)	415 (60)
-MCP-1-927 GC, n (%)	205 (30)	223 (33)	193 (29)	221 (31)
-MCP-1-927 CC, n (%)	23 (3)	21 (3)	44 (7)	35 (5)
-MCP-1-927 undetermined, n (%)	38 (6)	32 (5)	34 (5)	46 (6)
MCP-1+764 C/G (RS 2857657)				
-MCP-1+764 CC, n (%)	447 (64)	425 (62)	400 (60)	426 (59)
-MCP-1+764 CG, n (%)	188 (27)	204 (30)	201 (30)	212 (30)
-MCP-1+764 GG, n (%)	23 (3)	22 (3)	30 (5)	31 (4)
-MCP-1+764 undetermined, n (%)	37 (5)	35 (5)	34 (5)	48 (7)
MCP-1+3726 T/C (RS 2530797)				
-MCP-1+3726 TT, n (%)	251 (36)	232 (34)	235 (35)	266 (37)
-MCP-1+3726 TC, n (%)	305 (44)	324 (47)	283 (43)	302 (42)
-MCP-1+3726 CC, n (%)	100 (14)	90 (13)	107 (16)	94 (13)
-MCP-1+3726 55 undetermined, n (%)	39 (6)	40 (6)	40 (6)	55 (8)

Data are presented as numbers.

CCL2 genotype, haplotypes and the risk of CAD

For the CCL2 polymorphism MCP-1+3726 the CC genotype was associated with a significant odds ratio of 1.31 (95% CI 1.05-1.65; p=0.020) for CAD in a recessive genetic model (table 6). This association remained significant after correction for variables in table 1 and after correction of the Framingham Risk Score. The genotype combination haplotype 5 showed significant odds ratios for future CAD unadjusted, after multivariable adjustment according to significant variables in table 1 and after adjustment for the Framingham Risk Score (table 6). Since previous analyses reported differences in results depending on gender, we performed a subgroup analysis for the risk of future CAD according to sex. Analyses for males showed similar associations between both MCP-1-2835 AA + AC (OR 1.28; 95% CI, 1.05-1.57; p=0.017) and MCP-1-2578 GG + GA (OR 1.26; 95% CI, 1.03-1.53; p=0.027) in a recessive genetic model. These associations remained significant after correction for significant variables in table 1 and after correction of the Framingham Risk Score. For women, we found a significant odds ratio of 1.59 (95% CI 1.11-2.27; p-value 0.011) for future CAD testing MCP-1+3726 CC in a recessive genetic model, that remained significant after multivariable correction and correction for the Framingham Risk Score (table 6). Full numbers and percentages for individuals according to polymorphism and cases or controls are given in supplemental table 2.

Table 6. Risk of Future Coronary Artery Disease according to CCL2 Polymorphism and combinations for Men and Women Pooled and Separately.

CCL2 Genotype	Unadjusted		Multivariable adjustment		FRS adjusted	
	OR (95% CI)	P	OR (95% CI)	P*	OR (95% CI)	P
Pooled						
MCP-1-2835 AA + AC	1.11 (0.95-1.31)	0.193	1.15 (0.96-1.37)	0.126	1.13 (0.95-1.34)	0.157
MCP-1-2578 GG + GA	1.11 (0.94-1.30)	0.210	1.24 (1.01-1.52)	0.040	1.11 (0.94-1.32)	0.205
MCP-1+3726 CC	1.31 (1.05-1.65)	0.020	1.30 (0.97-1.73)	0.077	1.31 (1.03-1.66)	0.026
Male						
MCP-1-2835 AA + AC	1.28 (1.05-1.57)	0.017	1.34 (1.07-1.68)	0.010	1.32 (1.07-1.62)	0.011
MCP-1-2578 GG + GA	1.26 (1.03-1.53)	0.027	1.46 (1.13-1.89)	0.004	1.28 (1.04-1.57)	0.022
MCP-1+3726 CC	1.15 (0.85-1.55)	0.367	1.06 (0.73-1.55)	0.766	1.14 (0.84-1.55)	0.408
Female						
MCP-1-2835 AA + AC	0.86 (0.65-1.14)	0.293	0.86 (0.64-1.17)	0.339	0.85 (0.64-1.13)	0.269
MCP-1-2578 GG + GA	0.88 (0.67-1.16)	0.363	0.93 (0.65-1.31)	0.660	0.87 (0.65-1.15)	0.317
MCP-1+3726 CC	1.59 (1.11-2.27)	0.011	1.83 (1.16-2.91)	0.010	1.58 (1.09-2.27)	0.015
CCL2 genotype combination						
H5	0.77 (0.60-1.00)	0.047	0.75 (0.57-0.97)	0.030	0.76 (0.59-0.98)	0.037

Odds ratios (ORs) for the risk of future CAD events. * Adjustment for waist circumference, body mass index, diabetes mellitus, systolic blood pressure, diastolic blood pressure, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, C-reactive protein, apo-B/apo-AI ratio and smoking status. FRS= Framingham risk score.

Supplemental Table 2. Numbers of study participants in the control and cases group according to CCL2 Polymorphisms.

Total no. of study participants, n	Controls 1778	Cases 985
MCP-1-2835 C/A (RS 2857654)		
-MCP-1-2835 CC, n (%)	888 (50)	461 (47)
-MCP-1-2835 CA, n (%)	663 (37)	382 (39)
-MCP-1-2835 AA, n (%)	122 (7)	69 (7)
-MCP-1-2835 undetermined, n (%)	105 (6)	73 (7)
MCP-1-2578 A/G (RS 1024611)		
-MCP-1-2578 AA, n (%)	897 (50)	463 (47)
-MCP-1-2578 AG, n (%)	668 (38)	384 (39)
-MCP-1-2578 GG, n (%)	126 (7)	68 (7)
-MCP-1-2578 55 undetermined, n (%)	87 (5)	70 (7)
MCP-1-2136 A/T (RS 1024610)		
-MCP-1-2136 AA, n (%)	1094 (62)	585 (59)
-MCP-1-2136 AT, n (%)	505 (28)	280(28)
-MCP-1-2136 TT, n (%)	82 (5)	38 (4)
-MCP-1-2136 undetermined, n (%)	97 (6)	82 (8.3)
MCP-1-1811 A/G (RS 3760399)		
-MCP-1-1811 AG, n (%)	156 (9)	76 (8)
-MCP-1-1811 AA, n (%)	1538 (87)	840 (85)
-MCP-1-1811 undetermined, n (%)	84 (5)	69 (7)
MCP-1-927 G/C (RS 3760396)		
-MCP-1-927 GG, n (%)	1055 (59)	593 (60)
-MCP-1-927 GC, n (%)	553 (31)	289 (29)
-MCP-1-927 CC, n (%)	81 (5)	42 (4)
-MCP-1-927 undetermined, n (%)	89 (5)	61 (6)
MCP-1+764 C/G (RS 2857657)		
-MCP-1+764 CC, n (%)	1104 (62)	594 (60)
-MCP-1+764 CG, n (%)	513 (29)	292 (30)
-MCP-1+764 GG, n (%)	72 (4)	34 (4)
-MCP-1+764 undetermined, n (%)	89 (5)	65 (7)
MCP-1+3726 T/C (RS 2530797)		
-MCP-1+3726 TT, n (%)	647 (36)	337 (34)
-MCP-1+3726 TC, n (%)	802 (45)	412 (42)
-MCP-1+3726 CC, n (%)	237 (13)	154 (16)
-MCP-1+3726 55 undetermined, n (%)	92 (5)	82 (8)

Data are presented as numbers.

Discussion

In this large prospective case-control study we found evidence supporting the notion that the chemokine MCP-1 is a pathogenic factor in human CAD. First we found that individuals possessing the MCP-1-2136T alleles or the MCP-1+764G allele had higher MCP-1 levels. Second, we found that homozygotes of the MCP-1-3726C allele have an increased risk of CAD and that the presence of the MCP-1-2835A allele and the MCP-1-2578G allele is associated with an increased risk of CAD. Finally, we observed that CCL2 genotype is associated with both MCP-1 serum concentrations and the risk of future CAD.

At present, the relation between CCL2 polymorphisms, MCP-1 serum levels and the risk of cardiovascular disease remains unclear. Several studies demonstrate an association between MCP-1 serum concentrations and a higher incidence of CAD.[8,11-13] CCL2 genotype variations could potentially modulate MCP-1 serum concentrations and function. Indeed, several studies reported an association between MCP-1-2578G and increased circulating levels of MCP-1,[14-16] while this could not be shown in other large case-cohort studies.[17,18] The latter is in line with our findings that the MCP-1-2578G is not associated with MCP-1 serum concentrations in our cohort. It should be noted, however, that baseline unstimulated serum MCP-1 concentrations may not be a reliable reflection of local tissue levels, particularly in the vicinity of an atherosclerotic plaque where an abundance of inflammatory mediators are being produced.

In the community-based Framingham Heart Study Offspring Cohort, McDermott et al demonstrated that the MCP-1-2136 and the MCP-1+764 significantly correlate with MCP-1 serum concentrations. We found similar correlations between the MCP-1-2136T allele, the MCP-1+764G allele and MCP-1 serum concentrations. However since the MCP-2136 allele frequencies were not in full Hardy Weinberg equilibrium in the control group of the cohort, possible ascertainment bias cannot be excluded. In addition to the effects of CCL2 polymorphisms on MCP-1 serum concentration levels

two case control studies and one cohort study published associations between MCP-1-2578G allele and atherosclerosis.[8,19,20] This is in line with our findings that the MCP-1-2578G allele is associated with future CAD in males. To our knowledge, this is the first study showing an association between MCP-1 2835A, and MCP-1+3726C and future CAD. The exact mechanism by which MCP-1 2835A or 2578G might increase CAD risk remains uncertain. Surprisingly, despite the substantial amount of research into the role MCP-1 in atherogenesis, nothing is known with regard to the functionality of the MCP-1 protein affected by any of the known SNPs. Of note, the increased propensity to atherosclerotic cardiovascular disease in MCP-1 2835 C/A or 2578 A/G carriers was only significant in males, whereas the homozygote possession of the MCP-1+3726C allele is associated with futue CAD in the entire cohort and in females only. Gender differences with regard to the relation between MCP-1 concentration, genotypes and CAD have been reported previously[8] and remain to be elucidated.

To futher clarify the role of CCL2 genotype and the risk of future CAD, we performed a haplotype analysis estimating the effect of CCL2 genotype combinations on MCP-1 serum concentrations and CAD risk. Haplotype frequencies were comparable with earlier studies.[8] Previous haplotype analyses showed associations of CCL2 genotype combinations and MCP-1 serum concentrations. In our analysis we have used the most common haplotype as our reference haplotype, making comparison with different studies difficult. However, changing the reference haplotype would be associated with higher concentrations of MCP-1 for H4 and a higher risk for future CAD for H5. The effect of both haplotypes remained significant after multivariable adjustment and FRS adjustment indicating that CCL2 genotype combinations can have additional effects on MCP-1 serum concentrations and CAD.

Conclusion

This large community based prospective study investigating the role of CCL2 genotype, MCP-1 serum concentrations and the risk of future CAD, validates associations between CCL2 genotype, MCP-1 serum concentrations and the risk of

CAD. We therefore provide firm evidence that MCP-1 is involved in the pathogenesis of atherosclerosis and/or the progression into CAD. This study acknowledges MCP-1 as possible target for prevention and or treatment of atherosclerosis.

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Chapter 4

A single bolus infusion of C-reactive protein increases gluconeogenesis and plasma glucose concentration in humans

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Abstract

Objective: Recently we reported that CRP elicits inflammatory and procoagulant responses in humans. In addition, CRP has been associated with the development of type 2 diabetes mellitus. To further explore interactions between CRP and glucose handling, we evaluated the effects of CRP infusion on glucose metabolism in humans.

Methods: Seven healthy Caucasian male volunteers (age 39.3 ± 16.9 years) received a single bolus infusion of 1.25 mg/kg purified recombinant human (rh)CRP or CRP-free diluent in a cross-over design.

Results: CRP infusion induced an inflammatory response which was followed by increased plasma concentrations of norepinephrine (3 hours) and cortisol (4 hours). Concomitantly, plasma concentrations of insulin and C-peptide decreased transiently. These metabolic changes increased plasma glucose concentrations from 8 hours after CRP infusion, which was preceded by an increased rate of glucose appearance which was a direct consequence of increased gluconeogenesis.

Conclusions: CRP infusion induces an inflammatory response followed by increased norepinephrine and cortisol levels which results in increased gluconeogenesis. This finding implies that elevated levels of CRP in humans may in fact contribute to altered glucose metabolism and thereby may contribute to the induction of type 2 diabetes mellitus.

Introduction

Type 2 diabetes mellitus is a major risk factor for atherosclerotic disease. Even, nondiabetic subjects with an acute coronary syndrome exhibit a high prevalence of disturbed glucose tolerance.[1] Both conditions, chronic cardiovascular disease as well as acute coronary syndromes are characterized by increased C-reactive protein (CRP) levels. In fact, CRP has recently emerged as a strong and independent predictor for cardiovascular risk as well as for the development of type 2 diabetes mellitus.[2] However, since these are all observational studies they do not prove a causal relation between CRP and the development of type 2 diabetes mellitus. Interestingly, evidence from experimental studies has accumulated placing CRP within the atherosclerotic plaque, whereas other studies demonstrated that CRP elicits a wide array of atherothrombotic effects. All these findings were verified in human subjects, and infusion of highly purified recombinant human (rh)CRP has pronounced effects on pro-coagulant and inflammatory pathways.[3] In addition to this, clinical data also suggest that lowering of CRP translates into further cardiovascular benefit.[4] Taken together, these data gave birth to the controversial notion that CRP may actually be a mediator in cardiovascular disease, rather than merely a marker of cardiovascular risk. Given the strong base of epidemiological and experimental evidence linking CRP as causal agent of atherosclerosis as well as the development of type 2 diabetes mellitus, combined with the fact that nondiabetic subjects with an acute coronary syndrome and concomitant elevated CRP levels are associated with a high prevalence of glucose intolerance, we explored in a proof-of-principle study whether pathophysiologically relevant concentrations of CRP, as seen in patients with an acute cardiovascular event, exerts direct effects on glucose handling in humans.

Materials and methods

Ethical Issues and Safety Experiments

The study protocol was approved by the Institutional Review Board (IRB) at the Academic Medical Center in Amsterdam as well as the Central Committee on Research involving Human Subjects (CCMO) in the Netherlands. In view of the fact that the present study was performed before the European Union (EU) Clinical Trial Directive (CTD) came into law (1st march 2006) in the Netherlands the study was approved according to the procedures with national Dutch laws. Consequently, the IRB of our hospital together with the CCMO of our country functioned as the competent authority that evaluated at that time such clinical studies and carefully reviewed the study protocol which eventually lead to approval. In accordance with EU CTD regulations, national Dutch laws also require pre-human toxicology testing. Therefore, we performed these tests in mice and rabbits with CRP concentrations more than four times higher than peak concentrations obtained in humans, in which we observed no toxicological effects.[3]

Purification and safety control of the rhCRP solution

The rhCRP (BiosPacific, Emeryville, CA, USA) was supplied in 20 mM Tris, 140 mM NaCL, 2 mM $CaCl_2$, pH 7.5 and 0.05% (wt/vol) sodium azide and revealed a single 23 kDa band (>99%) after CBBR-staining (1 μg; SDS-polyacrylamide gel). Before purification, the host cell protein concentration was 85 p.p.m., as determined by a high-sensitive ELISA in accordance with manufacturers' instructions (Cygnus Technologies Inc., Southport, NC, USA). Subsequently, the rhCRP was purified using size exclusion chromatography to remove contaminants including endotoxin and sodium azide (Univalid Inc., Leiden, The Netherlands). Purity and stability was evaluated using sequential high-performance liquid chromatography and Time-of-Flight mass spectrometry, showing no other protein fractions besides the CRP-pentamer. The final concentration of endotoxin was below 1.5 endotoxin units (EU)/mL as evaluated by Limulus assay (Turbidimetric kinetic method; ACC Inc., East Falmouth, MA, USA).

The rhCRP was stored in a $CaCl_2$ containing buffer (pH 8.5) at 0-4°C degrees and all experiments were performed within 4 weeks after rhCRP-purification.

Study Design

Seven healthy Caucasian male volunteers (age 39.3 ± 16.9 years) were enrolled after written informed consent was obtained. Subjects did not have diabetes mellitus, hypertension, congestive heart failure or febrile illness and did not use any medication. Subjects abstained from alcohol and caffeine-containing beverages for at least 24 hours prior to the study. Subjects were randomly assigned to receive a single bolus of 1.25 mg/kg rhCRP or CRP-free diluent in a crossover design with a period of 4 weeks between both study visits. All subjects followed a diet with at least 250 grams carbohydrates for 3 days prior to the study.

Study Procedures

Approximately 14½ hours before rhCRP or diluent infusion, participants were instructed to have their last meal. At t = -14½ hours blood was drawn for the background enrichment of ^2H in body water, followed by ingestion of 1 g/kg body water ^2H_2O (99% pure, Cambridge Isotopes, Cambridge, MA, USA) at intervals of 30 minutes until a total dose of 5 g/kg body water was reached. The total body water content in males was estimated to be 60% of body weight.

The next morning a catheter was inserted into an antecubital vein of each arm. At 8.00 a.m. (t = -2½ hours), blood was drawn for assessment of background enrichment of [6,6-$^2H_2$]glucose. Subsequently, a primed (1.6 mg/kg), continuous (1.2 mg/kg/hr) infusion of [6,6-$^2H_2$]glucose (99% enriched, Cambridge Isotope Laboratories, MA, USA) dissolved in sterile isotonic saline, was initiated using a calibrated syringe pump (Perfusorâ Secura FT, B. Braun, Melsungen, Germany) through a Millipore filter (size 0.2 mm; Minisart, Sartorius, Göttingen, Germany). From 10.10 a.m. (t = -20 minutes) three blood samples were collected at intervals of 10 minutes for determination of plasma glucose concentration, [6,6-$^2H_2$]glucose enrichment and ^2H_2O enrichment in body water. Blood samples for the measurement of gluco-and counter regulatory

hormones were also collected. At 10.30 a.m. (0 hours), a bolus of rhCRP (1.25 mg/kg body weight) or CRP-free diluent was administered intravenously. Hereafter blood samples were collected on t = 1, 2, 3, 4, 6, 8 and 9 hours. After the last blood withdrawal at 19.30 pm (9 hours) the study ended.

Laboratory Analysis

Blood samples for measurement of gluconeogenesis were deproteinized by adding an equal amount of 10% perchloric acid. Blood for [6,6-$^2H_2$]glucose enrichment and hormone concentration measurements was collected in heparinized tubes. For determining levels of free fatty acids (FFA) plasma was collected in K-EDTA tubes. Samples were kept on ice, centrifuged, snap frozen and stored at -20 °C.

Enrichments of plasma [6,6-$^2H_2$]glucose, ^2H_2O and deuterium at the C5 position of glucose were determined as previously described.[5] Briefly, plasma samples for glucose enrichment of [6,6-$^2H_2$]glucose and plasma glucose concentration were measured as the aldonitril penta-acetate derivative of glucose in deproteinized plasma using xylose as an internal standard. Glucose was monitored at m/z 187 and 189. The enrichment of [6,6-$^2H_2$]glucose was determined by dividing the peak area of m/z 189 by the peak area of m/z 187 and correcting for natural enrichments. To measure deuterium enrichment at the C5 position, glucose was converted to hexamethylenetetramine (HMT).[6] HMT was injected into a gas chromatograph mass spectrometer. Separation was achieved on an AT-Amine column (30 m x 0.25 mm, d_f 0.25 μm). The deuterium enrichment in the plasma water was measured after conversion of water and carbide to acetylene. All isotopic enrichments were measured on a gas chromatograph mass spectrometer (model 6890 gas chromatograph coupled to a model 5973 mass selective detector, equipped with an electron impact ionization mode, Hewlett-Packard, Palo Alto, CA, USA).

Plasma insulin concentration was determined with a chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA). C-peptide was determined by RIA (RIA-coat C-peptide, Byk-Sangtec Diagnostica). Cortisol was measured by enzyme-immunoassay on an Immulite analyzer (DPC),

intra-assay CV 2–4%. Glucagon was determined by RIA (Linco Research, Mo, USA). Norepinephrine and epinephrine were determined by an in-house HPLC method. Plasma FFA were measured by an enzymic method (NEFAC; Wako Chemicals, VA, USA).

CRP was measured by a high-sensitivity immunoturbidimetric assay (Roche Diagnostics Corporation, Basel, Switzerland), while CRP concentrations in excess of 10 mg/L (after rhCRP- infusion) were assayed by immuno-nephelometry (P800 analyzer, Roche Diagnostic Corporation). Circulating cytokine concentrations were assessed with the luminex method (Bioplex Human Cytokines 1x96 wells, catalog number X500000 FFS, Bio-Rad Laboratories Inc, CA, USA).

Calculations

Glucose appearance rate (glucose R_a) was calculated from the dilution of labeled glucose in plasma. The Steele equation for steady-state conditions was used for the measurement of glucose R_a before rhCRP or diluent infusion and non-steady-state calculations were used after rhCRP or diluent infusion. The fraction of total extracellular glucose pool (pV) was assumed to be 40 mL/kg. The rate of GNG was calculated by multiplying the glucose R_a by fractional gluconeogenesis (GNG). The fractional GNG=100%x([^{2}H] enrichment on C5 of glucose)/([^{2}H] enrichment in plasma water). The rationale has been discussed in detail by Landau.[5] In brief, glucose produced during plasma ^2H_2O enrichment by gluconeogenesis will be labeled with deuterium at the C5 position. Glucose molecules produced by gluconeogenesis and glycogenolysis will be labeled with deuterium at the C2 position. The ratio of C5 and C2 enrichment of glucose constitutes fractional gluconeogenesis. Alternatively, fractional gluconeogenesis can be calculated by the ratio of C5 enrichment of glucose and plasma ^2H_2O enrichment. A requirement for the latter method is the complete equilibration of plasma ^2H_2O enrichment with C2 enrichment of glucose.

Statistical Analysis

Descriptive statistics between CRP and diluent infusion were compared by means of 2-tailed paired *t* tests or a non-parametric test (Wilcoxon test) was used in case of non normal distribution. Statistical analysis of glucose metabolism parameters for individual subjects between CRP and diluent infusion over time was performed using analysis of variance (ANOVA) for repeated measures. If such analysis revealed significant differences, a Wilcoxon test was used to locate the specific difference. All statistics were performed with SPSS software (SPSS for Windows 11.5.1, SPSS Inc., Chicago, IL, USA). Data are expressed as means ± SD.

Results

Clinical characteristics

Baseline characteristics were determined prior to CRP and diluent infusion (table 1). The seven healthy Caucasian male volunteers did not experience symptoms or side-effects during the study. Furthermore, body temperature, blood pressure and heart rate remained stable upon CRP infusion.

Plasma CRP and cytokine concentrations

After CRP infusion plasma concentrations of CRP increased to 23.9 ± 4.2 mg/L at 1 hour (figure 1A). TNFα concentrations did not change upon CRP infusion (figure 1B), whereas a transient rise in IL-6 as well as IL-8 was observed, peaking at 4 hours (figure 1B).

Table 1. Baseline Characteristics of the Seven Study Subjects on Both Study Days

	Control study day	CRP study day
Age, y	39.3 ± 16.9	-
BMI, kg/m^2	27.3 ± 5.2	27.4 ± 5.2
Body fat, %	24.7 ± 9.9	24.8 ± 10.2
Body temperature, ^{o}C	36.9 ± 0.5	36.8 ± 0.5
Heart rate, bpm	82 ± 11	79 ± 10
Systolic BP, mm Hg	123 ± 8	124 ± 11
Fasting plasma glucose, mmol/L	5.3 ± 0.6	5.4 ± 0.4
Fasting plasma insulin, pmol/L	90 ± 43	88 ± 45
Fasting plasma glucagon, ng/L	78 ± 28	73 ± 25
Fasting plasma cortisol, nmol/L	394 ± 83	374 ± 87
C-peptide, pmol/L	907 ± 180	881 ± 374
FFA, mmol/L	0.44 ± 0.15	0.45 ± 0.14
Epinephrine, nmol/L	0.08 ± 0.01	0.19 ± 0.07
Norepinephrine, nmol/L	1.15 ± 0.57	1.60 ± 1.03
*hs*CRP, mg/L	1.9 ± 2.0	1.8 ± 1.9
LDL cholesterol, mmol/L	3.1 ± 1.1	3.1 ± 1.1
HDL cholesterol, mmol/L	1.3 ± 0.2	1.3 ± 0.1
Triglycerides, mmol/L	1.0 ± 0.2	0.9 ± 0.2

Data are expressed as means±SD. BMI, body mass index; BP, blood pressure; bpm, beats per minute; FFA, free fatty acids; HDL, high-density lipoprotein; *hs*CRP, high sensitive C-reactive protein; LDL, low-density lipoprotein.

Glucose metabolism

After CRP infusion, plasma glucose concentrations increased with 10% from 8 hours onwards compared with diluent infusion (figure 2A). Baseline values for glucose R_a were comparable on both study days (figure 2B). At the same time there was a modest increase in glucose R_a of 11% compared to the control experiments, which showed a continuous decline in glucose R_a. Concomitantly, glucose R_d increased slightly by 4% after CRP infusion (figure 2C). These changes were preceded by increased gluconeogenesis from 6 hours after CRP infusion resulting in a 10% increase when compared with the control experiments (figure 2D). CRP infusion did not affect glycogenolysis (figure 2D).

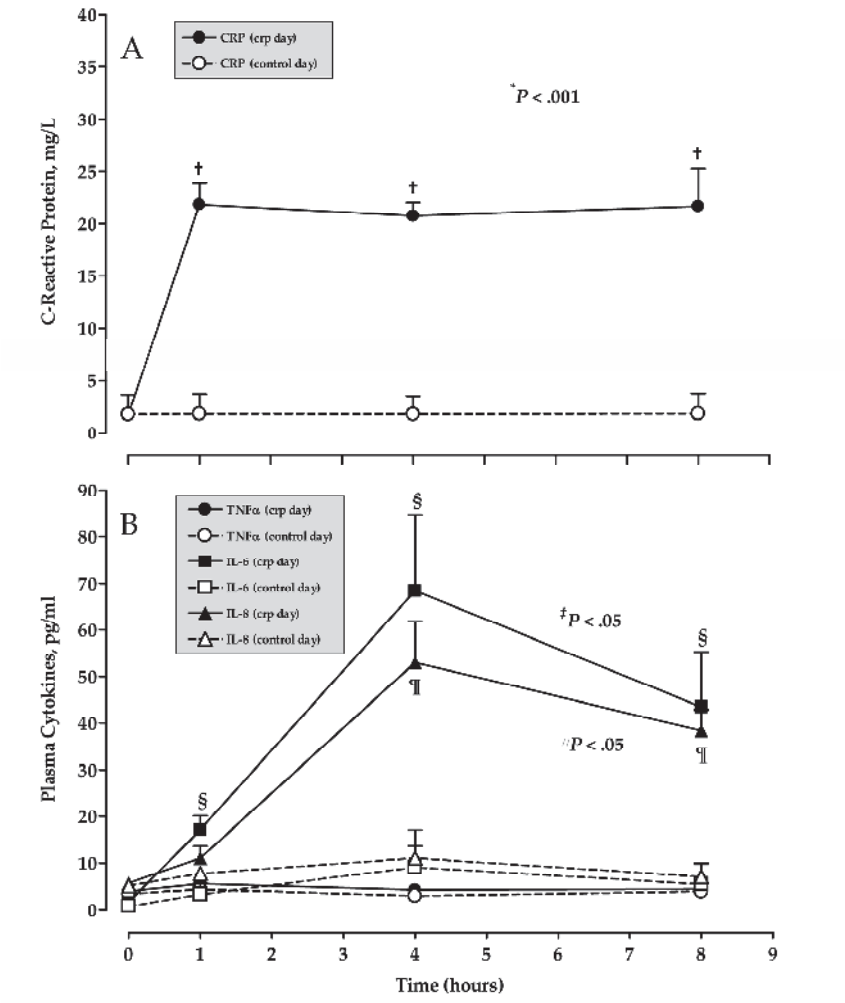

Figure 1. Effect of CRP infusion on inflammation activation.

(A) plasma CRP concentrations ($^{*}P < .001$ indicates difference between the CRP infusion day (●) and control day (○) by ANOVA for repeated meassures, † indicates difference between time points by Wilcoxon test, $P < .01$, **(B)** plasma cytokines concentrations: TNFα, non significant (NS) difference between interventions and time points on the CRP infusion day (●) and control day (○), IL-6 ($^{‡}P < .05$ indicates difference between the CRP infusion day (■) and control day (□), § indicates difference between time points, $P < .05$), IL-8 ($^{‖}P < .05$ indicates difference between the CRP infusion day (▲) and control day (Δ), ¶ indicates difference between time points, $P < .05$). Values are means±SD.

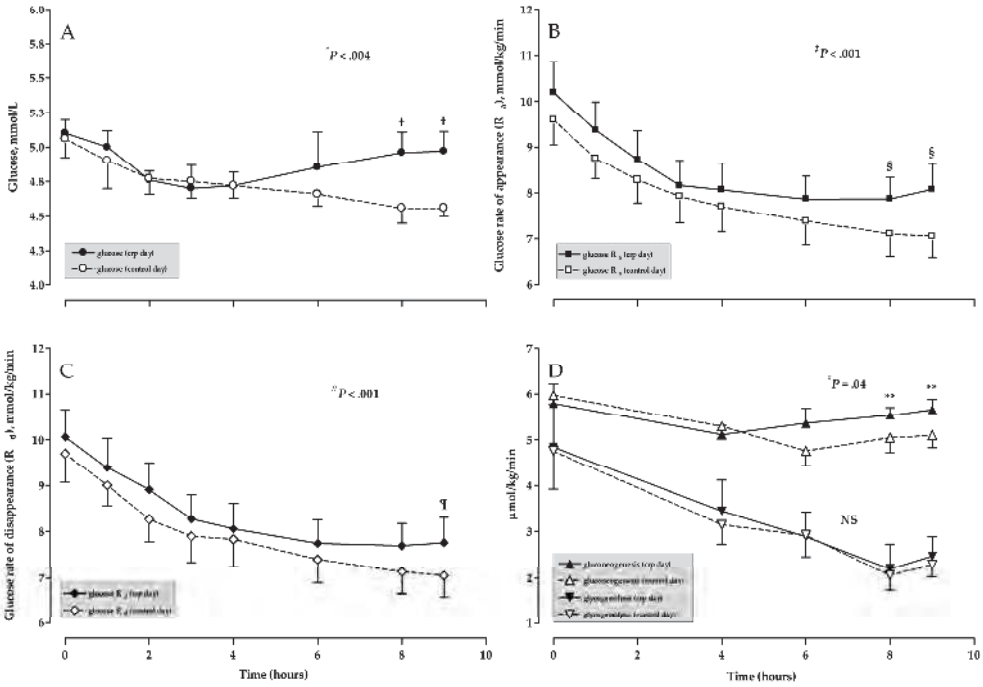

Figure 2. Effect of CRP infusion on glucose metabolism

(A) plasma glucose concentration (*P = .004 indicates difference between the CRP infusion day (●) and control day (○) by ANOVA for repeated meassures, † indicates difference between time points by Wilcoxon test, P < .05), **(B)** glucose R_a (‡P < .001 indicates difference between the CRP infusion day (■) and control day (□), § indicates difference between time points, P = .02), **(C)** glucose R_d (‖P < .001 indicates difference between the CRP infusion day (∀) and control day (*o*), ¶ indicates difference between time points, P < .05) and **(D)** gluconeogenesis (#P = .04 indicates difference between the CRP infusion day (▲) and control day (Δ), ** indicates difference between time points, P =.02) and glycogenolysis, NS difference between interventions and time points on the CRP infusion day (▼) and on the control day (∇). Values are means±SD.

Gluco- and counter regulatory hormones

Three hours after CRP infusion, there was a transient decline in plasma insulin concentrations which subsequently returned back to normal (figure 3A). In line, C-peptide concentrations closely followed insulin kinetics (figure 3B).

Glucagon concentrations were unaffected by CRP (Figure 4A). In contrast, plasma cortisol concentrations rose significantly peaking at 4 hours (figure 4B). Concomitantly, norepinephrine concentrations also peaked at 3 hours after CRP infusion (figure 4C). However, epinephrine concentrations were unaffected by CRP (figure 4D).

Other measurements

Baseline FFA- and adiponectin concentrations were comparable on both study days and were unaffected upon CRP administration.

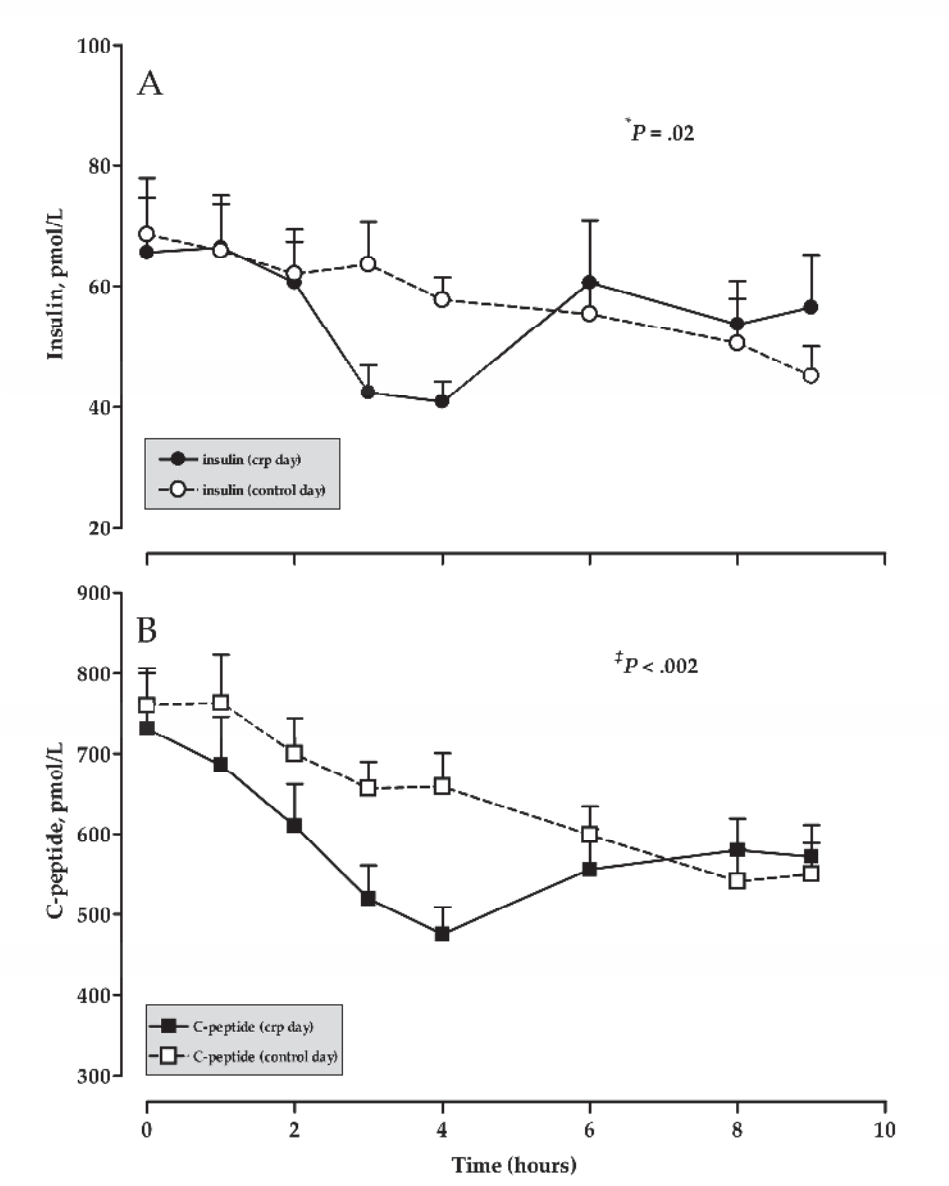

Figure 3. Effect of CRP infusion on insulin secretion

(A) plasma insulin concentrations (*P = .02 indicates difference between the CRP infusion day (●) and control day (○) by ANOVA for repeated meassures, † indicates difference between time points by Wilcoxon test, P < .02), **(B)** plasma C-peptide (‡P = .002 indicates difference between the CRP infusion day (■) and control day (□), § indicates difference between time points, P < .05). Values are means±SD.

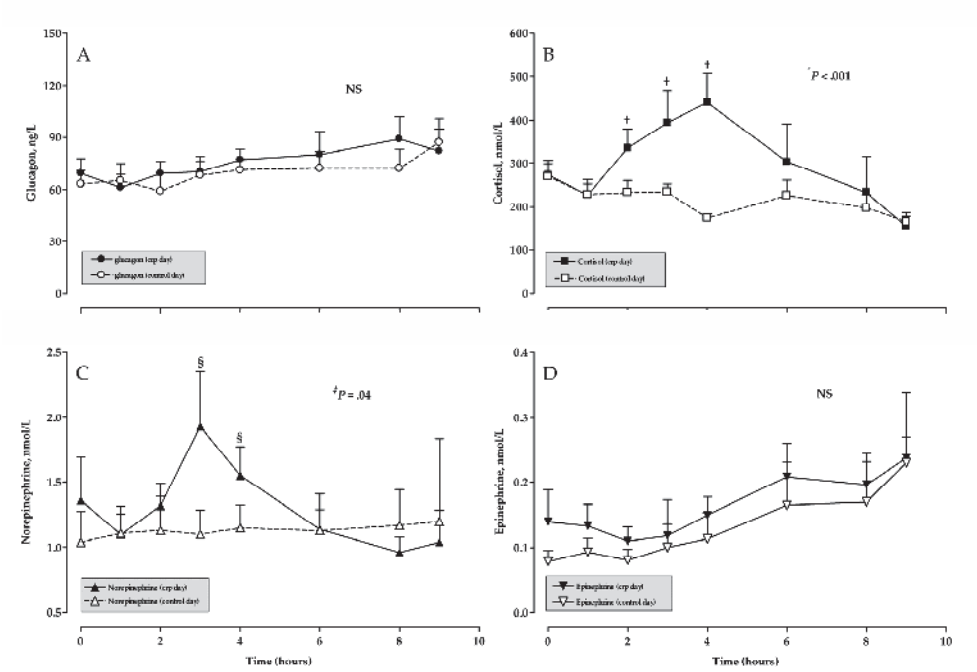

Figure 4. Effect of CRP infusion on counter glucoregulatory hormones

(A) plasma glucagon concentrations, NS difference between interventions and time points on the CRP infusion day (●) and control day (○), **(B)** plasma cortisol concentrations (*P < .001 indicates difference between the CRP infusion day (■) and control day (□), † indicates difference between time points, P < .05), **(C)** norepinephrine (‡P = .04 indicates difference between the CRP infusion day (▲) and control day (Δ), § indicates difference between time points, P < .05) and **(D)** epinephrine, NS difference between interventions and time points on the CRP infusion day (▼) and control day (∇).Values are means±SD.

Discussion

In the present study we show that a single bolus infusion of CRP affects glucose metabolism *in vivo* as illustrated by increased glucose production due to increased gluconeogenesis as well as an increase in plasma glucose concentration. Preceding these metabolic changes, CRP elicited an inflammatory response as well as an increase in counter-glucoregulatory hormones with a transient decline in insulin three to four hours after CRP. These findings suggest that CRP may have a direct effect on glucose handling *in vivo*.

Glucose metabolism

The changes in glucose metabolism during the control experiments were reflected by an initial decline in plasma glucose concentration and glucose $R_{a.}$ After CRP infusion, the decrease in plasma glucose concentration during the first hours was similar to control experiments. However, from 6 hours onwards plasma glucose concentrations rose significantly in CRP-infused subjects, preceded by an 11% increase in glucose R_a. Only a small increase in glucose R_d was observed. The combination of 10% increase in glucose, 11% increase in glucose R_a and a 10% increase in gluconeogenesis suggests that increased glucose production was the predominant factor responsible for the rise in plasma glucose levels after CRP infusion.

Glucose production

Several factors may have contributed to the observed increase in glucose production. First, CRP infusion induced a transient decrease in insulin and C-peptide concentrations, known to be associated with increased hepatic glucose production. Second, CRP infusion significantly increased plasma cortisol and norepinephrine concentrations. Particularly cortisol and to a lesser extent norepinephrine is known to induce increased glucose production.[7,8] Although norepinephrine has a relatively short duration of action, some studies have shown that infusion with norepinephrine may acutely increase hepatic glucose production.[9,10] Thus, cortisol together with

norepinephrine provide a plausible explanation for the CRP-induced effects on glucose metabolism.[11,12] Most likely, the increases in plasma cortisol and norepinephrine concentrations are secondary to increased IL-6 release peaking at 4 hours.[13-15] Since CRP levels remained elevated through the CRP study day, a direct effect of CRP on cortisol and norepinephrine seems unlikely but cannot be excluded with all certainty. Notably, in contrast to other inflammatory stimuli such as TNFα and IL-6, CRP infusion did not affect glucagon concentrations.[14,16] Finally, CRP infusion induced a modest but significant cytokine response. Especially, IL-6 has been shown to result in decreased insulin- and C-peptide.[13] These effects of IL-6 can be observed from concentrations of 400-600 pg/mL onwards, whereas IL-6 concentrations in the present study did not exceed the 60-80 pg/mL range. The latter makes it unlikely that IL-6 had a major contribution to the effect of CRP on glucose handling.

Purity of the rhCRP solution

Recently, the purity of commercially available rhCRP has been criticized in view of potential contamination of rhCRP with endotoxin and sodium azide.[17] Therefore, we used a modified purification procedure.[18] In line, several findings argue against a role of contaminants in the present study. First, we show a clear disparity in cytokine profiles between that mediated by CRP and endotoxin, lacking TNFα increase. Second, the trace amounts of endotoxin present in the purified rhCRP solution (1.5 EU/kg) did not induce inflammatory changes in humans, thereby excluding a causal role for endotoxin.[19] Third and foremost, the metabolic changes in the present study are slow, which is in contrast to the acute metabolic changes upon infusion of endotoxin.[20]

Study Limitations

Chronic elevations of CRP in the lower range (e.g. 1 to 5 mg/L) have been associated with metabolic sequelae and adverse cardiovascular outcome.[21] Since repeated administration of rhCRP is not ethical in view of potential sensitization, we performed acute CRP-infusion experiments only, aiming at pathophysiological CRP concentrations approximating those found in patients with an acute coronary syndrome

(20-25 mg/L).[22] Therefore the current effects of CRP on glucose metabolism must be interpreted as pathophysiological effects. These current methodological impediments need to be addressed in future studies, including dose-response experiments as well as evaluation of the impact of CRP infusion in high risk groups, such as severe metabolic syndrome.

Clinical implications

Whereas several observational studies have reported a relation between CRP and insulin resistance, the present study provides the first in vivo evidence that CRP interferes with glucose metabolism in man. Although the association between modestly elevated CRP concentrations and glucose metabolism does not necessarily reflect the effects observed upon single infusion with higher CRP concentrations, our findings lend further support to develop strategies aimed at lowering CRP concentrations and/or CRP bio-activity, particularly in subjects characterized by increased risk for development of insulin resistance or type 2 diabetes mellitu

Funding or Grant Support

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Chapter 5

ApoAI-phosphatidylcholine infusion neutralizes the atherothrombotic effects of C-reactive protein in humans

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Abstract

Background: High-density lipoprotein (HDL) exerts a variety of anti-atherothrombotic functions, including a potent anti-inflammatory impact. In line, the direct pro-inflammatory effects of C-reactive protein (CRP) can be attenuated by HDL *in vitro*.

Objective: To evaluate whether this also holds true in humans, we assessed the ability of reconstituted HDL to neutralize CRP-mediated activation of coagulation and inflammation.

Methods: Fifteen healthy male volunteers received an infusion of recombinant human (rh)CRP (1.25 mg/kg body weight). In 8 of these volunteers, an infusion of human apoAI reconstituted with phosphatidylcholine (apoAI-PC; 80 mg/kg body weight) preceded rhCRP infusion.

Results: Infusion of rhCRP alone elicited an inflammatory response, thrombin generation and activation of fibrinolysis. In individuals who received apoAI-PC prior to rhCRP, these effects were abolished. Parallel tests in primary human endothelial cells showed that apoAI-PC preincubation with rhCRP abolished the CRP-mediated activation of inflammation as assessed by IL-6 release. Although we were able to show that rhCRP co-eluted with HDL after size-exclusion chromatography, plasmon surface resonance indicated the absence of a direct interaction between HDL and CRP.

Conclusion: Infusion of apoAI-PC prior to rhCRP in humans completely prevents the direct atherothrombotic effects of rhCRP. These findings imply that administration of apoAI-PC may offer benefit in patients with increased CRP.

Introduction

Inflammation plays a major role in all phases of atherogenesis from the development of fatty streaks to plaque rupture and subsequent atherothrombosis. C-reactive protein (CRP) has emerged as an independent predictor of cardiovascular risk in various patient populations.[1,2] Prospective studies have shown a positive relation between plasma CRP levels and coronary atheroma burden[3] and cardiovascular events,[4] independent of statin-induced lowering of low density lipoprotein (LDL)-cholesterol. These observations have in part been attributed to direct pro-atherogenic effects of CRP itself on various cell types within the atherosclerotic plaque.[5] These direct effects are mediated by CRP binding to FcγRII (CD32)[6] for leukocytes and to CD32 and CD64 in human aortic endothelial cells.[7] In addition to these cell surface receptors, CRP has also been reported to have affinity for LDL[8] and high-density lipoprotein (HDL).[9,10] It is not known, however, whether these lipoproteins serve as a mere docking station for CRP or whether biological actions of CRP, through binding of these macromolecules, are affected. *In vitro*, many of the detrimental effects of CRP could be counteracted by HDL.[11,12] In line, Wadham et al. recently showed that CRP-induced upregulation of adhesion molecules by endothelial cells was attenuated by pre-incubation with HDL.[13] It is unknown whether this also occurs in humans. In previous experiments, we have shown that infusion of recombinant human CRP (rhCRP) elicits acute atherothrombotic effects.[14,15] In the present study we investigated the effect of pre-infusion with human apoAI reconstituted with phosphatidylcholine (apoAI-PC) on the downstream effects of a bolus injection of CRP in healthy human volunteers.

Methods

Study Protocol

Fifteen healthy, nonsmoking men were included in this study after obtaining written informed consent. None of the volunteers had febrile illness or cardiovascular disease

or were on medication. After an overnight fast, a bolus of highly purified rhCRP was given intravenously at a dose (1.25 mg per kg body weight) previously shown to result in activation of coagulation and inflammation.[15] Blood was drawn at baseline and 1, 4 and 8 hours after infusion. In eight of these volunteers this was preceded by intravenous administration of apoAI-PC disks at a dose of 80 mg/kg body weight over a period of 3 hours. Reconstituted HDL consists of apolipoprotein AI isolated from human plasma and phosphatidylcholine derived from soybean[16] and was kindly provided by CSL Limited (Sydney, Australia).

The rhCRP (BiosPacific), derived from *Escherichia coli* (K12, substrain NM522), was supplied in 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L $CaCl_2$, pH 7.5, and 0.05% (wt/vol) sodium azide and revealed a single 23-kDa band (>99%) after CBBR-staining (1 μg; SDS-polyacrylamide gel). Before purification, the host cell protein concentration was 85 ppm, as determined by a high-sensitive ELISA in accordance with manufacturer's instructions (Cygnus Technologies Inc., Southport, NC, USA). Subsequently, the rhCRP was purified using size exclusion chromatography to minimize contaminants including endotoxin and sodium azide (Univalid bv, Leiden, the Netherlands). Purity as well as stability was evaluated using sequential high-performance liquid chromatography and time-of-flight mass spectrometry, showing no other protein fractions, including the monomeric variant of CRP, besides the CRP pentamer. Endotoxin levels in the final sample were below 1.5 endotoxin units (EU)/mL as evaluated by Limulus assay (turbidimetric kinetic method; Bactimm bv, Nijmegen, the Netherlands). The rhCRP was stored in a $CaCl_2$-containing buffer (pH 8.5) at 0 to 4°C.

Endotoxin (*Escherichia coli* lipopolysaccharide, catalog number 1235503, lot G2B274, United States Pharmacopeial Convention Inc, Rockville, USA) was administered to five healthy volunteers. Blood was withdrawn prior to infusion and at 1, 4 and 8 hours after infusion.

Biochemical analysis

Blood samples were drawn from the subjects after a 12-hour overnight fast, immediately and 3 hours after apoAI-PC infusion. After centrifugation within 1 hour after collection, aliquots were snap-frozen in liquid nitrogen and stored at -80°C until the assays were performed. All measurements were carried out at the vascular and clinical laboratories of the Academic Medical Center, University of Amsterdam. CRP concentrations were measured with a high-sensitive immunonephelometric assays (Roche Diagnostics Corporation). IL-6 and IL-8 were assayed by cytometric bead array analysis (BD Biosciences). We measured prothrombin fragment F1+2 (Dade-Behring), d-dimer (Asserachrom D-dimer, Roche) and plasminogen activator inhibitor type-1 (PAI-1) antigen (Monozyme, Charlottelund, Denmark), using ELISAs.

Distribution of lipoproteins was measured by Fast Protein Liquid Chromatography (FPLC). The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, a FP-920 fluorescence and UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra reagent pump (Besta, Uppsala, Germany) was used for in-line enzymatic lipid reagent addition at 0.1 ml/min. Plasma lipoprotein separations were performed with a Superose 6 HR 10/30 column (Pharmacia Biotech, Sweden) with TBS, pH 7.4 as eluent at a flow rate of 0.31 ml/min.

Total cholesterol measurement was determined using PAP 250 cholesterol enzymatic methods (Biomerieux, Le Fontanille, France). Commercially available lipid plasma standards were used for quantitative analysis (SKZL, Nijmegen, the Netherlands) for the total cholesterol content of the main lipoprotein classes.

Rat Liver Slices

Precision-cut liver slices (10–14 mg) were prepared as described previously[17] and stored in UW solution on ice until incubation. Slices were incubated individually at 37°C in six-well plates (Greiner) in 3.2 ml Williams' medium E supplemented with glutamax I (GIBCO-BRL; Paisley, Scotland) and 50 mg/ml gentamicin (GIBCO-BRL) and saturated with 95% O_2-5% CO_2. Slices were incubated for 24 h with 1, 10 and 50 ng/ml LPS, and 25 and 75 µg/ml rhCRP for a period of five hours. These experiments were carried out in triplicate.

Surface Plasmon Resonance

Surface plasmon resonance was used to assess molecular interaction between HDL and CRP. Mouse mono anti-apoAI and goat poly anti-CRP antibodies (Acris, Hiddenhausen, Germany) were coupled at different concentrations to an activated CM-5 sensor chip according to the manufacturer's instructions. Specific binding to the anti-ApoAI channels was always corrected for nonspecific binding to the control channels (absence of HDL). Isolated native HDL at a protein concentration of 1 mg/ml in Hepes buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA) was injected for 600 s at a flow rate of 5 µl/min, and HDL capture was monitored in real time.

Binding of apoAI and apoAII antibodies to captured HDL was monitored in real time to validate the presence of HDL particles. CRP (average pentamer size, 115 kDa), at 25 mg/L in HEPES buffer, was subsequently injected at a flow rate of 20 µl/min for 180 s, and the binding of CRP to HDL was monitored in real time.

Regeneration of the sensor chip was achieved by a 5-min wash with a solution of 3 M potassium isothiocyanate followed by a 2-min wash with 10 mM glycine, pH 2.7, and it was finally equilibrated with HEPES buffer. All analyses were done at a constant temperature of 25°C.

Human endothelial cells experiments

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described[18] using culture medium 199 (GIBCO-BRL, Paisley, Scotland), supplemented with 20% (v/v) fetal bovine serum, 50 µg/ml heparin (Sigma), 6.5-25 µg/ml Endothelial Cell Growth Supplement (Sigma) and 100 U/ml penicillin/streptomycin (Gibco-BRL). Upon reaching confluency, the tissue culture medium was refreshed and cells were allowed to develop their characteristic 'cobble-stone' appearance for at least 24–48 hours prior to harvesting. The cells were stimulated with CRP (25 µg/ml), or with CRP preincubated with apoAI-PC (10 µg/ml; 1 and 16 hours at 37°C). The supernatant was harvested 24 hours after the CRP stimulation for IL-6 measurements (ELISA, Pelikine compact human IL-6, M1916, Sanquin, Amsterdam, the Netherlands).

Statistical Analysis

The results are expressed as mean ± SEM unless otherwise stated. Differences between the two groups over time were tested by 2-way analysis of variance (ANOVA) for repeated measures using SPSS for Windows (SPSS Inc., version 11.0, Chicago, Illinois, USA). Comparisons within groups were done by the Wilcoxon signed rank test. A probability (p) value of <0.05 was considered significant.

Results

Upon recruitment, 15 healthy male volunteers were randomly assigned to either a single infusion with rhCRP or to double infusion with apoAI-PC followed by rhCRP infusion. Table 1 summarizes the demographic characteristics and lipid profiles of both groups. After infusion of rhCRP or apoAI-PC plus rhCRP, hemodynamic parameters and temperature recordings were stable throughout the infusion studies and did not differ between the two groups (data not shown). No adverse effects were recorded throughout this study.

Plasma apoAI and CRP levels

In volunteers who only received rhCRP, apoAI levels remained unaltered during the course of the experiment (see figure 1A) which is in agreement with a previous study showing that levels are not affected when rhCRP is used at this concentration in healthy volunteers.[15] In the group that received apoAI-PC infusion (started at t=-3 hours) the average apoAI level rose from 1.29 (range: 1.03 to 1.55) to 2.66 g/L (range: 2.32 to 2.94).

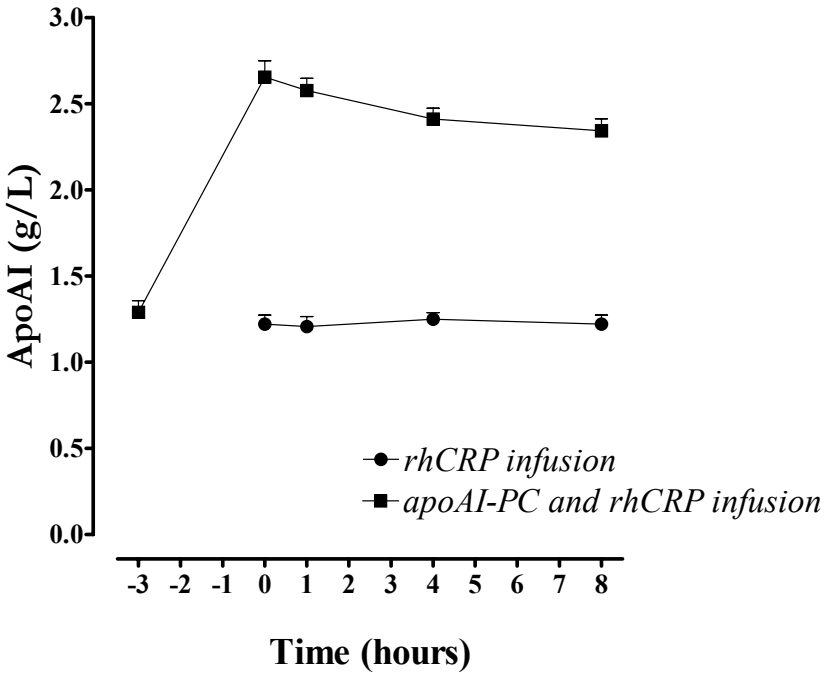
In volunteers receiving rhCRP mono-infusion, average CRP concentrations rose from 2.5 (range: 0.3 to 8.5) to 25.5 mg/L (range: 21.1 to 30.4; see figure 1B) at t=1, directly followed by a gradual decline until t=8. In volunteers who had been pretreated with apoAI-PC, subsequent rhCRP infusion resulted in a similar rise of CRP-concentrations from 1.7 at baseline (range: 0.3 to 5.0) to 26.6 (range: 21.4 to 32.2) at t=1, and fell as observed in the other group from t=1 until t=8.

Table 1. Baseline characteristics of the study subjects

	rhCRP (n=7) Mean ± SD	ApoAI-PC & rhCRP (n=8) Mean ± SD
Age, y	39.3 ± 16.9	27.6 ± 12.6
BMI, kg/m²	27.3 ± 5.2	22.3 ± 2.5
Systolic BP, mmHg	126 ± 10	131 ± 11
Diastolic BP, mmHg	82 ± 6	74 ± 7
Glucose, mmol/L	5.3 ± 0.4	5.0 ± 0.3
hsCRP, mg/L	2.49 ± 2.88	1.72 ± 0.74
TC, mmol/L	4.8 ± 1.1	3.8 ± 0.9
LDL, mmol/L	3.1 ± 1.1	2.1 ± 0.7
HDL, mmol/L	1.3 ± 0.2	1.5 ± 0.4
TG, mmol/L	1.0 ± 0.2	0.6 ± 0.3

BMI indicates body mass index; hsCRP, high-sensitivity CRP; TC, total cholesterol; LDL, low-density cholesterol; HDL, high-density cholesterol; TG, triglycerides.

1A



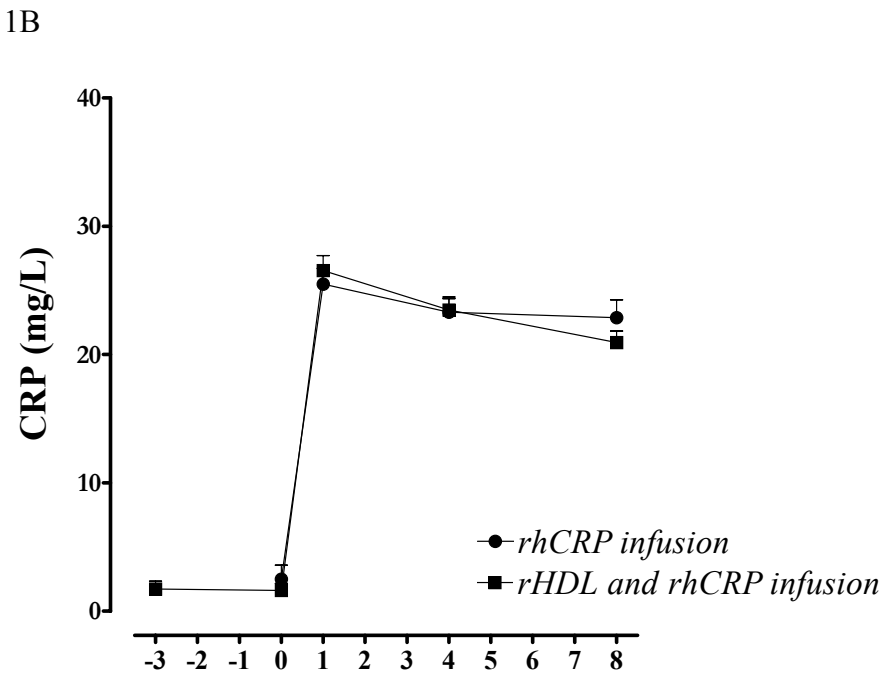


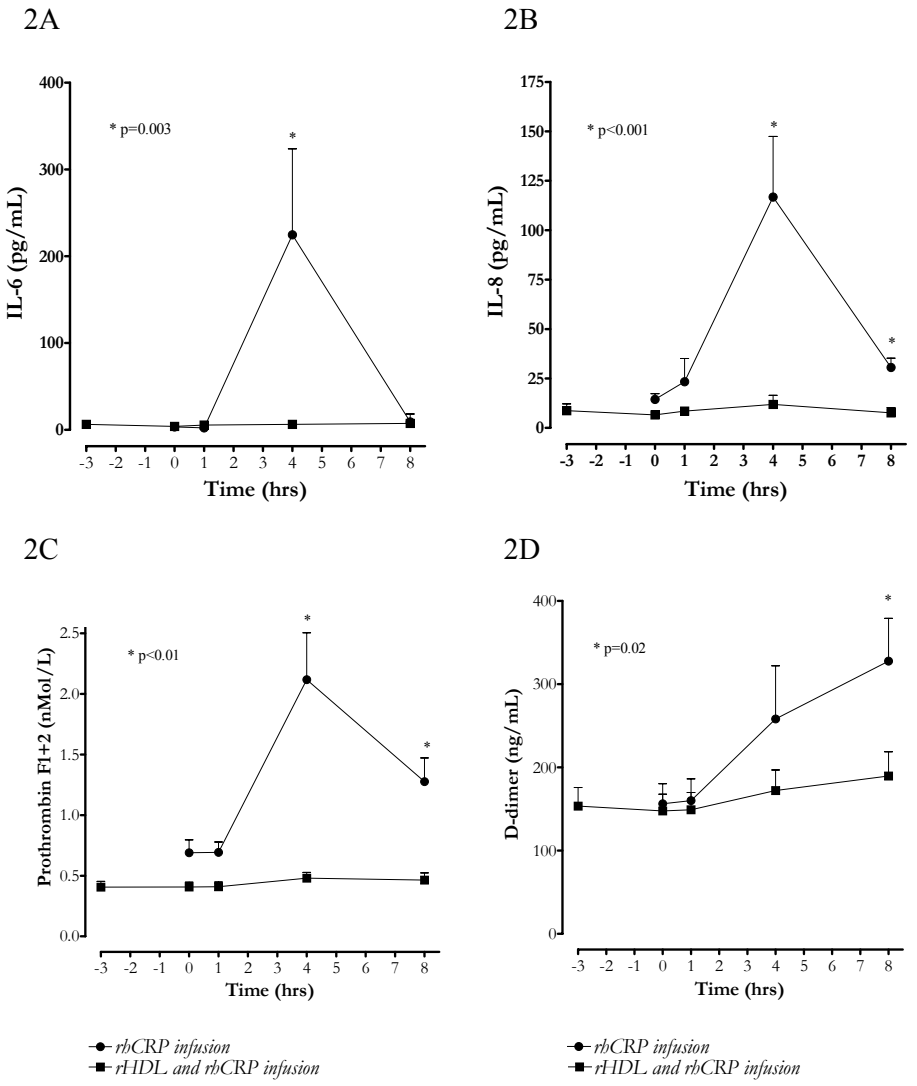
Figure 1. Circulating levels of CRP and ApoAI after dual infusion of apoAI-PC and CRP compared to CRP mono-infusion.

Mean (± SEM) concentrations of (A) CRP and (B) ApoAI in response to rhCRP infusion (●) and rhCRP infusion preceeded by apoAI-PC infusion(■). ApoAI-PC pretreatment resulted in a significant rise in ApoAI levels whereas levels remained unaltered in volunteers receiving rhCRP mono-infusion. Infusion of rhCRP led to an acute rise in CRP plasma levels.

ApoAI-PC blunts rhCRP-mediated activation of inflammation and coagulation

In line with previous findings,[14,15] infusion of rhCRP elicited a systemic proinflammatory response as reflected by a monophasic rise in plasma IL-6 and IL-8 levels peaking at 4 hours after rhCRP-infusion (figure 2A and B). Such increases in IL-6 and IL-8 were abolished in volunteers that had been pre-treated with apoAI-PC. Mono-infusion of rhCRP elicited thrombin generation as reflected by elevated levels of F1+2 (figure 2C). Although increased levels of D-dimer observed in volunteers receiving rhCRP

(figure 2D) may be indicative of activation of fibrinolysis this may also be secundary to enhanced thrombin formation. Indeed, rhCRP infusion induced a significant increase of PAI-1 antigen levels (figure 2E), indicative of reduced fibrinolysis. In the group pretreated with apoAI-PC, the F1+2 levels were unaffected while D-dimer and PAI-1 antigen concentrations were markedly lower compared to the group that received CRP mono-infusion.



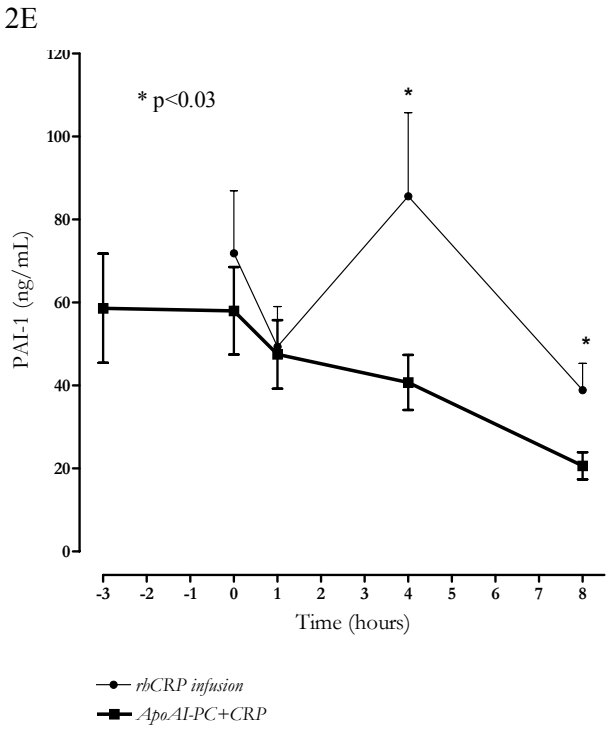


Figure 2. Effects on inflammation and coagulation of dual infusion of apoAI-PC and CRP compared to CRP mono-infusion.

Mean (± SEM) concentrations of (A) IL-6, (B) IL-8, (C) F1+2, (D) D-dimer and (E) PAI-1 antigen in response to rhCRP infusion (●) and rhCRP infusion preceeded by apoAI-PC infusion(■). Activation of inflammation and coagulation induced by rhCRP infusion was prevented by apoAI-PC pretreatment.

Atherothrombotic effects are directly mediated by CRP, not endotoxins

It has been suggested that in some studies the observed effects of recombinant CRP may have been due to contamination of the CRP solution with lipopolysaccharide (LPS).[19] To exclude the possibility that the observations in the current study could be ascribed to LPS contamination, we carried out a series of control experiments.

Using a sensitive turbidimetric kinetic method, we observed trace amounts of LPS in the rhCRP solution, i.e.1.5 EU/ml. To evaluate whether such minimal LPS exposure has the ability to elicit an inflammatory response in humans, we infused an equivalent amount of LPS in 5 healthy male volunteers. At this concentration, LPS did not induce a systemic proinflammatory response (mean IL-6 at baseline: 7.0 pg/mL, at t=4 hours: 8.9 pg/mL; mean TNF-α at baseline: 5.6 pg/mL, at t=4 hours: 6.0 pg/mL).

We took a second approach to investigate whether LPS in our rhCRP preparation would confound our data using a sensitive biological readout system, i.e. murine liver slices.[17] In line with others,[20] we showed that LPS at concentrations between 1 to 50 ng/ml is invariably associated with a dose-dependent release of TNF-α in this system (figure 3). In contrast, incubation with our rhCRP preparation at concentrations of 25 up to 75 μg/ml did not elicit a detectable TNF-α release. Of note, the CRP concentrations used in these *in vitro* experiments exceed the observed CRP levels in our human studies threefold.

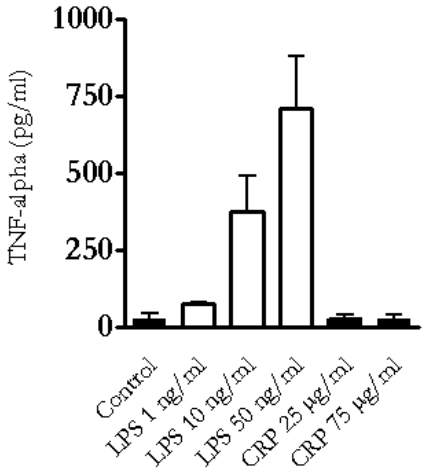


Figure 3. Effects of stimulation of rat liver slices with CRP and LPS.

Mean (± SEM) concentrations of TNF-α after stimulation of rat liver slices for a period of five hours. Stimulation with LPS at various doses (1, 10 and 50 ng/ml) resulted in significantly increased TNF-α production. In contrast, stimulation of rat with CRP liver slices failed to induce TNF-α production.

In vitro effects of rhCRP

We also assessed the effects of rhCRP, apoAI-PC and combinations of these compounds in primary human umbilical vein endothelial cells (HUVECs), cultured under static conditions with IL-6 production as a readout parameter. The data were expressed as percentage IL-6 production observed in PBS-treated control cells. Exposure to 25 µg/ml rhCRP for 24 hours resulted in a 25% increase in IL-6 production (figure 4). Heat-inactivated rhCRP (30 minutes, 55°C) did not increase IL-6 production (-4.5% compared to PBS controls). Preincubating of CRP with apoAI-PC for 1 hour at 37°C neutralized CRP-mediated production of IL-6 (3.5%). Finally, preincubation of rhCRP with apoAI-PC for 16 hours even reduced endogenous IL-6 production in this model system.

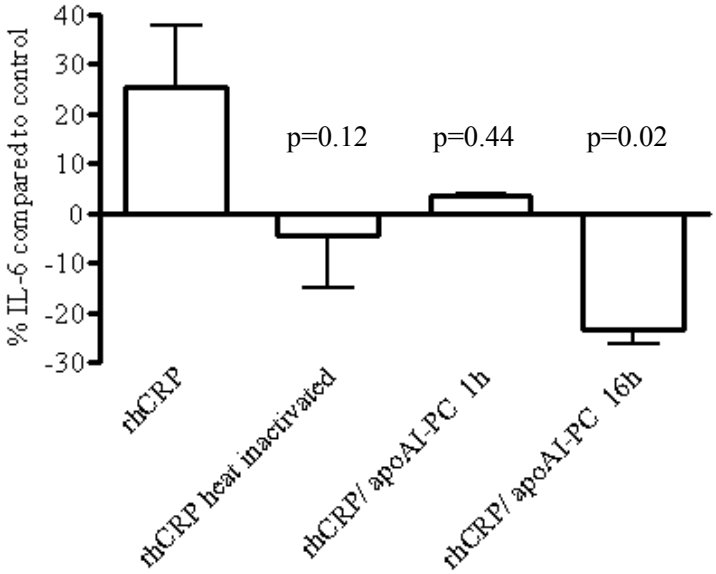


Figure 4. Effects of stimulation of HUVECs with CRP, in presence and absence of apoAI-PC. Stimulation of HUVECs with rhCRP increased IL-6 production with 25% compared to baseline. HUVECs stimulated with rhCRP and apoAI-PC that were added together for one hour to allow aggregation, produced a similar levels of IL-6 when compared to baseline. CRP stimulation of HUVECs which had been pretreated with apoAI-PC for 16 hours resulted in reduced IL-6 production of 24% compared to baseline levels. P-values are relative to stimulation with rhCRP.

Interaction of rhCRP and HDL

Fast Protein Liquid Chromatography (FPLC)

To elucidate the molecular background of the observed capacity of apoAI-PC to abolish the effects of rhCRP infusion *in vivo*, we hypothesized that apoAI-PC binds rhCRP thereby neutralizing its biological activity. Plasma lipoproteins were isolated using FPLC from plasma obtained from subjects at t=4 hours after receiving the bolus-infusion of rhCRP. In volunteers who only received the rhCRP bolus, 5.5% (range: 1.1 to 10.8) of total serum CRP was found in the HDL fraction (figure 5). In the volunteers who had been pre-treated with apoAI-PC, 27.8% (range 8.8 to 40.7) of total serum CRP was located in the HDL fraction. These data suggest an association between exogenously added apoAI-PC (equilibrated with endogenous lipoproteins) and CRP.

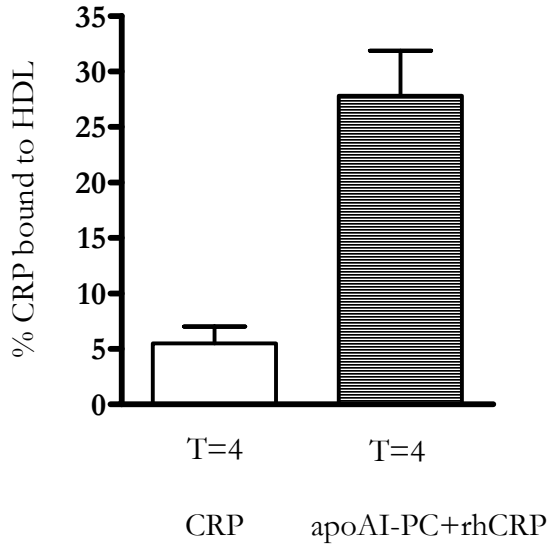


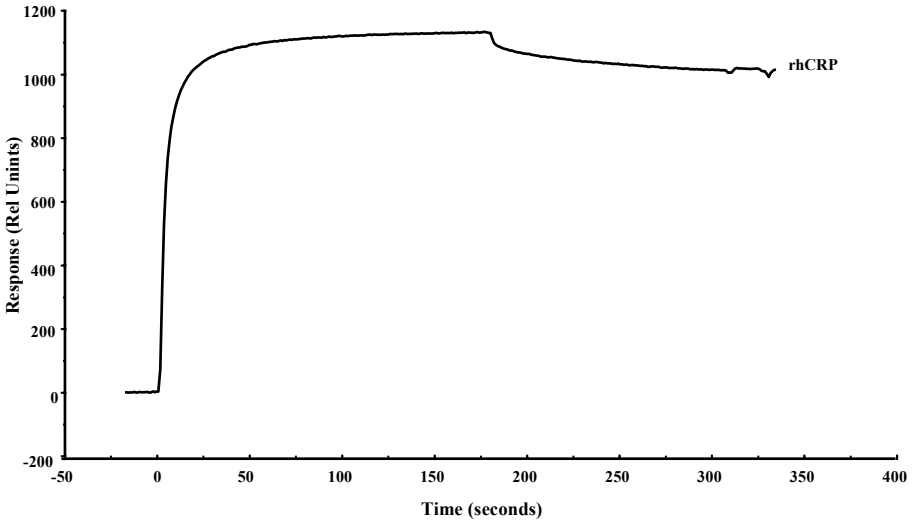
Figure 5. Amount of CRP detectable in the HDL fraction after dual infusion with apoAI-PC and CRP compared to CRP mono-infusion.

Four hours after infusion of CRP, the amount of CRP in the HDL fraction of volunteers that had been pretreated with apoAI-PC was five-fold higher when compared to volunteers that did not receive apoAI-PC.

Plasmon Surface Resonance

To obtain insight whether CRP and HDL interact at the molecular level we used plasmon surface resonance. First, CRP antibodies were coupled to a sensor chip. Subsequenly, rhCRP was injected and CRP capture was monitored in real time which showed a clear positive response indicative of direct molecular interaction (figure 6A). Second, we isolated HDL from plasma (FPLC) that was obtained at t=4 hours after apoAI-PC treated subjects had received the bolus-infusion of CRP. This HDL was captured on the surface of a sensor chip (CM-5) via immobilized polyclonal anti-apoAI antibodies. Quantitative binding of HDL was found proportional to the concentration of antibody present on the sensor chip and dissociation of HDL was found to be negligible (k_d = 10^{-5} s^{-1}). To validate appropriate binding of native HDL to the chip, we used antibodies against apoAI and apoAII which showed clear positive responses indicative of direct molecular interaction between the captured HDL and these antibodies (figure 6B). However, injection of anti-CRP antibodies did not show any response indicating that CRP was not present on the captured HDL. To further study complex formation of rhCRP and HDL in real-time, HDL that was obtained at t=0 hours was isolated and captured on the chip. Injection with rhCRP only showed minimal binding to HDL.

6A



6B

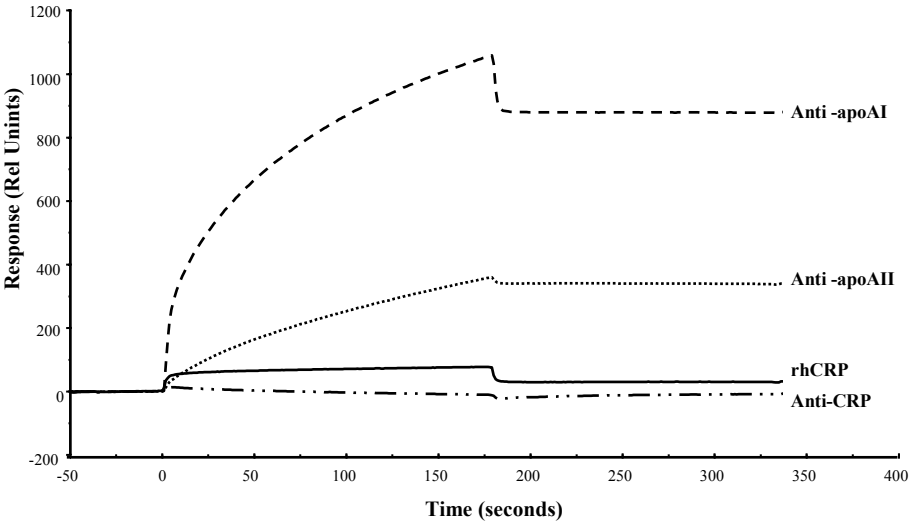


Figure 6. Compilation of sensorgrams representing the interaction characteristics of HDL and CRP.

A. Shown is a control experiment demonstrating a strong interaction of antibodies directed against rhCRP. The sensor-chip was coated with a goat polyclonal anti-CRP antibody (3700 RU) followed by an injection of 250 nM rCRP. A strong interaction of the rCRP with this antibody was observed

B. Shown here are individual experiments with HDL obtained from t=4 hours after rhCRP infusion, demonstrating a strong interaction of antibodies directed against apo AI (_ _ _ _) and AII (.....) and no interaction of antibodies directed against CRP (___ - - ___) to immunocaptured HDL. Very minor direct interaction between HDL obtained at baseline (t=0 hours after rhCRP infusion) and rhCRP was observed (solid line).

Discussion

We previously reported that rhCRP infusion in humans elicits atherothrombotic effects.[14,15] The current study shows that infusion with apoAI-PC prior to rhCRP administration neutralizes these effects. Specifically, apoAI-PC blunted the release of the pro-inflammatory cytokines IL-6 and IL-8 and diminished thrombin generation and fibrinolysis activation as illustrated by decreased levels of F1+2 and D-dimer, respectively. The present findings lend support to a direct anti-inflammatory and anticoagulant effect of apoAI-PC infusion in man.

Mechanism

Regarding the potent interaction between CRP and phospholipids in LDL,[8,21,22] it is conceivable that the interaction between CRP and HDL particles also depends on the presence of phospholipids. To analyze whether the neutralizing effect of apoAI-PC were related to sequestration of CRP by HDL, we isolated lipoprotein subfractions using FPLC. Four hours after administration of CRP, the amount of CRP within the HDL fraction of volunteers having received apoAI-PC was 5 times higher compared to volunteers who only received CRP.

In vitro, such interactions between CRP and apoAI-containing lipoproteins have been reported[10] and proteomic analyses also show the presence of CRP on HDL.[9] To study putative molecular interactions between CRP and HDL, we subsequently used plasmon surface resonance. These experiments indicate that rhCRP does not interact directly with native HDL when the latter is immobilized to the chip by means of anti-apoAI antibodies. Also, when using HDL isolated from plasma of the subjects who were infused with apoAI-PC and rhCRP (t=4 hours), antibodies against CRP failed to detect CRP on HDL. Although we cannot exclude a direct interaction between HDL and CRP *in vivo*, these data argue against such a phenomenon. Moreover, only 30% of the rhCRP could be detected in the HDL fraction after FPLC indicating that direct molecular neutralization of CRP by HDL is unlikely to account for the very strong anti-inflammatory and anti-coagulant effects that were observed. Speculating on how

apoAI-PC exactly protects against the acute inflammatory reponse induced by rhCRP infusion, it is interesting to note that HDL can induce significant changes in target tissues, which renders them resistant to a subsequent inflammatory stimulus. It has e.g. been shown that HDL pretreatment prevents subsequent CRP-mediated upregulation of adhesion molecules by endothelial cells *in vitro*[13] an effect that we could reproduce when using apoAI-PC, CRP and HUVEC. Similarly, it has been reported that HDL can interfere with the expression of Fcγ receptors,[23-25] via which CRP has been shown to mediate its effects. Such studies are however, difficult if not impossible to conduct in man.

Use of rhCRP and the danger of LPS contamination

Some of the reported atherothrombotic effects of CRP[5-7,13-15,26] have been suggested to be caused by endotoxin contamination of the commercially available CRP preparation[27] which, however, has been refuted in more recent studies.[28,29] In support, rhCRP stimulation of endothelial cells missing the LPS receptor (toll-like receptor 4) induced atherothrombotic effects,[30] confirming direct biological effects of CRP. Since the rhCRP solution used in the present study did contain trace amounts of endotoxin (less than 1.5 EU/mL), we evaluated a potential role of LPS in contributing to the pro-inflammatory effects following rhCRP infusion in a series of separate experiments. Whereas very low concentrations of LPS (1 ng/ml) in murine liver slices already elicited a strong TNF-α release, the rhCRP used in our *in vivo* study showed no effect at 3 times the maximum rhCRP concentration that was observed in the healthy volunteers. Further support for a direct effect of CRP comes from the observation that heat-inactivation of rhCRP solution completely abolished the IL-6 increase in HUVECs, confirming the absence of LPS-mediated effects. We also performed a separate LPS infusion experiment in 5 healthy subjects using a total amount of LPS that was identical to the amount which was co-infused with the rhCRP infusion. These low concentrations did not induce inflammatory effects. In view of these equivocal *in vitro* and *in vivo* tests, we feel confident to conclude that the presence of LPS contamination at less than 1.5 endotoxin units/ml in our rhCRP batch has not contributed to the biological effects observed in the present study.

Clinical implications

Data on the pro-atherogenic effects of CRP, combined with the correlation between CRP lowering and improved cardiovascular outcome in the PROVE-IT[4] study has stimulated thought on inhibiting CRP. Promising candidates include a specific small-molecule inhibitor of CRP[31] as well as CRP antisense treatment.[32] In the present study we show that also apoAI-PC infusion abolishes the pro-inflammatory effects of rhCRP. It is tempting to compare the present findings to those of the ERASE (Effect of rHDL on Atherosclerosis- Safety and Efficacy) study,[33] in which weekly infusion of apoAI-PC was performed in patients with acute coronary syndromes characterized by elevated CRP levels. This study revealed favourable changes in both plaque characterization index using intravascular ultrasound as well as coronary score using quantitative coronary angiography. Whereas there was a reduction in coronary atheroma volume of 3.4% compared to baseline measurements, this change did not reach statistical significance compared to placebo infusion (1.6% reduction).[33] Unfortunately, it cannot be dissected from this study whether and to what extent anti-inflammatory effects of apoAI-PC may have contributed to a favourable outcome. Based on the present study, it seems warranted to study the effects of apoAI-PC infusion particularly in pro-inflammatory states addressing specifically the beneficial impact of the anti-inflammatory effects of apoAI-PC.

Acknowledgements

This study was in part funded by a grant from EU STREP HDLomics, contract number 037631. We would like to thank Prof. G.M. Groothuis for helping us establishing the precision cut liver slice model, Arnoud Marquart for his excellent assistance in the Biacore experiments and analysis and Geesje Dallinga-Thie for her expertise.

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Chapter 6

High-Density Lipoprotein Attenuates Inflammation and Coagulation Response upon Endotoxin Challenge in Humans

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Abstract

Objective: Low high-density lipoprotein (HDL) cholesterol is a strong independent cardiovascular risk factor, which has been attributed to its role in reverse cholesterol transport (RCT). Whereas HDL also has potent anti-inflammatory effects, the relevance of this property remains to be established in humans. In the present study, we evaluated whether there is a relation between HDL and sensitivity towards a low-dose endotoxin challenge.

Methods and Results: Thirteen healthy men with genetically determined isolated low HDL-cholesterol (averaging 0.7±0.1 mmol/L) and 14 age-, and body weight-matched healthy men with normal/high HDL-cholesterol levels (1.9±0.4 mmol/L) were challenged with low dose endotoxin i.v. (1 ng/kg bodyweight). The incidence and severity of endotoxin-associated clinical symptoms was increased in the low HDL-group. Accordingly, both the inflammatory response (TNFα, IL-1β, IL-6, IL-8 and MCP-1) as well as thrombin generation (prothrombin activation fragments F_{1+2}) were significantly increased in the low HDL-group upon endotoxin challenge.

Conclusions: Low HDL in healthy males is associated with increased sensitivity towards inflammatory stimuli as reflected by enhanced inflammatory and coagulation responses upon endotoxin challenge. These anti-inflammatory effects of HDL in humans may lend further support to HDL increasing interventions particularly in pro-inflammatory conditions, such as acute coronary syndromes.

Introduction

Following the first report on the potential anti-atherogenic effect of high-density lipoprotein (HDL) almost sixty years ago, HDL is now generally acknowledged as a potent anti-atherogenic mediator.[1] The impact of isolated low HDL-cholesterol on atherogenesis was recently underscored by the finding that carotid intima-media thickness (IMT) in patients with genetically-determined low apolipoproteinA-I (apoA-I) was comparable to that in patients with familial hypercholesterolemia.[2] In line, HDL increasing drugs now are prime candidates for combined use with statins in high risk subjects.

Traditionally, the protective effect of HDL was considered to be confined to its role in the reverse cholesterol transport (RCT) pathway. However, recent evidence supports a wide array of anti-atherogenic effects by HDL, comprising anti-oxidative, anti-thrombotic as well as anti-inflammatory effects.[3] The latter has attracted special interest, since inflammation has been acknowledged to underlie atherosclerotic lesion formation. At the same time, HDL-cholesterol consistently shows an inverse relation with systemic markers of inflammation.[4] Interestingly, HDL-increasing compounds (e.g. reconstituted HDL) have recently been shown to attenuate systemic inflammation in humans[5] as well as vessel wall inflammation in experimental animal models.[6] However, it remains to be established whether HDL also exerts anti-inflammatory effects in the human setting. In the present study, we evaluated the impact of plasma HDL-cholesterol level on the sensitivity towards a low dose endotoxin challenge in subjects with genetically determined low- versus normal/high-HDL-cholesterol levels.

Methods

Study participants

Study subjects were recruited from a study designed to identify genes that control HDL-cholesterol levels.[2] Healthy male subjects with plasma HDL-cholesterol levels below the 10th percentile (low HDL-group, n=13) and healthy male subjects with plasma HDL-cholesterol levels above the 90th percentile (high HDL-group, n=7) matched for age and sex were recruited from families in which an autosomal dominant phenotypic trait for low- or high HDL-cholesterol was established in at least 3 first degree relatives. Subjects in the low HDL-group with known genetic causes for low HDL-cholesterol were excluded from the study, including carriers of the apoA-I (L178P) mutation.[2] Subjects with secondary dyslipidemias, such as familial combined hyperlipidemia, were excluded. We also excluded low HDL as part of the metabolic syndrome or secondary to hypertriglyceridemia. Unaffected healthy male relatives with normal (40 to 60th percentile) HDL-cholesterol levels, matched for age and gender were also recruited from families included in the database (n=7). Since the primary objective was to evaluate increased sensitivity towards inflammatory challenge in individuals with low HDL-cholesterol, data from subjects with normal and high HDL levels (n=14) were combined in the analyses.

Written informed consent was obtained from all subjects. The study protocol was approved by the institutional review board at the Academic Medical Center in Amsterdam. Subjects with cardiovascular disease and risk factors for cardiovascular disease such as impaired fasting glucose, diabetes mellitus, hypertension, hypercholesterolemia, hypertriglyceridemia, CRP levels above 5 mg/mL, elevated Lp(a) and smoking were excluded during the screening visit. Other exclusion criteria were a history of alcohol and/or drug abuse, vaccination in the previous six months, previous exposure to endotoxin experiments, use of medication such as lipid-modifying drugs (resins, statins, niacin, fibrates e.g.) , non-steroidal anti-inflammatory drugs, paracetamol and anti-oxidants. All study subjects were free from signs of acute

infection or febrile illness during the month preceding the study. One subject in the low HDL-group was excluded because he had elevated hepatic enzymes (> 2-times upper limit of normal) and was suspect of alcohol abuse.

Study design

Study participants were required to refrain from alcohol and caffeine-containing beverages at least 24 hours prior to the study. The incidence, time and severity of clinical symptoms associated with endotoxemia, were recorded as follows: 0 = absent, 1 = mild, 2 = moderate and 3 = severe. Other clinical parameters such as blood pressure, heart rate and body temperature were also recorded. Carotid IMT measurements were performed at baseline as previously described.[2]

In the morning of the study day at 7.30 a.m. after an overnight fast, study participants were admitted to the research unit. At 7.45 a.m. a catheter was inserted in an antecubital vein of each arm. At 8.00 a.m. (t=0), blood was drawn for baseline measurements. Subsequently, subjects received a bolus infusion of 1 ng/kg body weight of endotoxin (Escherichia coli lipopolysaccharide, catalog number 1235503, lot G2B274, United States Pharmacopeial Convention Inc, Rockville, USA) in the antecubital vein of the contralateral arm. Blood samples were collected at t = 0, 1, 2½, 4, 6 and 8 hours after endotoxin challenge. The next morning at 8.00 a.m., 24 hours after endotoxin infusion study participants returned after an overnight fast for final blood withdrawal.

Biochemical Analysis

Blood was collected in EDTA, citrate and heparin anticoagulated aliquots as well as serum tubes which were kept on ice and centrifuged at 1600 g for 15 minutes at 4 °Celsius, snap frozen and stored at -80 °Celsius until analysis. Plasma total cholesterol was measured with an enzymatic colorimetric procedure (CHOD-PAP, Boehringer Mannheim, Mannheim, Germany). HDL-cholesterol was determined after precipitation of apoB containing lipoproteins by $MnCl_2$. LDL-cholesterol was calculated using the Friedewald formula. ApoA-I and apoB were measured using

Beckman reagents and array nephelometry (Beckman, Brea, California, USA). Triglycerides were measured using an enzymatic colorimetric method using lipase, glycerol kinase and glycerol-3-phosphate 3 oxidase. Hematology parameters were assessed by standard laboratory techniques.

Baseline lipid measurements were repeated at least three times. First, general practitioners selected patients on the basis of HDL-cholesterol values. Next, genetic field workers collected blood samples from these patients and first degree relatives which were assayed for lipid profiles. Subsequently, eligible patients were invited for a screening visit. Finally, on the study day the research physician took baseline lipid measurements. All samples were obtained after a 12 hour overnight fast.

Analysis of the Inflammatory Response

CRP was measured by a high-sensitivity immunoturbimetric assay (Roche Diagnostics Corporation, Basel, Switzerland), while CRP levels in excess of 10 mg/L were assayed by immunonephelometry (P800 analyzer, Roche Diagnostic Corporation). Circulating levels of tumor necrosis factor-alpha (TNFα), interleukin-1β (IL)-1β, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) were assessed with the luminex method (Bioplex Human Cytokines 1x96 wells, catalog number X500000 FFS, Bio-Rad Laboratories Inc, CA, USA). LPS Binding protein (LBP) was measured with a commercially available ELISA (Human LBP ELISA, Cell Sciences Inc, catalog number HK 315, Canton, MA, USA).

Paraoxonase-1 activity: Serum paraoxonase-1 (PON-1) activity was measured as previously described.[7]

Analysis of the Procoagulant Response

Coagulation activation was assessed by measuring plasma levels of prothrombin fragment 1+2 (F_{1+2}) as a marker of *in vivo* thrombin generation (ELISA; Dade-Behring, Marburg GmbH, Germany). ELISAs were used to measure markers of endogenous fibrinolysis, i.e. plasma levels of tissue-type plasminogen activator (tPA; Asserachrom

tPA; Diagnostic Stago, Asnieres-sur-Seine, France), plasminogen activator inhibitor type-1 (PAI-1) antigen (Monozyme, Charlottelund, Denmark) and the fibrin split product D-dimer (Asserachrom, D-Di, Diagnostic Stago, Asnieres-sur-Seine, France).

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Differences between the low HDL-group versus the normal/high HDL-group were tested by analysis of variance (ANOVA) for repeated measures. Linear regression analysis was used to evaluate correlations between HDL-cholesterol as well as apoA-I levels versus inflammation parameters and coagulation parameters. The SPSS software package for Windows was used for statistical analysis (SPSS Inc., version 12.0, Chicago, Illinois, USA).

Results

Baseline Characteristics

The demographic and biochemical characteristics of study participants in the low HDL-group and the normal/high HDL-group are listed in table 1. The two groups were carefully matched for age and body mass index (BMI), lipids and lipoproteins except for plasma HDL-cholesterol and apolipoproteinA-I (apoA-I) levels.

Clinical Symptoms

Endotoxin infusion caused typical endotoxin-induced symptoms.[8] Clinical symptoms such as backache, chills, headache, myalgia, nausea and vomiting occurred frequently, earlier and more intensive in the low HDL-group (table 2). In the low HDL-group, heart rate increased from 69 beats per minute (bpm) at baseline to 80 bpm at 4 hours versus 67 to 76 bpm in the normal/high HDL-group (P = .03, between groups). Blood pressure did not change throughout the experiment in both groups.

Table 1. Demographic and Biochemical Characteristics of the Study Subjects

	Low HDL-group n=13	Normal/high HDL-group n=14
Age, years	35.4 ± 2.5	35.1 ± 5.2
BMI, kg/m^2	24.9 ± 1.4	24.7 ± 1.5
Systolic BP, mmHg	125 ± 6	125 ± 9
Temperature, °C	36.6 ± 0.8	36.7 ± 0.7
Alcohol use, (%)	6/13 (46)	6/14 (43)
Smoking (previous), (%)	9/13 (69)	10/14 (71)
Fam history CVD, (%)*	6/13 (46)	1/14 (7)
HDL, mmol/L†	0.7 ± 0.1	1.9 ± 0.4
LDL, mmol/L	2.7 ± 0.6	2.7 ± 0.8
Triglycerides, mmol/L	1.1 ± 0.5	1.0 ± 0.6
ApoA-I, g/L‡	1.0 ± 0.2	1.7 ± 0.2
ApoB, g/L	1.1 ± 0.2	1.0 ± 0.2
hsCRP, mg/L	1.4 ± 1.1	1.3 ± 1.4
Plasma glucose, mmol/L	5.3 ± 0.5	5.2 ± 0.5
Carotid IMT, mm	0.57 ± 0.07	0.52 ± 0.06

ApoA-I indicates apolipoproteinA-I; apoB indicates apolipoproteinB; BMI indicates body mass index; BP indicates blood pressure; CVD indicates cardiovascular disease, HDL indicates high-density lipoprotein; hsCRP indicates high sensitive C-reactive protein; IMT indicates intima-media thickness; LDL indicates low-density lipoprotein. Data are expressed as mean±SD. *P = .02 by chi-square (χ^2) test; †P < .001 and ‡P < .001 by independent student's t-test.

Table 2. Effect of HDL levels on Endotoxin-Induced Clinical Symptom

	Low HDL-group n=13			Normal/high HDL-group n=14			*P	†P
Symptoms	No.	Mean Severity	Peaking Time	No.	Mean Severity	Peaking Time		
Backache	5	0.5 ± 0.8	1.8 ± 0.2	2	0.2 ± 0.4	2.6 ± 0.2	.05	.04
Chills	10	1.1 ± 0.9	1.4 ± 0.1	6	0.4 ± 0.6	2.1 ± 0.1	.03	<.01
Fever*	10	38.3±0.9	3.5 ± 1.1	8	37.8±0.8	4.1 ± 2.0	.11	.43
Headache	10	1.4 ± 0.9	2.0 ± 0.3	6	0.5 ± 0.7	3.2 ± 0.3	.01	<.01
Myalgia	5	0.6 ± 0.8	3.1 ± 0.2	2	0.2 ± 0.4	4.0 ± 0.1	.03	<.01
Nausea	10	1.1 ± 0.8	2.9 ± 0.3	5	0.4 ± 0.6	2.9 ± 0.3	.02	.78
Vomiting	5	0.7 ± 1.0	2.6 ± 0.2	2	0.2 ± 0.6	3.8 ± 0.4	.02	<.01
Total AE	55	-	-	29	-	-		

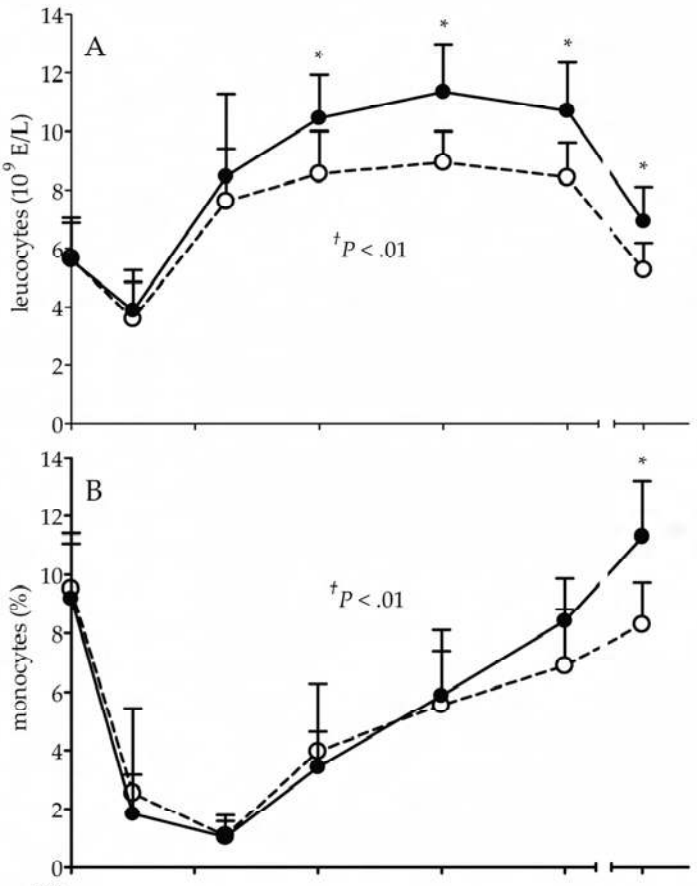
The total number of volunteers suffering from a specific symptom, mean maximum severity (0 = absent, 1= mild, 2= moderate, 3 = severe) and time of maximum severity (hours, relative to endotoxin infusion) are summarized. AE indicates adverse events. *Mean severity for fever is denoted as temperature in °C. *Fever is defined as a body temperature of > 38 °C. Data are expressed as mean±SD. *P indicates difference between the mean maximum severity by independent student's t-test between both groups, †P indicates difference between the time of maximum severity by independent student's t-test between both groups.

Lipid and Lipoprotein Response

Endotoxin challenge induced modest decreases in LDL-cholesterol from 2.7±0.6 to 2.4±0.5 mmol/L (P < .01) and from 2.7±0.8 to 2.4±0.9 mmol/L (P < .01), TG levels from 1.1±0.5 to 0.8±0.6 mmol/L (P < .01) and from 1.0 ± 0.6 to 0.6±0.3 mmol/L (P < .01) and apoB from 1.1±0.2 to 0.9±0.2 (P < .01) and from 1.0±0.2 to 0.7±0.2 mmol/L (P < .05), in the low- and normal/high HDL-group, respectively. Notably, HDL-cholesterol and apoA-I levels did not change upon endotoxin infusion over time. The lipids and lipoprotein response after endotoxin challenge was not significantly different between both groups.

Inflammatory Response

Leukocyte Response: After endotoxin infusion, early leukocytopenia and monocytopenia was comparable between both groups. The subsequent increase in leukocytes, monocytes, however, was significantly higher in the low HDL-group (figure 1A+B). In addition, endotoxin infusion significantly increased the neutrophil response in the low HDL-group (figure 1C).



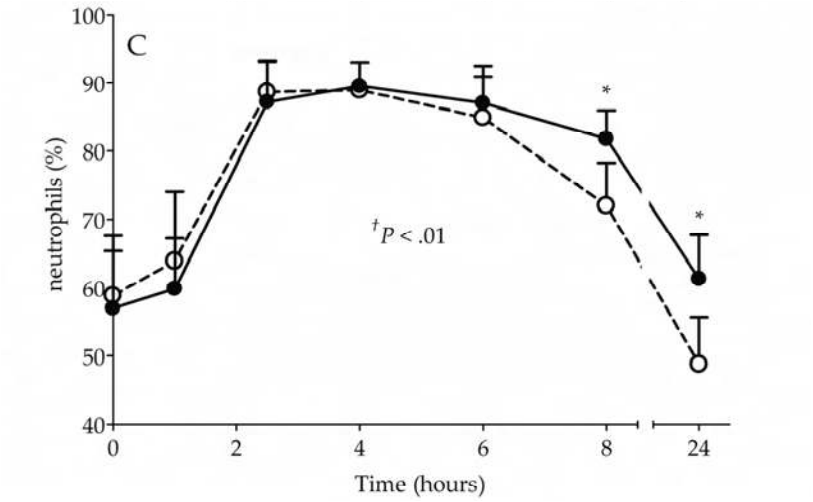


Figure 1. Effect of Inflammatory Challenge on Leukocyte Response

(A) leucocytes, (B) monocytes and (C) neutrophils curves in the low HDL-group (●) and the normal/high HDL-group (○). * indicates difference between timepoints by unpaired Student's *t* test ($P < .05$), †$P < .01$ indicates difference between the low HDL-group versus the normal/high HDL-group using ANOVA repeated measures analysis. Data are expressed as means±SD.

Cytokines: Baseline cytokine levels were similar in both groups. The effects upon endotoxin infusion on each cytokine are shown in figure 2. At 1 hour post-infusion TNFα levels were significantly increase in the low HDL-group compared to the normal/high HDL-group (figure 2A). A similar effect was noted for IL-1β, IL-6, IL-8 and MCP-1 levels at 2½ hours post endotoxin infusion (figures 2B-2E).

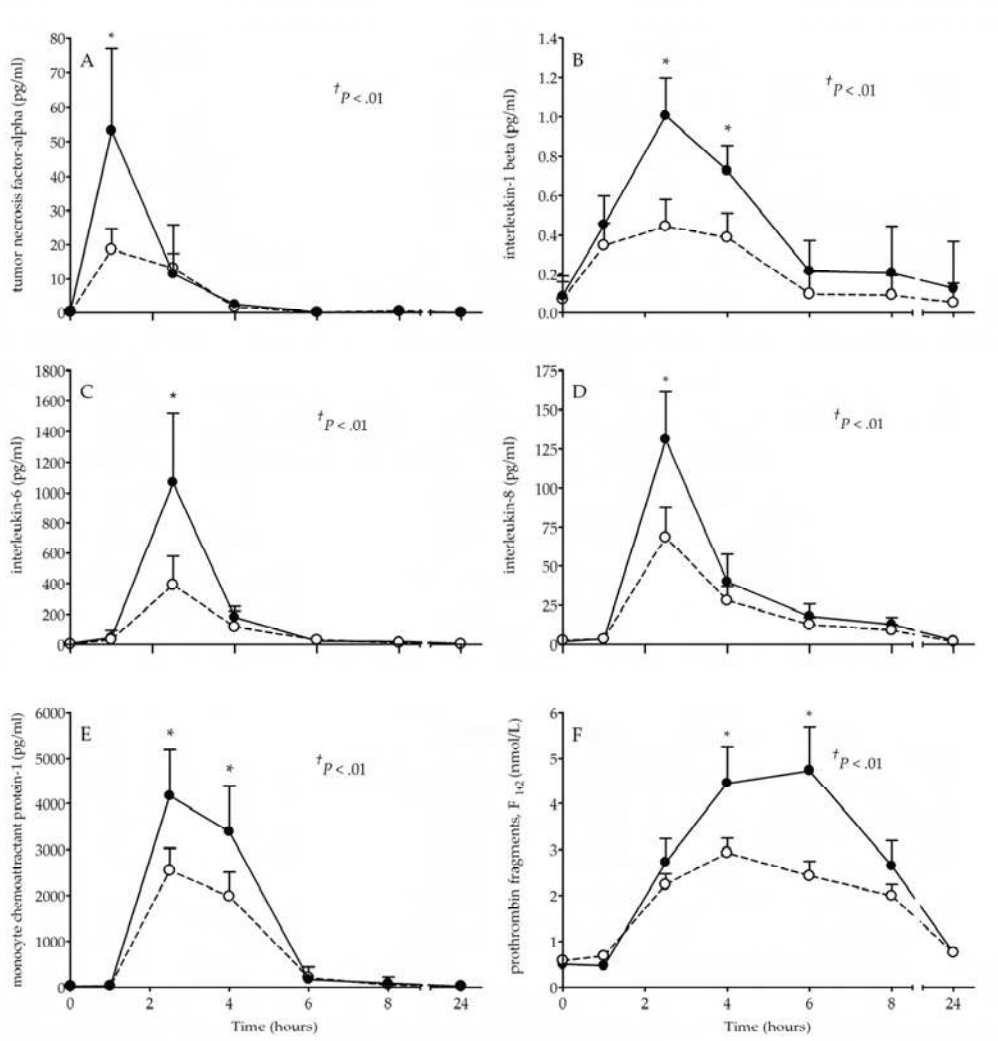


Figure 2. Effect of Inflammatory Challenge on Inflammation and Coagulation Activation

(A) TNFα, (B) IL-1β, (C) IL-6, (D) IL-8, (E) MCP-1 and (F) F_{1+2} curves in the low HDL-group (●) and the normal/high HDL-group (○). * indicates difference between timepoints by unpaired Student's *t* test ($P < .05$), †*P* value indicates difference between the low HDL-group versus the normal/high HDL-group using ANOVA repeated measures analysis. Data are expressed as means±SD.

Acute phase proteins: Although the groups had similar baseline hsCRP levels (table 1), CRP was significantly elevated in the low HDL-group compared to the normal/high HDL-group at 24 hours (43.2±6.5 mg/L versus 27.2±5.9 mg/L, $P < .01$). Baseline LBP levels were elevated in the low HDL-group (15.8±6.0 μg/ml) as opposed to the normal/high HDL-group (12.4±5.3 μg/ml, $P = .03$) and remained significantly elevated after endotoxin infusion through the 24 hour period (37.7±11.3 μg/ml versus 29.0±12.0 μg/ml, $P < .01$ respectively).

Paraoxonase-1 activity: Serum PON-1 activity was similar at baseline in both groups and was unaffected by endotoxin challenge (data not shown).

Coagulation Response

Starting out with similar prothrombin fragments (F_{1+2}) in both groups, F1+2 levels were significantly increased the low HDL-groups at timepoints 4 and 6 but returned to baseline in both groups at 24.

Fibrinolysis

Fibrinolytic markers (D-dimer, tPA and PAI-1) were similar between groups both at baseline and after endotoxin infusion, peaking at 2½ hours following endotoxin infusion and returning to normal levels at 24 hours (data not shown).

Linear Regression Analysis

Linear regression analysis revealed a significant inverse relation between HDL-cholesterol as well as apoA-I levels versus leukocytes (figure 3A), pro-inflammatory cytokines (figure 3B) as well as pro-thrombin fragments (data not shown).

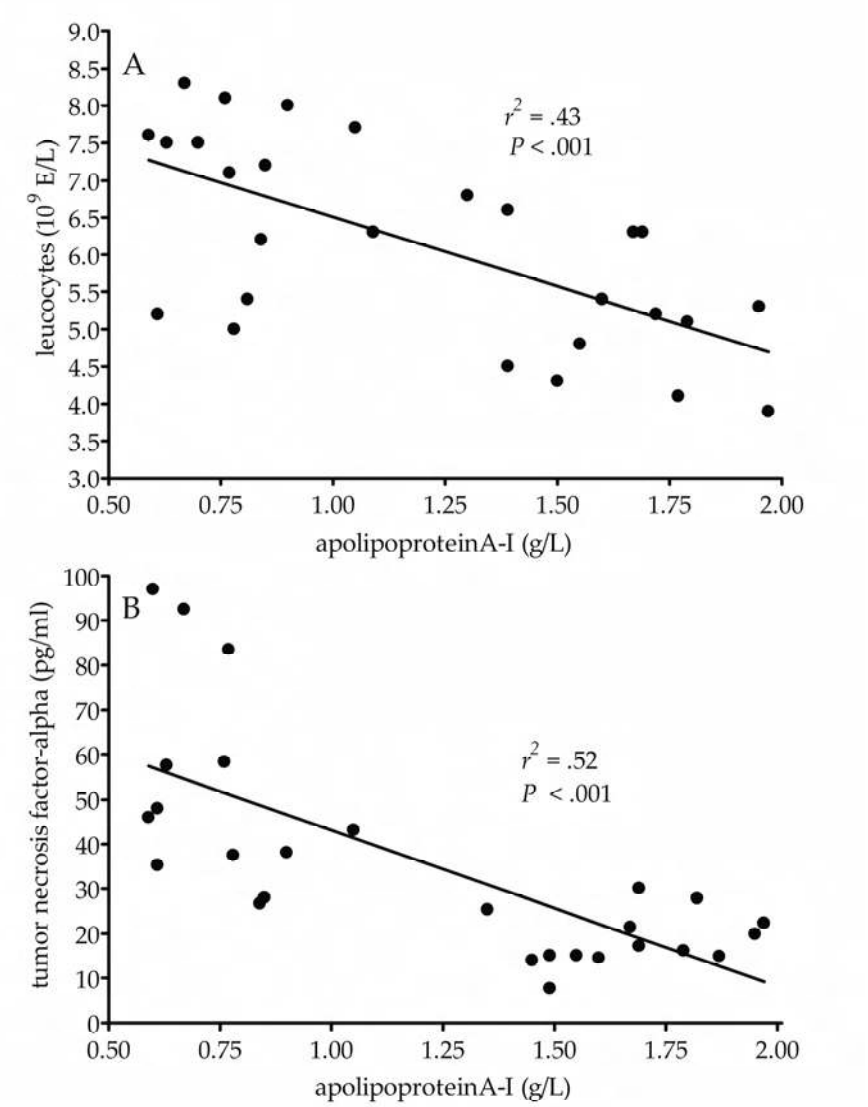


Figure 3. Correlation between apoA-I levels and endotoxin-induced systemic inflammatory response in all 27 study subjects

(A) leucocytes and (B) TNFα. Lines indicate linear regression analysis between apoA-I versus leucocytes at 24 hours and TNFα at 1 hour.

Discussion

In the present study, we demonstrate that apparently healthy males with genetically determined isolated low HDL-cholesterol levels are characterized by an increased sensitivity towards a low dose endotoxin challenge compared to subjects with normal/high HDL-cholesterol levels. Throughout the whole range of HDL-cholesterol levels, there was an inverse relation between apoA-I levels and sensitivity towards this inflammatory challenge. These findings lend further support to the relevance of HDL as an anti-inflammatory mediator in vivo.

Study Population

In order to evaluate the interaction between HDL and inflammation, we recruited participants from a study designed to identify genes that control HDL-cholesterol levels, excluding subjects with known genetic causes for low HDL, such as apoA-I mutations.[2] In line with a primary selection on low HDL-cholesterol levels, difference in HDL-cholesterol was more pronounced than the difference in apoA-I, implying the abundance of smaller HDL particles in the low HDL-group. In the control group, both subjects with normal HDL-cholesterol levels were included as well as 7 subjects with genetically determined high HDL-cholesterol levels. Importantly, the low and normal/high HDL-groups were carefully matched for parameters known to affect HDL-cholesterol levels or inflammatory state such as BMI and smoking.

Clinical Parameters and Lipid Changes

Endotoxin-induced symptoms like backache, chills, body temperature rise, hearth rate increase, headache, myalgia and nausea were increased and noted more severe in the low HDL-group compared to the normal/high HDL-group. In line with these results, low HDL-cholesterol level is associated with an increased mortality and severity of septic disease.[9] The sequential changes in lipids and (apo)lipoproteins following endotoxin challenge were comparable between groups. Within the 24 hours observation period, neither HDL-cholesterol nor apoA-I changed significantly upon endotoxin challenge, which is in line with previous analyses employing a 1 ng/kg bodyweight LPS infusion in healthy volunteers.[10]

Inflammatory Response

Early leukocytopenia, monocytopenia and decreased neutrophil count were comparable between groups (Figure 1). The magnitude of the "early" leukocyte margination has been shown to closely reflect the dose of endotoxin infused.[8] Hence, a similar degree of early leucocytopenia implies comparable exposure to endotoxin in both the low- as well as normal/high HDL-group. In contrast, endotoxin challenge elicited augmented leukocytosis, monocytosis and increased neutrophil count in the low HDL-group at later time points (Figure 1). Similarly, the increase of pro-inflammatory cytokines as well as acute phase reactants was also elevated in the low HDL-group compared to the normal/high HDL-group. Linear regression analysis revealed a strong inverse relation between HDL-cholesterol as well as apoA-I levels versus leukocyte response, pro-inflammatory cytokines and plasma CRP levels, supporting an anti-inflammatory effect of HDL throughout a wide concentration range (Figure 3).

Mechanism of HDLs' anti-inflammatory effects

Theoretically, the increased in vivo anti-inflammatory effect of HDL in the normal/high HDL-group could be adjudicated solely to increased scavenging of endotoxin, thereby minimizing the amount of endotoxin available to elicit inflammatory activation.[11] However, several other facts must be taken into account. First, the scavenging of endotoxin by HDL does not equal HDL-mediated neutralization of endotoxin bio-activity. Endotoxin triggers the inflammatory cascade by binding of the TLR4 receptor on monocytes and endothelial cells which occurs within seconds,[12] whereas HDL requires several minutes to scavenge endotoxin within its lipid moiety.[13] Even after endotoxin sequestration, HDL needs hours to fully neutralize the bio-activity of 'trapped' endotoxin.[14] Second, endotoxin elicits rapid sequestration of leucocytes and monocytes, the magnitude of which closely mirrors the dose of endotoxin exposure.[8] Since leukocyte and monocyte margination were identical in low- and normal/high HDL-group, decreased exposure towards endotoxin is less likely. Combined, these data imply that HDL scavenging cannot fully account for the differences in inflammatory and coagulation responses observed in the low- versus normal/high HDL-group. In

this respect, HDL may also have direct anti-inflammatory effects, independently from endotoxin scavenging.[6,15] First, the HDL-particle harbors protective enzymes, such as PON-1 which has been shown to inhibit monocyte migration.[16] However, in the present study, PON-1 activity was similar in both groups. Other moieties within HDL have also been implicated to exert anti-inflammatory effects. Thus, apoA-I itself has a direct inhibitory effect on several pro-inflammatory loops.[17] In fact, the potent, inverse relation between apoA-I and endotoxin-induced systemic response may highlight a role for apoA-I as anti-inflammatory mediator in vivo.

Procoagulant Response

Endotoxin infusion resulted in a significantly larger increase in thrombin generation in the low HDL-group as compared to the normal/high HDL-group, implying that HDL may also attenuate coagulation activation.[18] Mechanistically, HDL may attenuate coagulation either indirectly by modulating the inflammatory cascade via its associated proteins,[19] but also by directly affecting the coagulation system. Previous in vitro studies have already shown that HDL enhances the activity of the important physiological anticoagulant protein C pathway.[20] In line with these findings, population-based studies have shown an inverse relationship between HDL-cholesterol levels and tissue factor pathway inhibitor (TFPI) levels.[21] Furthermore, HDL have been shown to inhibit the expression of tissue factor by endothelial cells.[22] An inverse relation between HDL and coagulation has been supported by clinical observations. Indeed, low levels of HDL have been associated with increased risk for venous thrombosis.[23] HDL may also be entangled with arterial thrombosis in the setting of acute coronary syndromes. In the MIRACL study, it was shown that HDL-cholesterol levels at the time of acute coronary syndromes had strong prognostic impact on short-term clinical outcome, the latter being driven by arterial thrombosis on ruptured plaques

Summary

In the present study we provide evidence that endogenous HDL attenuates the inflammatory and coagulation response towards low-dose endotoxin challenge. These

findings provide a further impetus for implementation of HDL-increasing strategies in the acute, often inflammatory setting of e.g. myocardial infarction and acute coronary syndrome.[25]

Acknowledgments

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PART 2

The Echo of Inflammation

Chapter 7

Measurement of carotid intima–media thickness to assess progression and regression of atherosclerosis

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Abstract

Imaging modalities have been developed to assess atherosclerosis in vivo in the arterial wall because large clinical end-point studies are time-consuming and costly. Historically, in-hospital angiography and Doppler ultrasonography have been used to assess atherosclerosis development. Investigations of the arterial lumen are, however, increasingly being replaced by modalities that can measure changes in the arterial wall itself—intravascular ultrasonography, MRI and multislice CT. The fact that intravascular ultrasonography is invasive, CT involves substantial radiation exposure and requires contrast agents, and that MRI is time-consuming and technically challenging all limit the widespread use of these techniques. Moreover, all modalities have high associated costs. B-mode ultrasonographic imaging of the carotid arterial walls occupies a unique position in atherosclerosis research. This method enables sensitive, reproducible and noninvasive assessment of intima-media thickness (IMT) as a continuous variable. Epidemiological and clinical trial evidence as well as digitization and standardization have made carotid IMT a validated and accepted marker for generalized atherosclerosis burden and vascular disease risk.

Here we describe the application of carotid IMT measurements as a tool in risk evaluation of individuals and in studies of atherosclerosis progression and regression.

Introduction

Atherosclerosis is an inflammatory disease that often begins early in life with impairment of endothelial function, which leads to the formation of lesions in large and medium elastic and muscular arteries.[1,2] The disease process is dynamic and is associated with remodeling of the arterial wall. In the early stages of arterial wall thickening and plaque formation, no luminal changes are seen, because the arterial wall expands to compensate. This process, known as the Glagov effect, can ensure the size of the arterial lumen is preserved until plaque formation becomes extensive. This compensatory mechanism is considered the best explanation for the fact that the early stages of atherosclerosis are clinically asymptomatic.[3]

Although atherosclerosis can remain below the clinical horizon for a long time, it can manifest clinically as acute vascular disease at almost any stage of the disease process. Evaluation of atherosclerosis by means of clinical end points—morbidity and mortality—requires large study populations and necessitates considerable human and financial resources.[4,5] As atherosclerosis is a slow process, collecting clinical end-point data takes a long time and provides information mainly on the late stages of vascular disease, which makes cause and effect relationships difficult to unravel. New insights into the disease process are, therefore, difficult to obtain and rapid evaluation of novel therapies can prove difficult—unless techniques to assess early atherosclerotic changes are used.

The use of surrogate markers to measure atherosclerosis burden in vivo has become widespread. To be useful as a validated tool for risk assessment in cardiovascular disease the method needs to be technically sound, but must also meet a number of methodological requirements. Disease risk and effects of treatment need to be identified with greater sensitivity and speed than clinical end points.[6] The method must be widely available and preferably noninvasive. Furthermore, the association between risk and the disease marker must have been established statistically and be relevant

to the study question. The surrogate marker should also model pathophysiological information.[7] Finally, for clinical research in humans, regulatory requirements must be met.

As atherosclerosis is a multifactorial disease with distinct progression and regression behavior, cause and disease progression might not always be straightforward. A standardized and validated method is crucial for generating reproducible results that are predictive in nature. Brightness or 'B-mode' ultrasonography can depict all stages of atherosclerotic arterial wall changes as a continuous variable. The entire continuum of ultrasound-derived atherosclerosis measurements (arterial wall thickness, including the plaque) is referred to as intima–media thickness (IMT), although, strictly speaking, this nomenclature is incorrect from a histological perspective. IMT is a surrogate marker for atherosclerosis burden as well as cardiovascular disease risk assessment.[8-20] It could be argued, however, that rather than being a surrogate marker, IMT is part of the disease process itself. Notably, the FDA has approved IMT as a surrogate marker of atherosclerotic disease for application in clinical trials; however, the addition of IMT assessment to prevention guidelines is still under discussion. [8,21-25]

In this review we discuss the role of carotid IMT as a marker for progression and regression of atherosclerosis. We explore how the measurements can be used to assist clinical management and to advance atherosclerosis research.

Imaging atherosclerosis

Techniques to visualize atherosclerosis in humans originate from the refinement of available clinical imaging techniques such as Doppler ultrasonography and coronary angiography. Doppler ultrasonography can only identify arterial stenosis if the lumen area is reduced by at least 40–50%.[26] Traditionally, quantitative coronary angiography was the predominant imaging modality to assess the progression or regression of atherosclerosis. With quantitative coronary angiography, the cross-sectional coronary anatomy is depicted as a planar silhouette of a contrast-filled vessel lumen.

Both modalities are highly relevant in a clinical setting, but do not provide useful information on the early stages of arterial wall thickening before lesion formation.[27,28]

Multislice CT is rapidly outgrowing its angiographic heritage and is being developed into a completely noninvasive technique. This method will undoubtedly replace coronary angiography for assessment of coronary stenosis and calcification. Electron-beam CT is also noninvasive, but is not widely available. This technique also predominantly highlights calcified tissue,[29] which makes it unsuitable for the evaluation of the early stages of atherosclerosis. High-resolution MRI is also being used for volumetric and spectrometric measurement of the carotid arterial wall. That these modalities hold promise is undisputed; however, prospective epidemiologic and human trial data validating these techniques are not yet available. Moreover, an expensive infrastructure will be required to implement these techniques.

Intravascular ultrasonography (IVUS) depicts the arterial lumen and, most importantly, the arterial wall. This modality can, therefore, provide information on both plaque burden and coronary atheroma volume.[30,31] IVUS is now increasingly used in conjunction with noninvasive B-mode ultrasonographic imaging of the carotid artery to assess the treatment effects of antiatherosclerotic therapies.[32-34]

Measurement of carotid intima–media thickness

In essence, B-mode ultrasound IMT measurement involves a simple distance measurement between the leading edges of the lumen–intima and media–adventitia ultrasound interfaces (Figure 1). With this imaging method, the typical 'double-line' pattern of the normal arterial wall of the large peripheral arteries can be seen. Pignoli et al. investigated the double-line pattern in depth, and established the relationship between the leading edges of the ultrasound interfaces and the boundaries of the intima-media seen in an aorta specimen.[35] This relationship formed the basis of the present ultrasonographic carotid IMT measurements. Initially, B-mode images were used to guide the Doppler sound beam for investigating blood velocity. With the

availability of computerized technology, B-mode ultrasonography was developed to produce real-time, high-resolution images. In the late 1980s, the assessment of small-vessel wall structures throughout the different stages of atherosclerosis became feasible. The technique can be used to monitor atherosclerosis by measurement of IMT as a continuous variable from the healthy and thin arterial wall to total occlusion.[36] As a reference, the lumen of the common carotid artery in healthy individuals measures 6–9 mm in diameter with an average IMT of 0.4 mm at birth and 0.8 mm by the age of 80 years, if no risk factors are present. With an increased number of cardiovascular risk factors, IMT grows more rapidly over a lifetime and the probability of emerging lesions increases.[37]

It should be realized that noninvasive assessment of atherosclerosis in humans investigates submillimeter structures and structural changes measured in hundredths of millimeters. These small changes are clinically significant; a meta-analysis of statin intervention studies using IMT as a surrogate marker indicated that a reduction in IMT thickening of 0.012 mm per year is congruent with a significant odds ratio of 0.48 for the reduction of cardiovascular events.[38]

Practical considerations

The use of imaging techniques to visualize small structures of the peripheral arterial wall poses major technical challenges. First, the accuracy of measurements must be as high as possible. Although an increase in the ultrasound frequency can improve axial resolution, it must be noted that the measurement of IMT is a distance measurement in which the structure measured (approximately 0.55-0.95 mm) is much larger than the wavelength of the ultrasound produced by the commonly used near-field linear array transducer (approximately 0.12 mm). As the signal sampling rate is much higher than the wavelength, accurate measurement of IMT is not dependent on the frequency used but on the pixel resolution of the digitized image. As a consequence, digital representation of the imaged structures depends, in part, on pixel resolution. To visualize small structures such as the intima and media layers, the digital images

should be spread out over as many pixels as possible. Although this issue of accuracy is similar for every imaging technique, understanding how the ultrasonographic instrument displays information on the monitor and optimization of the image is particularly important when submillimeter-sized structures and even smaller changes in size are evaluated.

Second, the emphasis should be on high-end equipment that enables maximal spatial resolution at the time of image acquisition, and can scan with a low signal: noise ratio (a high dynamic range) and high tissue differentiation (i.e. relatively high frequencies, if depth allows). The latter element is of particular importance if early atherosclerosis and soft plaques are to be discriminated from signal noise in the vessel wall (Figure 1).

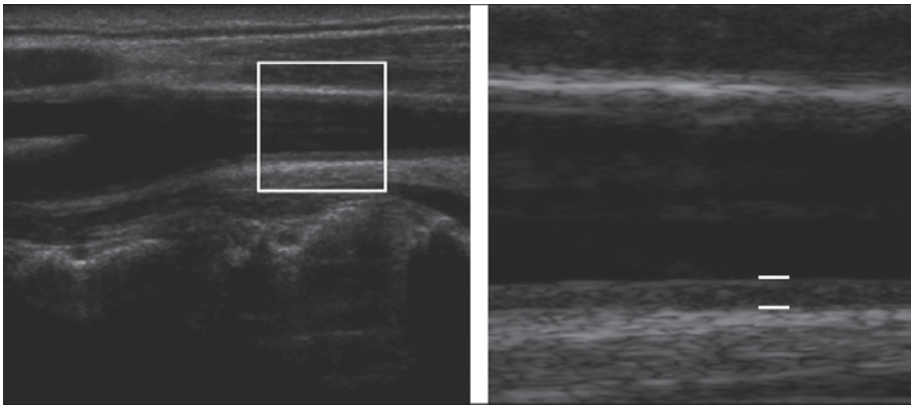

Figure 1. Brightness-mode ultrasound images of carotid artery wall segments

Brightness-mode ultrasound images of carotid artery wall segments from a 35-year-old asymptomatic male with a 14-year history of type 1 diabetes mellitus. (A) The sonographer selects the region of interest from an overview image of the carotid artery. (B) The expanded high-resolution 1.2 × 1.2 cm image of the region of interest is used for intima–media thickness measurements. The sonographer selects the best per-protocol image possible as a high resolution DICOM still. The distance between the arrows from the lumen–intima interface to the media–adventitia interface indicates an intima–media thickness of 1.12 mm. Abbreviation: DICOM, Digital Imaging and Communications in Medicine.

Another technical challenge involves the reproducibility of measurements, which should be as high as possible and, according to methodological and good clinical practice guidelines, must be reported for every specific study, in order to prove a priori that the measurements meet predefined protocol requirements. For this purpose, sonographers should undergo training specific to each new trial protocol, followed by certification and quality control to guarantee quality-assured data.[39,40] The aim of standardized scan protocols is to ensure that comparable and reproducible IMT measurements are acquired within and throughout studies.

Imaging protocol

Different protocols do exist, but essentially all comprise the following elements. At the start of a carotid ultrasound scan, the individual being assessed is placed in a comfortable reclining position. As during writing, the sonographer should support the elbow of the arm holding the ultrasound transducer. The transducer angles for each of the cross-sections at which images are obtained have to be predefined. Arterial wall segments are defined anatomically by the anatomic landmarks of carotid dilatation and the carotid flow divider (Figure 2).

For each cross-section, the best image obtained from the segment is selected by the sonographer at the time of the scan. The selected images and metafile information, as well as the corresponding digital clip for dynamic information, are then saved in the Digital Imaging and Communications in Medicine (DICOM) file format.[41]

Most large vascular clinics have ultrasonography equipment and trained vascular sonographers. For clinical purposes, when relatively large arterial wall structures are imaged, extensive plaques and symptomatic stenosis can be evaluated by Doppler flow and 'onscreen' measurements. For study purposes, however, particularly for pharmaceutical trials, the 'onscreen' method is inadequate, because measurement is inaccurate, administratively cumbersome, and hence difficult, if not impossible, to quality-control.

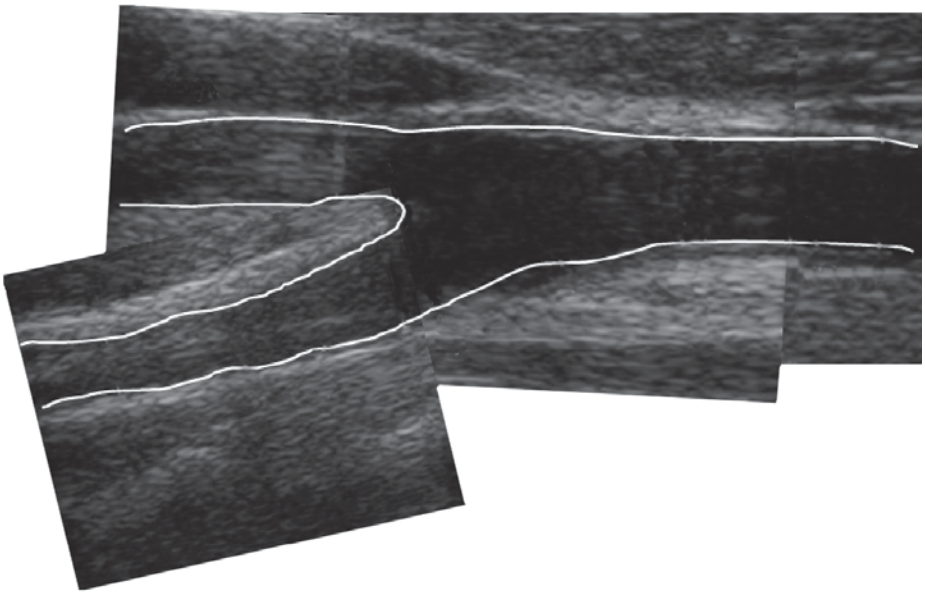

Figure 2. A composite ultrasonographic image of the carotid artery.

A composite ultrasonographic image of the carotid artery. For each arterial segment a high-resolution still image, as indicated by the dashed boxes, is selected by the sonographer. The carotid dilatation (arrowhead) and the carotid flow divider (arrow) are indicated. The following anatomical features are indicated: the common carotid artery1 cm proximal to the dilatation; the carotid bulb between the dilatation and flow divider; and the internal carotid artery1 cm distal to the flow divider. The scan protocol includes Doppler signal analysis to distinguish the external from the internal carotid artery and to exclude clinically relevant vascular stenoses. A bilateral carotid scan will, therefore, provide six DICOM stills and, to provide the image analysts with dynamic information, six associated DICOM clips. Abbreviations: CB, carotid bulb; CCA, common carotid artery; DICOM, Digital Imaging and Communications in Medicine; ECA, external carotid artery; ICA, internal carotid artery.

Taking the approach of analyzing DICOM-structured files in a core lab has the advantage that the source file cannot be altered once the image is saved by the sonographer, and an audit trail is recorded that includes traceability and change

control. In multicenter trials, in order to meet the necessary quality, administrative and regulatory requirements, images are handled and IMT measurements recorded off-line in a core ultrasound laboratory.[42]

Variability

Even though this modality is technically optimized and sonographers are trained, measurements in biological systems are always subject to a certain degree of variability. Variability can be introduced through the population studied, by the sonographer and image analyst, and by random error.[43] Once the images have been acquired, however, the contribution of the on-line and off-line IMT measurements to variability is minor, since the image is fixed.

Whereas observing the change of population variability can be the goal of some IMT measurements, the sources of measurement variability should be reduced as much as possible. If thin artery walls, such as those in children, are imaged (Figure 3A), population and measurement variability is small. When imaging thicker structures (Figure 3B and C), total measurement variability increases because of the difficulty of reproducing a slice through extensive plaque structures (Figure 4). In follow-up studies, measurement variability is, therefore, mostly sonographer-dependent. Intrasonographer variability (expressed as SD of the mean absolute difference of paired replicate scans) of IMT measurements is around 0.04 mm in children,[44] and 0.2 mm in older patients with peripheral vascular disease.[45,46]

In controlled clinical trials, measurement variability is decreasing. This decrease is most likely to be a result of technical improvements, standardization and training. In studies carried out between 1985 and 1990, the measurement SD as calculated above was 0.2 mm in patients with coronary artery disease,[45,47] whereas currently this figure is approximately 0.09 mm.[48] Moreover, two decades ago intraclass correlations of 0.60-0.75 mm were reported, whereas currently they are often 0.90 mm or higher.[40] The sources of variability of IMT measurements have been described extensively elsewhere. [43]

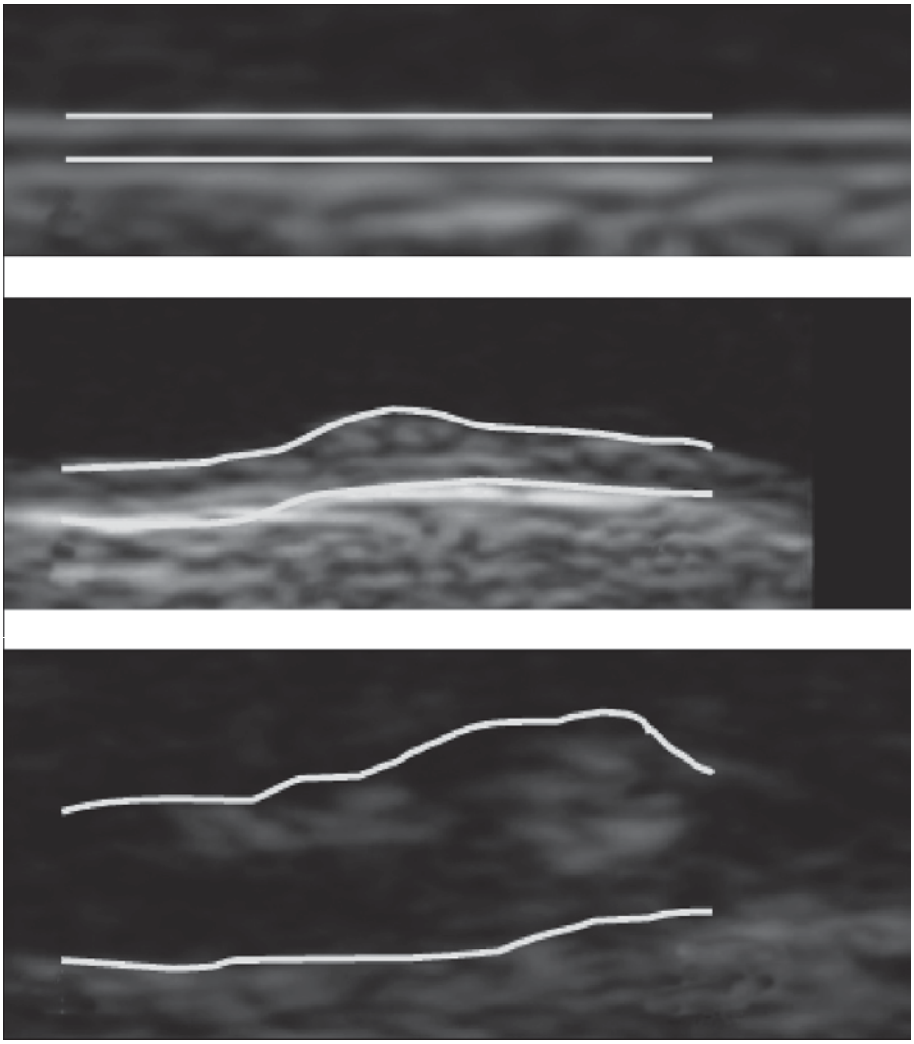

Figure 3. Examples of images of the arterial wall of the common carotid artery

The arterial wall of the common carotid artery of an asymptomatic individual throughout life and the various stages of arterial wall thickening. (A) Double lines in the common carotid artery during childhood (age 8 years). (B) Slight arterial wall thickening at the carotid bulb during middle age (age 52 years). (C) Extensive plaque formation at the carotid bulb at old age (age 85 years). The white lines delineate the lumen–intima and the media–adventitia interfaces drawn along the wall for approximately10 mm.

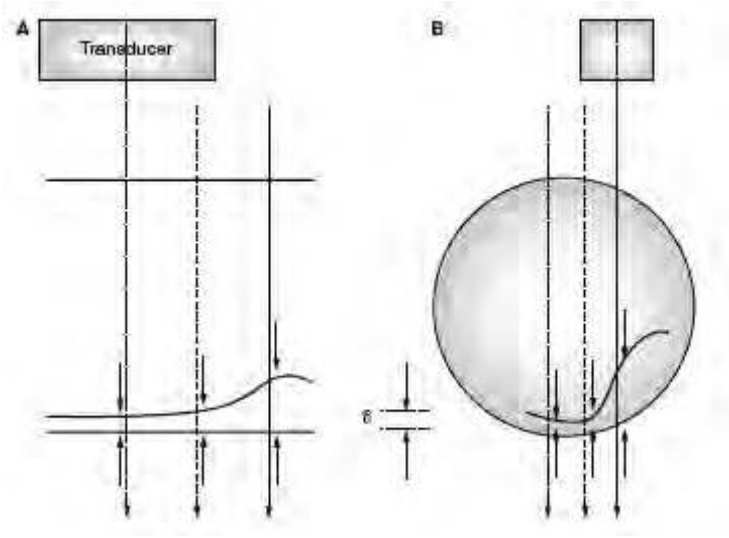

Figure 4. Schematic representation of sources of variability in intima–media measurement. The main sources of variability are differing features between patients, and between-sonographer and within-sonography variability in measurements. (A) Variation in measurements caused by plaque differences in the longitudinal plane. (B) Variation in measurements caused by plaque area differences in the transverse plane. Lack of adherence to predefined transducer angulations and landmark identification can influence results in comparative and follow-up studies. The dotted lines represent the transducer axis. δ indicates the distance between the lumen–intima and media–adventitial interfaces.

Carotid intima-media thickness: observational studies

Large follow-up studies such as the Rotterdam Study[10,11] and the Atherosclerosis Risk in Communities Study (ARIC)[12-15] have used B-mode ultrasonography to measure IMT to investigate the determinants of atherosclerotic disease in the general population.[9] The Rotterdam Study was a single-center, prospective, follow-up study of 7,983 individuals older than 55 years. The main objective of this study was to identify the determinants of atherosclerosis progression in the carotid artery wall. This ultrasound study provided solid evidence that IMT measurements can be used to indicate the degree of existing generalized atherosclerosis and future cardiovascular

disease risk. The investigation also provided evidence for associations between carotid IMT and stroke, angina pectoris, myocardial infarction, intermittent claudication and essential hypertension.[10,11] In ARIC, a US-based study that involved 15,800 adults, high-resolution B-mode ultrasonography was able to identify atherosclerotic lesions at all stages of development.[12-15] A seemingly small increase in mean carotid IMT of 0.2 mm was associated with an increase in relative risk for myocardial infarction and stroke of 33% and 28%, respectively—a link that has been confirmed subsequently by many other studies.

Studies that used multiple measurements of carotid IMT to investigate the determinants of disease progression showed that risk factors such as age, smoking, dyslipidemia and hypertension are the main predictors of increased carotid IMT.[16-18] Furthermore, carotid IMT measurements have predictive value not only for adverse cerebral events, but also for cardiac and peripheral vascular events.[9,10] The presence of plaques or stenoses in the carotid artery tree drastically increases this cardiovascular event risk.[16-18] At present, vascular clinics are increasingly using IMT to define an individual's cardiovascular risk. Although more standardized evaluation is necessary, results from recent studies indicate that individual risk-profile assessment might improve with the addition of this noninvasive parameter.[19,20]

Carotid intima–media thickness: Assessing success of therapy

Studies have used measurement of IMT has to assess the efficacy of drugs designed to lower lipid concentrations, antihypertensive agents, hormone replacement therapy, antioxidant supplements and lifestyle interventions.[49-56] An early study that used ultrasound-derived IMT was the 4-year, placebo-controlled Cholesterol Lowering Atherosclerosis Study (CLAS), which assessed the effects of therapy with colestipol and nicotinic acid in men who had previously undergone CABG surgery.[57] The investigators found that drug treatment had beneficial effects and reduced carotid IMT after 2 and 4 years of therapy ($p < 0.0001$). The Asymptomatic Carotid Artery Progression Study (ACAPS) was a 3-year trial in which the effects of therapy with a

daily dose of 20–40 mg lovastatin was compared with placebo in asymptomatic men and women aged between 40 and 79 years who had early carotid atherosclerosis.[58] The IMTs of 12 carotid artery wall segments were recorded per patient. Lovastatin significantly reduced IMT over the trial period compared with placebo ($p < 0.001$). The Kuopio Atherosclerosis Prevention Study (KAPS) investigated the 3-year efficacy of pravastatin in hypercholesterolemic men aged between 44 and 65 years.[59] In this trial, the primary outcome measure of disease progression (increase in IMT) in both carotid and femoral artery segments was significantly lower in pravastatin-treated patients than in those who received placebo ($p = 0.02$).

In the Regression Growth Evaluation Statin Study (REGRESS), the effects of 40 mg pravastatin were assessed in men with angiographically proven coronary artery disease and a total cholesterol level in the range 4.0-8.0 mmol/l (154-309 mg/dL).[45,60] In this 2-year atherosclerosis regression trial, the efficacy of pravastatin was demonstrated by coronary angiography[60] and B-mode ultrasonography of the peripheral arteries.[45] The ultrasonography substudies were conducted at three trial sites and included 255 of the 885 patients enrolled in REGRESS. Interestingly, the ultrasonography findings showing regression of IMT with treatment were highly significant ($p < 0.0001$), a significance level not obtained using coronary angiography in the overall REGRESS study.

In the 2-year Atorvastatin versus Simvastatin on Atherosclerosis Progression (ASAP) trial, the effects of 80 mg atorvastatin and 40 mg simvastatin once daily were investigated in 325 patients with familial hypercholesterolemia.[47] Aggressive cholesterol lowering was found to be more effective than conventional statin therapy. Specifically, the study showed an actual decrease in carotid IMT in the more-aggressive therapy group (who achieved an average LDL cholesterol reduction of 51%), whereas the less-aggressive treatment (resulting in a 41% reduction) was associated only with inhibition of atherosclerosis progression. The outcome of the 1-year Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol (ARBITER) study

in 161 patients with cardiovascular disease is in line with the ASAP trial findings.[61] To investigate whether lowering LDL cholesterol to below the National Cholesterol Education Program Adult Treatment Panel III criterion of 2.6 mmol/L (100 mg/dL) for secondary prevention would further reduce the burden of atherosclerotic disease, the effects on carotid IMT of 80 mg atorvastatin and 40 mg pravastatin once daily were compared. Atorvastatin reduced LDL cholesterol levels by an average of 49.0% to 2.0 (± 0.6) mmol/l (76 (± 23) mg/dL); pravastatin by 27% to 2.8 (± 0.7) mmol/L (110 (± 30) mg/dL). In the pravastatin group, IMT stabilized; in the atorvastatin group, IMT decreased, demonstrating atherosclerosis regression ($p = 0.03$).

The 2-year Long-term Intervention with Pravastatin in Ischaemic Disease Study (LIPIDS) also investigated whether lipid lowering to below the guideline threshold was beneficial.[44] In this study, 214 children aged between 8 and 18 years who had familial hypercholesterolemia were randomized to either 20 mg or 40 mg pravastatin or placebo once daily. Carotid IMT showed a reduction of 0.014 mm (± 0.046 mm; $p = 0.02$) in the pravastatin arm relative to placebo. Cross-sectional baseline data showed significant differences in IMT between children with familial hypercholesterolemia and unaffected siblings, even at a young age (Figure 5). Long-term follow-up showed that ultrasound imaging allows observation of atherosclerosis progression even in thin arterial walls (average 0.49 mm (± 0.06 mm)). These results indicate that the observation of treatment effects depends on the difference in IMT change between treatment groups, regardless of the stage and extent of the disease. The efficacy of antiatherosclerotic agents is, therefore, best tested in populations with a high atherosclerosis progression rate, but not necessarily in a population with a high burden of atherosclerosis.

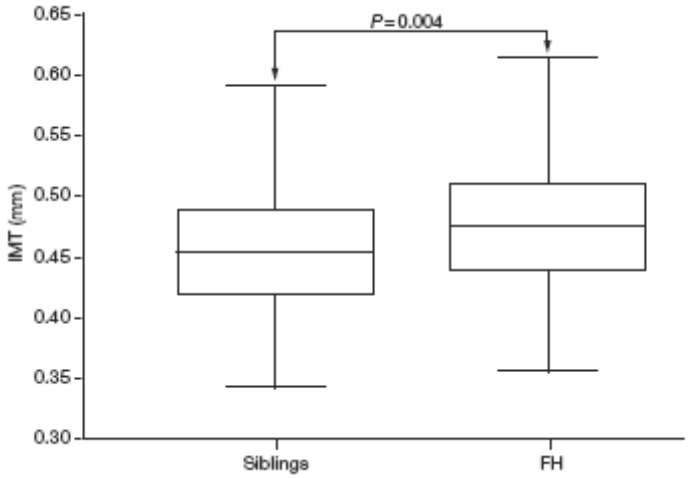

Figure 5. Intima–media thickness data from unaffected siblings and children with familial hypercholesterolemia

Intima–media thickness data from 95 unaffected siblings and 214 children with familial hypercholesterolemia aged 13.0 years (SD 3.0; range 7.9–18.9). Intima–media thicknesses are 0.46 mm (SD 0.054 mm) and 0.48 mm (SD 0.054 mm), respectively (p = 0.004). Even at a young age, intima–media thickness measurements can detect early increases in arterial wall thickness in those at high cardiovascular disease risk. In the same children, using the same ultrasonography imaging protocol, statin therapy for 2 years yielded favorable effects when compared with placebo. The strength of the standardized intima–media measurement lies in its potential to noninvasively identify cardiovascular disease risk and show treatment effects in populations in need of atherosclerosis prevention before the outbreak of disease. Abbreviations: FH, familial hypercholesterolemia; IMT, intima–media thickness.

Carotid and coronary ultrasonography: complementary imaging modalities

The results of the previously mentioned studies underline the utility of carotid ultrasonography as a tool to assess cardiovascular drug effects. To be convincing clinically, a novel drug has to prove its antiatherosclerotic effects in the vascular bed that is directly related to its clinical symptoms—for cardiovascular disease this means the coronary arteries.

IVUS of the coronary arteries is a validated and standardized tool for observing the effects of novel antiatherosclerotic drugs on coronary atheroma volume.[30-32] The findings of the ARBITER carotid IMT study,[61] the IVUS-based Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial[62] and the large outcome study Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis in Myocardial Infarction 22 (PROVE IT–TIMI 22)[63] in patients with acute coronary syndromes were similar, in that they all showed 80 mg atorvastatin (i.e. aggressive lipid lowering) to be superior to 40 mg pravastatin (i.e. moderate lipid lowering).

More recently, the results of the Rating Atherosclerotic Disease Change by Imaging with a New CETP Inhibitor (RADIANCE) 1 trial[64] in heterozygous patients with familial hypercholesterolemia and the RADIANCE 2 trial[65] in patients with mixed dyslipidemia, in addition to the Lipid Level Management Using Coronary Ultrasound to Assess Reduction of Atherosclerosis by CETP Inhibition and HDL Elevation (ILLUSTRATE) IVUS-based trial,[66] showed no benefit of 60 mg torcetrapib on arterial wall parameters despite impressive and beneficial lipoprotein changes. These findings indicated that this agent had off-target toxic effects and were corroborated by the results of the large morbidity and mortality study of this drug—the Investigation of Lipid Level to Understand Its Impact in Atherosclerotic Events (ILLUMINATE) trial.[67]

Conclusions

Surrogate markers of atherosclerosis such as carotid IMT can identify populations and individuals at increased cardiovascular disease risk and can guide future treatment in cardiovascular disease prevention. Observational studies and clinical trials have demonstrated the strong relationship between carotid IMT progression and regression, and cardiovascular events. With IMT data from many epidemiological studies and clinical trials accumulating, there is increasing evidence that IMT measurement can be used at individual level to refine cardiovascular disease risk scores. As B-mode ultrasonography is a relatively new technique and involves training and craftsmanship,

official acceptance of carotid IMT as a routine measurement in risk evaluation is still under discussion. Carotid IMT in fact meets the criteria of a validated marker for the assessment of atherosclerotic vascular disease and can evaluate novel agents in the true spirit of prevention before the emergence of clinical disease.

Key points

- In atherosclerosis research, surrogate markers are important in the early identification of disease, and in risk assessment and the evaluation of drug efficacy
- If imaging modalities are to be used in clinical studies and pharmaceutical trials, technical optimization and stringent standardization are required
- Carotid ultrasonography is a noninvasive method for measuring carotid intima–media thickness—a validated surrogate marker of atherosclerotic disease—that allows atherosclerosis assessment in individuals across the entire cardiovascular risk spectrum
- Carotid intima–media thickness measurements can be used to assess the consequences of cardiovascular disease risk reduction in patients and to investigate novel antiatherosclerotic strategies in clinical trials

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Chapter 8

Enhanced Atherogenesis And Altered High-Density Lipoprotein in Patients with Crohn's Disease

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Abstract

Background: A chronic inflammatory state is a risk factor for accelerated atherogenesis. The aim of our study was to explore whether Crohn's disease (CD), characterized by recurrent inflammatory episodes, is also associated with accelerated atherogenesis.

Methods: In 60 CD patients and 122 matched controls, carotid intima media thickness (IMT), a validated marker for the burden and progression of atherosclerosis, was assessed ultrasonographically. Additional subgroup analyses including plasma levels of acute phase reactants and HDL protein profiling were performed in 11 consecutive patients with CD in remission, 10 patients with active CD and 15 healthy controls.

Results: Carotid IMT in patients with CD was increased compared to healthy volunteers; 0.71 (0.17) versus 0.59 (0.14) mm ($p<0.0001$), respectively. In the subgroup analysis, HDL levels in controls and patients in remission were identical ((1.45 (0.48) and 1.40 (0.46)mmol/L: $p=0.797$), whereas HDL during exacerbation was profoundly reduced; 1.02 (0.33): $p=0.022$. HDL from patients with active CD and CD patients in remission was characterized by a reduced ability to attenuate oxidation compared to controls ($p=0.008$ and $p=0.024$ respectively).

Conclusions: Patients with CD have increased IMT compared to matched controls, indicative of accelerated atherogenesis. The changes during CD exacerbation in terms of HDL concentration as well as composition imply a role for impaired HDL protection in these patients.

Introduction

In the last decade it has become increasingly clear that inflammation plays a pivotal role in the pathogenesis of atherosclerosis. Leukocytes can adhere to activated endothelium and transmigrate to the subendothelial space. Subsequently, immune cell activation leads to plaque progression and eventually plaque rupture resulting in atherothrombotic disease.[1,2] Conversely, systemic inflammation itself has been suggested to promote the atherosclerotic process.[2] Indeed, in several chronic inflammatory disorders such as systemic lupus erythematosus (SLE),[3] rheumatoid arthritis (RA),[4] human immunodeficiency virus (HIV)[5] or even periodontitis,[6] systemic inflammation has been linked to enhanced atherogenesis illustrated by an increased incidence of cardiovascular disease (CVD). Several mechanisms by which a systemic inflammatory state can accelerate the atherosclerotic process have been suggested. Cytokine mediated damaging of the endothelium, immune cell activation and activation of the coagulation cascade have all been implicated. In addition, inflammation can also induce changes in lipoprotein metabolism. Particularly, inflammation can lower high-density lipoprotein (HDL) concentrations as well as qualitatively affect HDL.[7] During systemic inflammation, specific enzyme and protein components of HDL, contributing to HDL's anti-atherogenic potential, are modified thereby impeding its anti-atherogenic functions, and even may render it pro-atherogenic.[8] In several chronic inflammatory disorders, dyslipidemic changes have been linked to enhanced atherogenesis. The current exploratory study was designed to evaluate whether Crohn's disease (CD) is associated with an increased progression of the atherosclerotic process and whether inflammatory exacerbations are associated with alterations in HDL metabolism.

Methods

Patients

CD patients were recruited at the outpatient inflammatory bowel disease (IBD) clinic at the Academic Medical Centre, Amsterdam. During study visits, disease activity was assessed using the Harvey Bradshaw Index (HBI). The HBI is a research tool composed of clinical parameters (general well-being, abdominal pain, number of liquid stools per day, abdominal mass and complications) used to quantify the symptoms of patients. Patients with HBI ≥ 4 were considered to have active CD, patients with scores <4, were considered to be in remission. Blood samples were collected for CRP and lipid profiling. CRP measurements were used for the second criteria defining active CD and CD in remission: CRP ≥ 10 mg/l and CRP < 10 mg/l respectively. All study patients were asked to participate in ultrasound carotid IMT measurements. Healthy controls matched for age and gender were recruited at the department of vascular medicine and participated in the analysis of lipid profiles and IMT measurements. Patients gave written informed consent, and the study was approved by the local Medical Ethical Committee.

Ultrasound measurements of the carotid intima-media thickness (IMT)

As is described in extenso elsewhere, IMT measurements allow for the investigation of arterial wall morphology and can describe the status as well as the present and future cardiovascular disease risk, non-invasively.[9] In summary, B-mode ultrasound cIMT measurements depict the intima-media complex of carotid arterial walls. As was shown in prospective epidemiological studies already a modest increase of IMT substantially increases the relative risk for myocardial infarction and stroke. IMT measurements have also shown the benefit of cholesterol lowering and anti-hypertensive agents. IMT is an accepted validated surrogate marker for the status of atherosclerosis and present and future atherosclerotic disease risk.[10]

Laboratory measurements

All blood samples were immediately stored at -80º C and thawed once before measurement. C-reactive protein (CRP) was measured using a commercial high sensitivity-CRP reaction kit for human CRP (hsCRP, HemoIL, Lexington, MA, USA) according to manufacturer's protocol. The minimum detectable CRP concentration of the assay was 2.0 mg/l. Samples with CRP concentrations higher than 8 mg/l were diluted using CRP diluent. Serum amyloid A (SAA) analysis was carried out using a commercial available Human SAA ELISA kit from Anogen (Mississauga, Ontario, Canada) according to the manufacturer's protocol, ApolipoproteinAI (apoAI) and apoB were determined by nephelometric immunochemistry (Beckman, USA).

Lipoprotein Composition

Total cholesterol (TC) distribution amongst the lipoproteins was measured by Fast Phase Liquid Chromatography (FPLC). In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and an UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line enzymatic reagent (Biomerieux, Marcy l'Etoile, France) addition at 0.1 ml/min. Plasma lipoprotein separations were performed using a Superose 6 HR 10/30 column (Pharmacia Biotech, Upsala Sweden) with TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Total cholesterol was determined quantitatively using PAP 250 cholesterol enzymatic method (Biomerieux, Le Fontanille, France). Computer analyses of the chromatograms for quantitative peak integration of the lipoproteins were carried out using Borwin Chromatographic software, version 1.23 (JMBS Developments, Le Fontanil, France).

Sample preparation and SELDI-TOF MS analysis

HDL protein profiling was carried out as described previously.[11] Coating of antibodies: A 5 µL mixture containing 2.8 nM anti-apo A-I monoclonal antibodies, 3 µM ethylenediamine and 0.1 M Na_2SO_4 was added per spot of a PS-20 protein chip and covalent binding of antibodies through primary amine-epoxide chemistry

was achieved by incubating the chip in a humid chamber overnight at 4 ˚C. Excess antibody was removed by 1 wash with distilled water and subsequently free amine-binding places were blocked by incubating the chip for 30 min at RT with 1 M Tris buffer (pH 8.0).

HDL capture: After mounting the PS-20 protein chip(s) in a 96 wells bioprocessor, 100 µL diluted plasma aliquots (1:2 diluted with TBS buffer; 50 mM Tris, pH 7.4, 150 mM NaCl) or purified HDL were applied onto single SELDI spots and were allowed to bind for 2 hours at RT on a horizontal shaker. The protein chips were washed 4 times with TBS for 10 minutes, followed by a 5 minutes TBS-Tween (0.005%) rinse unless indicated otherwise. A final wash step with Hepes solution (5 mM) was carried out to remove the excess of salt. All spots were allowed to dry and subsequently 1.2 µL sinapinic acid (10 mg/ml) in a 50/49.9/0.1 % acetonitril/H_2O/trifluoric-acid mix was applied on each spot. All chips were air dried and stored at room temperature in the dark.

SELDI-TOF analysis

Analysis was carried out using a PBS IIc protein chip reader (Ciphergen Biosystems, Fremont, CA, USA) using an automated data collection protocol within the ProteinChip Software (version 3.1). Data was collected up to 200 kDa. Laser intensity was set in a range from 190 to 220 arbitrary units and the focus mass was set to 28 kDa specific for the anti A-I capture. Measurement of the spectra was performed with an average of approximately 100 shots at 13 positions per SELDI spot. Calibration was done using a protein calibration chip (Ciphergen). Spectra were normalised on total ion current. Detected peaks having a signal/noise ratio > 5 were recognized as significant peaks.

HDL anti-oxidant score

The assay was performed as described previously with slight modifications[12] using historical controls. Briefly, oxidized PAPC (oxPAPC) is a pro-inflammatory phospholipid, which triggers vascular inflammation processes. The respective HDL_oxPAPC assay measures the potential of plasma-derived HDL to reduce/inactivate previously (air)oxidized phospholipids. HDL for this assay is isolated from plasma

using dextran-sulphate coated magnetic beads. These beads precipitate apoB-containing lipoproteins. The cholesterol content of the HDL-containing supernatant is determined. HDL cholesterol (25uM final concentration) is added to a reaction mixture that contains oxPAPC. Adding DCF – a fluorochrome – to the reaction mixture produce a fluorescence signal dependent of the concentration of oxPAPC. Reduction of oxPAPC due to pre-incubation with HDL results in loss of fluorescence, the readout parameter of this assay. As such, HDL with an anti-oxidant score>1 is considered pro-inflammatory whereas a score <1 is considered anti-inflammatory.

Statistical analysis

SPSS statistical program 11.0.1(SPSS Inc., Chicago, USA) was used. Standard descriptive and comparative analyses were undertaken. Results are expressed as mean and standard deviations (SD). Student's *t*-test for unpaired data was used to compare CD patients with healthy volunteers. Linear regression analyses were performed to asses the relationships between CRP, SAA, HDL, ApoAI and ApoB. One-way ANOVA tests were used to investigate differences between lipid, lipoprotein and acute phase protein values. A p-value <0.05 was considered statistically significant.

Results

Characteristics of the Study Groups

A total of 60 CD patients participated in this study, of which 12 suffered from active disease and 48 were in clinical remission (table 1). In total 122 healthy controls were included in the reference group. No differences were observed in smoking behavior or the presence of hypertension or diabetes mellitus between the three groups.

IMT measurements

IMT measurements were performed in 60 CD patients and 122 healthy controls. Both groups exhibited a wide age range (Table 1), but nevertheless the IMT of CD patients

(0.71(0.17) mm) was increased when compared to healthy controls (0.59(0.14) mm): p<0.001. The estimated IMT increase in CD and control subjects with age is graphically displayed in figure 1. The regression line for CD differed significantly from that of controls (p<0.05).

Table 1. Baseline Characteristics of the Study Subjects.

	Patients with active CD n=12	Patients with CD in remission n=48	Patients with CD n=60	Healthy controls n=122
Age, years	34.4 ± 9.0	44.4 ± 12.9	42.4 ± 12.8	40.5 ± 16.4
Male, n (%)	7 (58.3)	20 (41.7)	27 (45.0)	55 (45.1)
BMI	25.6	24.1 ± 3.9	24.4 ± 3.9	25.0 ± 8.1
HT, n(%)	1 (8.3)	4 (8.3)	5 (8.3)	7 (5.7)
DM , n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Smoking, n (%)	4 (33.3)	9 (18.8)	13 (21.7)	22 (18.0)
HBI	8.1 ± 3.2	1.3 ± 0.7	2.7 ± 4.8	NA
Total cholesterol	3.79 ± 0.89	4.73 ± 1.11	4.54 ± 1.12	5.04 ± 0.99
LDL cholesterol	2.46 ± 0.91	2.62 ± 0.97	2.59 ± 0.96	2.99 ± 0.81
HDL cholesterol	1.01 ± 0.30	1.66 ± 0.43	1.53 ± 0.48	1.47 ± 0.53
Triglycerides	0.70 ± 0.88	0.98 ± 0.54	0.92 ± 0.62	1.34 ± 1.25
hsCRP	94.5 ± 108.3	2.7 ± 2.5	21.0 ± 59.7	1.9 ± 1.7
IMT	0.62 ± 0.13	0.73 ± 0.17	0.71 ± 0.17	0.59 ± 0.14

Values are given as means ± SD. HT=hypertension, DM= Diabetes Mellitus, HBI= Harvey Bradshaw Index, LDL= Low Density Lipoprotein, HDL= High Density Lipoprotein, hsCRP= high sensitivity C-Reactive Protein, NA=not applicable. Lipid values are in mmol/L, hsCRP in mg/L, carotid IMT in mm.

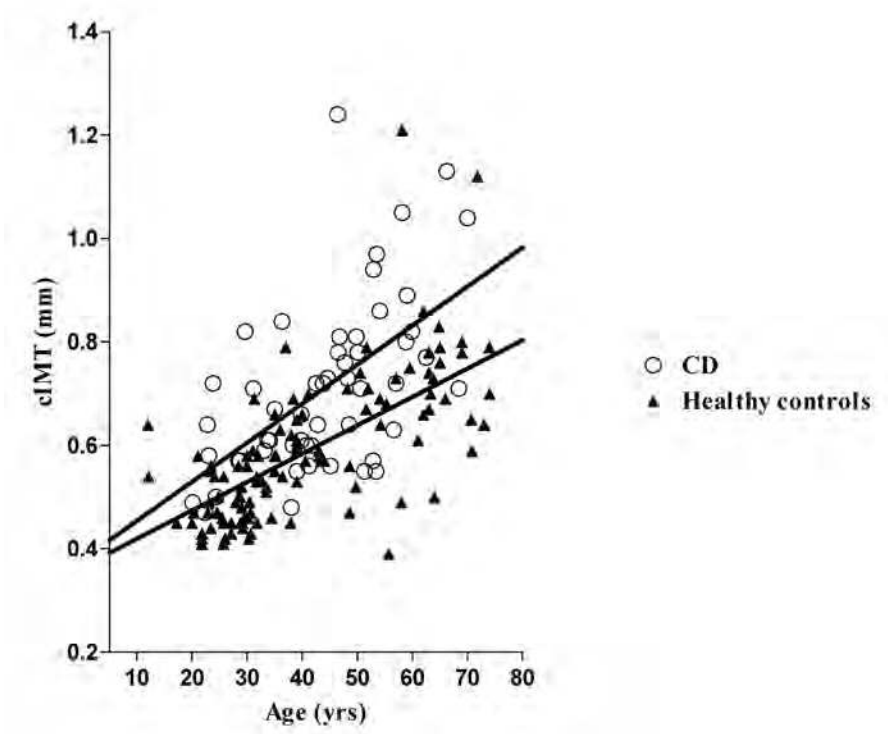

Figure 1. Carotid intima media thickness (cIMT) and age in CD patients and healthy controls.

The probability value indicates the difference in slope between the two lines.

Lipid profiles

Additional analyses were performed in the first consecutive 11 patients with CD in remission, 10 patients with active CD and 15 healthy controls (table 2). Mean HDL concentrations were higher in controls and patients in remission compared to patients with active CD (p=0.022 and p=0.043, respectively). HDL concentrations did not differ between controls and patients in remission. Reduced VLDL concentrations were found in active patients compared to controls and patients in remission (p=0.019 and 0.028, respectively). Concentrations of LDL did not differ among the groups. With respect to apolipoproteins, controls and patients in remission had significantly higher serum apoAI compared to CD patients with active disease (table 2) whereas apoB levels were similar in all groups.

Table 2. Lipid values, acute phase proteins and apolipoproteins of consecutive subjects in a subgroup analysis.

Parameter	Controls n=15	Active CD patients n=10	CD patients in remission n=11
HDL	1.45±0.48	1.02±0.33*†	1.40±0.46
VLDL	0.37±0.20	0.18±0.13*†	0.37±0.22
LDL	2.64±0.66	2.57±0.96	2.54±0.70
CRP	0.8±0.4	108.8±113.8**††	2.6±2.7
SAA	2.6±1.4	530.8±655.3**††	25.1±61.3
ApoAI	1.48±0.27	1.11±0.27**††	1.52±0.22
ApoB	0.87±0.21	0.88±0.26	0.90±0.27

Values are presented as mean ± SD; n= number of subjects, HDL, High Density Lipoprotein; VLDL, Very Low Density lipoprotein; LDL Low Density Lipoprotein; CRP, C-reactive-protein, SAA, Serum Amyloid-A; ApoAI, ApolipoproteinAI; ApoB, ApolipoproteinB. Lipid values are in mmol/L, acute phase reactants in mg/L and apolipoprotiens in g/L. * $p<0.05$ compared to controls.† $p<0.05$ compared to patients in remission.** $p<0.005$ compared to controls.†† $p<0.005$ compared to patients in remission

Acute phase proteins

Mean CRP concentrations were increased significantly in patients with active CD when compared to patients in remission and controls (table 2). A linear regression analysis (log transformed) showed that CRP and HDL were correlated: $r^2= 0.24$, $p=0.002$. Another acute phase protein, SAA, was not significantly different between controls and patients in remission, $p=0.870$, while SAA concentrations in active CD were significantly elevated compared to controls and patients in remission (table 2).

SELDI-TOF analyses

After SELDI-TOF analyses, protein spectra from HDL were obtained. Within each of the groups, patients with active CD or CD in remission and healthy controls, all spectra showed virtually similar profiles. Comparing the fingerprints of the healthy controls and CD in remission group with those of HDL from active CD patients statistically significant deviations in relative intensity on 5 different markers; 3602, 4634, 11695, 13843 and 14106 M/Z were observed (figure 2). No statistically significant differences between the HDL fingerprints from healthy controls and patients in remission were seen.

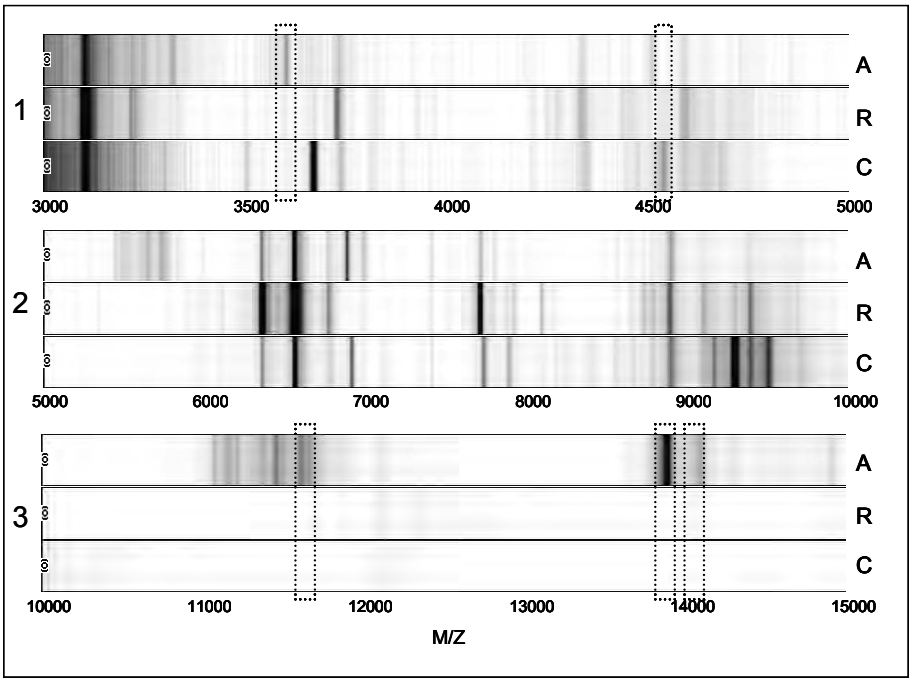

Figure 2. A representative example of HDL spectra in gelview of patients with Crohn's disease and controls respectively.

Depicted are the spectra in the M/Z mass range between 3000 –5000 Da (panel 1), 5000-10000 Da (panel 2) and 10000-15000 Da (panel 3). Within the dotted boxes the statistically significant deviations after Bonferroni correction between active Crohn (A) and remission (R) or controls (C) are seen. All spectra were normalised on total ion current.

Anti-oxidant potential of HDL

The anti-oxidative indeces of HDL of controls and patients with CD, active and in remission, are displayed in figure 3. The anti-oxidative capacity of HDL did not differ between CD patients in remission compared to those with active disease (p=0.53). HDL isolated from patients with active CD but also from CD patients in remission was characterized by a reduced ability to attenuate oxidation when compared to that of controls (p=0.008 and p=0.024 respectively).

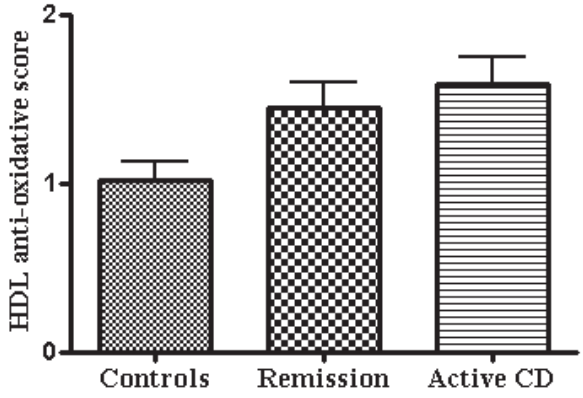

Figure 3. The anti-oxidative index of HDL isolated from controls, patients with CD in remission and patients with active CD.

Discussion

In the current exploratory analysis we show that CD is associated with acceleration of the atherosclerotic process as illustrated by an increased carotid IMT in CD patients compared to healthy controls. In addition, CD patients were characterized during an inflammatory exacerbation by profoundly decreased levels of HDL combined with biochemical changes of the HDL particle. These data suggest that early detection of atherosclerosis and subsequent cardiovascular prevention in patients with CD might be warranted.

Intima Media Thickness in Crohn's Disease

Arterial wall thickness can be measured as continuous variable from childhood into old age and can be used in patients as well as in controls. Consequently, carotid IMT measurements are used on a broad scale to assess risk in patients with an increased cardiovascular risk such as patients with chronic inflammatory disease such as SLE and RA but also familial hypercholesterolemia (FH). In the latter group IMT measurements have also been used to evaluate the effectiveness of various therapeutic interventions. As such, IMT is now an validated surrogate marker for the assessment

of atherosclerotic vascular disease.[9,10] In the current study we also assessed progression of the atherosclerotic process in CD by measuring IMT. Indeed, IMT was significantly increased in patients with CD when compared to healthy controls. Due to the plethora of risk factors that are of influence on IMT in CD, a CVD risk assessment based on IMT data of other populations at risk in parallel risk assessment is premature. It must be noted however, that the on average IMT increase of 0.12 mm in CD patients on top of 0.59 mm in the unaffected is of similar order to the estimated IMT increase in other patients groups characterized by a pro-atherogenic state. This strongly implies CD patients are at increased CVD risk. Long term follow up studies investigating the causality of inflammatory, dyslipidemic and other causes of accelerated atherosclerosis in these patients, as well as clinical trials to evaluate preventive drug therapy, are therefore warranted.

Cardiovascular disease in Crohn's Disease

For up to seventy years, it has been known that inflammatory bowel disease is associated with venous but also arterial thrombosis.[13-21] The incidence ranges from 1.2% to 6.1% according to different studies and up to 39% in autopsy studies.[22] The same risk factors that have been suggested to underly this atherothrombotic state in CD are also risk factors for atherosclerosis such as hyperhomocysteinemia,[23] antiphospholipid antibodies[24] and a procoagulant state.[25] Interestingly, atherosclerosis and CD also share a common pathway in the CD40/CD40L system[26] and while circulating activated platelets underlie a procoagulant milieu in CD[27] they have also been shown to exacerbate atherosclerosis.[28] Furthermore, systemic inflammation has emerged as a causal factor for accelerated atherogenesis in inflammatory disease states such as SLE and RA.[29,30] Recently, evidence for such an association has emerged in other chronic inflammatory disorders as well. Indeed, several reports have also suggested that CD is associated with premature atherosclerosis.[31-35] In addition, our findings of accelerated atherosclerosis correspond well with several other studies which showed evidence of subclinical atherosclerosis in IBD by demonstrating IMT thickening[36,37] or endothelial dysfunction.[38] Although numbers on cardiovascular morbidity are lacking,

cardiovascular mortality does not appear to be increased in patients with CD. However, there is significant disparity in reported mortality rates in Crohn's disease ranging from 30% lower than expected to 70% higher than expected.[39] Most of the patients in these cohorts were identified retrospectively and were diagnosed before improved medical treatment became available. In a more recent study, mortality risk of CD patients was significantly increased and a standardized mortality ratio for cardiovascular mortality was 1.49.[40] Long term follow up studies are required to resolve these issues.

HDL and Crohn's Disease

The outcome of the atherosclerotic process is determined by the balance between pro- and anti-atherogenic stimuli. HDL is amongst the most powerful endogenous mediators in atheroprotection which is illustrated by the strong inverse relationship between HDL levels and the incidence of CVD.[41,42] Via the reverse cholesterol transport (RCT) pathway, HDL can transport cholesterol from peripheral tissues, such as the arterial wall, back to the liver. Several additional properties of HDL contribute to its anti-atherogenic potential. Firstly, HDL exerts various anti-inflammatory effects and can reduce vascular inflammation in atherogenesis. For instance, HDL reduces the endothelial expression of adhesion molecules and chemokines thereby reducing recruitment of leukocytes to the subendothelial space.[43] Oxidative modification of lipoproteins plays a pivotal role in atherogenesis. HDL has potent anti-oxidant properties and can reduce oxidative stress by a transport mechanism that binds oxidant molecules and carries anti-oxidative enzymes as well.[43] Consistent with inflammation, there is increased oxidative stress in CD patients resulting in increased lipid peroxidation.[44] This will stimulate atherogenesis in patients with CD but can be neutralized by HDL. Thirdly, HDL has several antithrombotic properties which are of interest since CD is associated with a procoagulant state illustrated by an increased risk of venous thromboembolism. Thus, distinct changes in HDL are likely to contribute to the pro-atherogenic state in CD, particularly during exacerbation of the disease. With regard to the effects of HDL beyond its role in RCT, it has been advocated that changes in HDL's functional characteristics may provide information on its

vasculoprotective effects over and beyond merely focusing on HDL levels. In line with this, 'dysfunctional' HDL particles have been demonstrated in patients with overt cardiovascular disease.[45] Interestingly, HDL from patients with active CD, without cardiovascular disease, was characterized by a higher anti-oxidative score compared to healthy volunteers, indicating an attenuated anti-atherogenic potential.[45] The fact that there was still a higher anti-oxidative score of HDL in CD patients in remission compared to controls may imply that even in remission the low-grade inflammatory state has an impact on HDL quality in spite of normalization of HDL levels. These findings bear close resemblance to those reported in patients with SLE and RA, in whom pro-inflammatory changes in HDL have been observed.[46] In line, Navab et al. have elegantly demonstrated that during acute phase reactions the concentration of 'protective' proteins on the HDL particle actually decreases significantly.[47] Overall, our findings imply that during CD exacerbation, loss of HDLs' atheroprotective effects, on top of the drop in HDL levels, may contribute to accelerated atherogenesis.

Simultaneously, HDL protein profiling has emerged as a promising tool to unravel the biochemical composition of HDL[11,48,49] with which functional characteristics are intertwined. In addition to showing diminished levels of HDL during an inflammatory exacerbation of CD, using SELDI-DOF analysis we were able to show alterations in HDL composition. Interestingly, it has been suggested that HDL during an inflammatory episode can lose its protective properties and can even enhance atherogenesis.[8] In the present study, we observed changes in the biochemical composition of the HDL particle. Even though SELDI TOF ms is not a direct approach for identification of proteins, the 11695 marker can be identified as SAA. This has already been confirmed in previous reports by using SELDI TOF combined with MALDI TOF ms for identification of this specific marker.[50-52] The acute phase response in patients with active CD, characterized by increased SAA levels, thus seems to underlie an increased presence of SAA within the HDL particle. In fact, it has recently been shown that pro-inflammatory cytokines such as TNF-α and IL-6, which are increased in patients with CD, induce SAA expression in hepatocytes.[53] It is already known that SAA has the capacity to replace apoAI in the HDL particle which renders them less 'protective'.[54,55]

In conclusion, these data suggest atherogenesis is enhanced in patients with CD. Changes in HDL concentration as well as in HDL's compositional and functional characteristics during exacerbations of CD imply that attenuation of this anti-atherogenic mediator during these exacerbations might contribute to the progression of atherogenesis. The present findings call for consideration of the implementation of cardiovascular disease detection and prevention in patients with CD.

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Chapter 9

Atherogenesis during the early stages of rheumatoid arthritis: evidence from carotid artery imaging

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Interim analysis

Abstract

Background: The increased risk for cardiovascular disease (CVD) in patients with rheumatoid arthritis (RA) has been largely ascribed to the accompanying chronic inflammatory state. Whether this CVD risk is restricted to RA patients with 'longstanding' disease and whether the RA-associated autoantibodies also associate with accelerated atherogenesis remains to be elucidated.

Methods*:* We therefore measured carotid intima-media thickness (cIMT) in arthritis patients (n=17) with disease duration <6 months, diagnosed with RA according to ACR criteria or with undifferentiated arthritis with elevated serum IgM rheumatoid factor (RF) and/or anti-citrullinated antibody levels (ACPA), as well as in a group of individuals with elevated serum RF and/or ACPA levels without arthritis (n=40) and healthy controls (n=66). To evaluate the relationship between cIMT and RA associated autoantibodies as well as other parameters uni- and multivariate regression analyses were performed.

Results: Individuals with elevated serum levels of RA specific autoantibodies were characterized by increased mean cIMT compared to healthy controls, indicative of accelerated atherosclerosis (0.71 ± 0.19 vs. 0.57 ± 0.13, p=0.029). In contrast, mean cIMT was not increased in patients recently diagnosed with rheumatoid or undifferentiated arthritis compared to healthy controls (0.64 ± 0.13 vs. 0.57 ± 0.13, p=0.193). In subjects with elevated serum levels of RA specific autoantibodies, mean cIMT was associated with age, ESR and ACPA levels in a univariate analysis. Upon multivariate linear regression analysis only age and ACPA levels remained significantly associated with cIMT.

Conclusion: The atherosclerotic process is accelerated during the early stages of RA. These findings lend further support to close monitoring of CVD-risk in all patients in whom RA has been diagnosed.

Introduction

A systemic inflammatory state is a risk factor for accelerated atherosclerosis in patients with rheumatoid arthritis (RA).[1-3] This is illustrated by the observation that carotid intima-media thickness (cIMT) is increased and that it correlates with inflammatory status.[4,5] Moreover, an increased cardiovascular morbidity and mortality has been observed in these patients[6] which can, in fact, be reduced by potent, anti-inflammatory treatment.[7] Mechanistically, cytokines are released from the affected synovial tissue(s) into the systemic circulation which alter the functioning of distant tissues, including skeletal muscle and vascular endothelium, to generate a spectrum of proatherogenic changes that include insulin resistance, endothelial dysfunction and damage.[1] Moreover, circulating inflammatory mediators may also stimulate leukocytes and smooth muscle cells within the atherosclerotic plaque thereby promoting plaque growth or rupture.[8]

Although accelerated atherosclerosis has thus been well-characterized to be promoted by a systemic inflammatory state in patients with established RA, it has not been elucidated from which point in time RA patients are subjected to an increased risk of developing a cardiovascular event. Furthermore, it remains unclear whether autoantibodies associated with RA, IgM rheumatoid factor (RF) and anti-citrullinated antibodies (ACPA), play a role in promoting atherogenesis in RA patients.[9-11] To investigate the atherogenicity of the early stages of RA as well as the role of RA-associated antibodies we measured cIMT in RA patients who had been diagnosed with arthritis within the previous 6 months, in a group of individuals with elevated serum levels of RA specific autoantibodies who did not fulfill the American College of Rheumatology (ACR) criteria for RA, in a group of individuals with elevated serum levels of RA specific autoantibodies without arthritis, as well as in age- and gender-matched controls.

Methods

Patients

All participants were included at the outpatient department of Clinical Immunology and Rheumatology at the Academical Medical Center in Amsterdam, the Netherlands. Patients who had been diagnosed with RA according to the ACR criteria within the last six months as well as patients who had been diagnosed with undifferentiated arthritis (UA) with elevated serum levels of RA specific autoantibodies (RF and/or ACPA antibodies) were invited to participate (study group 1). In addition, individuals with elevated RF and/or ACPA serum levels without arthritis were included (study group 2). Healthy controls matched for age and gender were recruited at the department of vascular medicine and participated in the analysis of lipid profiles and cIMT measurements. Study participants were invited for a clinical study visit during which demographics, medication use, and clinical disease activity parameters were recorded, blood was withdrawn and cIMT measurements were performed. Written informed consent was obtained from all participants. The study protocol was approved by the institutional review board at the Academic Medical Center in Amsterdam.

Arthritis disease activity parameters

Arthritis disease activity was assessed by a 68 tender and swollen joint count, the patient's assessment of global disease activity and pain on a visual analog scale (VAS) of 0-100 mm, Disease Activity Score in 28 joints (DAS(28)),[12] morning stiffness in minutes, the erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) levels.

Laboratory measurements

Blood was collected in EDTA, citrate, and heparin anticoagulated aliquots, as well as serum tubes, which were kept on ice and centrifuged at 1600*g* for 15 minutes at 4°C, snap-frozen, and stored at –80°C until analysis. Plasma total cholesterol was measured with an enzymatic colorimetric procedure (CHOD-PAP; Boehringer Mannheim, Mannheim, Germany). HDL cholesterol was determined after precipitation of apoB-

containing lipoproteins by $MnCl_2$. Low-density lipoprotein cholesterol was calculated using the Friedewald formula. Triglycerides were measured using an enzymatic colorimetric method using lipase, glycerol kinase, and glycerol-3-phosphate 3 oxidase. ACPA were determined by the cyclic citrullinated peptide (CCP)-2 ELISA kit (Eurodiagnostica) (cutoff 25 kAU/L in serum). Rheumatoid factor (RF) IgM was determined with the RF IgM ELISA (Sanquin) (cutoff 12.5 kU/L in serum).

Carotid IMT

As shown in prospective epidemiological studies, a modest increase of cIMT substantially increases the relative risk for myocardial infarction and stroke. cIMT is an accepted validated surrogate marker for the status of atherosclerosis and present and future atherosclerotic disease risk.[13,14] B-mode ultrasound imaging was used to visualise three carotid arterial wall segments comprising common carotid, bulb and internal of the left and right carotid arteries. All scans were performed by the same sonographer. Both the sonographer and the image analyst were blinded to the clinical status of the subjects. Mean cIMT was defined as the mean cIMT of the right and left common carotid, the carotid bulb and the internal carotid far wall segments. For a given segment, cIMT was defined as the average of the right and left cIMT measurements. The per-patient averaged means of the cIMT values of segments was used for the primary analysis.

Statistical analysis

Standard descriptive and comparative analyses were undertaken using SPSS statistical program 16.0 (SPSS Inc., Chicago, USA). Results are expressed as mean and standard deviations (SD). Mean values of continuous variables between patients and subjects were compared using Student's *t* test for independent samples. The relation between the dependent variable cIMT on the one hand and other parameters on the other hand was first explored univariably using linear regression analysis. In addition, several multivariate analyses were performed to explore the effect of age and the statistically significant variables on cIMT. Throughout these analyses a p-value <0.05 was considered statistically significant.

Results

Clinical characteristics

Table 1 summarizes traditional risk factors for cardiovascular disease and cIMT values of the study participants. In total 17 patients who had recently been diagnosed with RA or UA with elevated serum levels of RA specific autoantibodies were included. In addition, 40 individuals with elevated RF and/or ACPA serum levels without arthritis were included as well as 66 apparently healthy controls matched for age and gender. There were no significant differences between the study groups with regard to age, sex, BMI, systolic and diastolic blood pressure and lipid profile. One individual with elevated serum levels of RA specific autoantibodies also had diabetes mellitus. Two individuals with RA specific autoantibodies had previously suffered from a TIA.

Table 1. Baseline Characteristics of the Study Subjects.

	Patients with recent onset RA or UA n=17 (Group 1)	Subjects with RA specific antibodies n=40 (Group 2)	Group 1 & 2 combined n=57	Healthy controls n=66
Age, years	48.9 ± 10.0	48.5 ± 11.5	48.6 ± 10.9	42.5 ± 15.1
Male/female	6/11	9/31	15/42	23/43
BMI	25.7 ± 3.7	25.4 ± 4.6	25.5 ± 4.3	24.7 ± 3.8
SBP	130 ± 20	129 ± 22	130 ± 22	129 ± 23
DBP	80 ± 12	78 ± 10	79 ± 11	77 ± 11
DM, n	0	1	1	0
Prior CVD, n	0	2	2	0
Total cholesterol	5.01 ± 0.94	5.25 ± 0.96	5.18 ± 0.95	5.26 ± 0.99
LDL cholesterol	3.05 ± 0.93	3.23 ± 0.82	3.18 ± 0.85	3.08 ± 0.84
HDL cholesterol	1.39 ± 0.43	1.53 ± 0.32	1.49 ± 0.36	1.60 ± 0.39
Triglycerides	1.27 ± 0.92	1.08 ± 0.48	1.13 ± 0.64	1.09 ± 0.66
cIMT	0.64 ± 0.13	0.71 ± 0.19	0.69± 0.18	0.57 ± 0.13

Values are given as means ± SD. BMI=Body Mass Index, SBP=Systolic Blood Pressure, DBP=Diastolic Blood Pressure, DM= Diabetes Mellitus, LDL= Low Density Lipoprotein, HDL= High Density Lipoprotein, CRP= C-Reactive Protein. Blood pressure values are in mmHg, lipid values in mmol/L, CRP in mg/L, carotid IMT in mm.

Characteristics related to inflammation and RA

Individuals with RA associated antibodies without arthritis were characterized by increased levels of CRP compared to patients who had been diagnosed with rheumatoid or undifferentiated arthritis within the previous six months (10.6 ± 22.8 vs. 6.2 ± 6.9, p=0.038). As expected, 68 swollen joint count was significantly higher in patients recently diagnosed with rheumatoid or undifferentiated arthritis (4.3 ± 6.3 vs. 0.0 ± 0.0, p<0.001). Whereas the level of RF did not differ significantly (74.2 ± 128.1 vs. 233.8 ± 1243.5, p=0.272) the level of ACPA was more then 3-fold higher in patients recently diagnosed with rheumatoid or undifferentiated arthritis as compared to subjects with RA associated antibodies (2907.7 ± 2299.3 vs. 770.4 ± 1384.2, p=0.012).

Table2. Characteristics related to inflammation and RA.

	Patients with recent onset RA or UA n=17 (Group 1)	Subjects with RA specific antibodies n=41 (Group 2)	P-value
CRP	6.2 ± 6.9	10.6 ± 22.8	0.038
ESR	21.1± 15.7	14.4 ± 12.8	0.193
Tender joint count	8.7 ± 8.1	6.6 ± 8.3	0.422
Swollen joint count	4.3 ± 6.3	0.0 ± 0.0	<0.001
Disease activity (VAS)	37.9 ± 26.6	37.8 ± 27.0	0.982
Pain (VAS)	35 ± 26	42 ± 33	0.189
DAS (28)	3.7 ± 1.6	2.9 ± 1.3	0.241
Morning stiffness	31 ± 40	23 ± 34	0.254
RF	74.2 ± 128.1	233.8 ± 1243.5	0.272
ACPA	2907.7 ± 2299.3	770.4 ± 1384.2	0.012

Values are given as means ± SD. CRP= C-Reactive Protein, ESR= erythrocyte sedimentation rate, VAS=visual analog scale, DAS= Disease Activity Score, RF= rheumatoid factor, ACPA= anti citrullinated antibodies.

cIMT measurements

Mean cIMT of patients recently diagnosed with rheumatoid or undifferentiated arthritis was comparable to that of individuals with elevated serum levels of RA specific autoantibodies (0.64 ± 0.13 vs. 0.71 ± 0.19; p=0.266). Subjects with elevated serum levels of RA specific autoantibodies were characterized by increased mean

cIMT compared to healthy controls, indicative of accelerated atherosclerosis (0.71 ± 0.19 vs. 0.57 ± 0.13, p=0.029). In contrast, mean cIMT was not higher in patients recently diagnosed with rheumatoid or undifferentiated arthritis compared to healthy controls (0.64 ± 0.13 vs. 0.57 ± 0.13, p=0.193). In addition, mean cIMT of patients recently diagnosed with rheumatoid or undifferentiated arthritis combined with mean cIMT values of individuals with elevated serum levels of RA specific autoantibodies was increased compared to healthy controls (0.69 ± 0.18 vs. 0.57 ± 0.13; p=0.045).

Determinants of cIMT

Mean cIMT was only determined by age in patients recently diagnosed with rheumatoid or undifferentiated arthritis. In subjects with elevated serum levels of RA associated autoantibodies, mean cIMT was determined by age, ESR and ACPA levels in univariate analysis (see table 3). Upon multivariate linear regression analysis, age and ACPA levels remained significantly associated with cIMT. When evaluating the two groups combined cIMT was significantly associated with age (p=0.006), LDL cholesterol (p=0.030) and ESR (p=0.041). Only age (p=0.003) remained significantly associated with cIMT in multivariate analysis.

Table 3. Determinants of cIMT in subjects with RA associated antibodies as well as in patients with recent onset RA or UA combined with patients with RA associated antibodies.

Parameter	P-value in univariate analysis	P-value in multivariate analysis
Subjects with RA associated antibodies		
Female sex	0.222	
Age	0.001	0.001
BMI	0.088	
SBP	0.079	
LDL cholesterol	0.076	
HDL cholesterol	0.087	
CRP	0.811	
ESR	0.012	0.103
IgMRF	0.814	
ACPA	0.031	0.030
Group 1 and 2 combined		
Female sex	0.311	
Age	0.006	0.003
BMI	0.140	
SBP	0.170	
LDL cholesterol	0.030	0.370
HDL cholesterol	0.187	
CRP	0.901	
ESR	0.041	0.072
IgMRF	0.751	
ACPA	0.625	

Values are given as means ± SD. BMI=Body Mass Index, SBP=Systolic Blood Pressure LDL= Low Density Lipoprotein, HDL= High Density Lipoprotein, CRP= high sensitivity C-Reactive Protein, ESR= erythrocyte sedimentation rate, RF= rheumatoid factor, ACPA= anti citrullinated antibodies.

Discussion

In the current study we show that mean cIMT of individuals with RA associated antibodies is increased compared to healthy controls, indicative of accelerated atherogenesis in these subjects. In univariate regression analyses, cIMT of individuals

with RA associated antibodies was determined by age, ESR and ACPA levels whereas after multivariate analysis only age and ACPA levels remained statistically significant. cIMT was not increased in a small group of patients recently diagnosed with RA or UA with elevated serum levels of RA specific autoantibodies as compared to healthy volunteers.

Preclinical RA and inflammation

It is well documented that the pathophysiological mechanisms of RA initiate long before symptoms occur or the actual diagnosis of RA is made,[15] similar to what has been shown in other autoimmune disorders.[16,17] In fact, several studies have shown that autoantibodies can be present more than 10 years before the clinical onset of RA.[18-20] Interestingly, immunohistological studies showed signs of inflammation in synovial tissue from clinically uninvolved joints in RA[21,22] which underlines the pathophysiological relevance of this preclinical phase. Moreover, serum levels of CRP[23] and sPLA2[24] are increased in preclinical RA patients in comparison with healthy controls. Thus, increased serum levels of CRP and sPLA2, which coincidentally are known to predict future coronary artery disease in apparently healthy individuals,[25,26] during the preclinical phase of RA indicate that an acute phase response can develop years before symptoms of RA occur. Similarly, ten years before onset of their symptoms, future RA patients already have a significantly more atherogenic lipid profile.[27] In these years prior to the clinical onset of RA, patients are thereby exposed to risk factors which can accelerate the atherosclerotic process significantly. Indeed, we not only showed that cIMT is increased in the early stages of RA, but also that cIMT is associated with ESR, lending further support to a potentially causal relationship between the systemic pro-inflammatory state and acceleration of the atherosclerotic process during the early stages of RA.

Autoantibodies and atherogenesis

Contradictory results with regard to the role of autoantibodies in RA-mediated acceleration of the atherosclerotic process have been reported. Indeed, increased

cIMT in 82 RA patients was associated with elevated levels of IgG anticardiolipin (IgG aCL) antibodies.[9] Moreover, RF positivity was associated with an increased likelihood of ischemic heart disease in a cross-sectional study in 567 apparently healthy men in a general population.[10] In contrast, cIMT was not associated with various autoantobodies (e.g. ACPA, IgG aCL, IgM aCL) in a recent study with 71 RA patients. It should be noted however that cIMT in RA patients was not increased compared to healthy controls.[11] In the current study we find a positive correlation between serum ACPA levels and cIMT in subjects with RA associated autoantibodies. This is in line with recent studies demonstrating an association with serum ACPA and increased cIMT[28] as well as premature death due to cardiovascular disease.[29] Further studies are needed to evaluate the potential effects of autoantibodies on atherogenesis. In a recent study, IgG antibodies to β2 GP-I were obtained from a patient with antiphospholipid syndrome.[30] Administration of these antibodies to apoE$^{-/-}$ mice significantly enhanced atherogenesis compared to apoE$^{-/-}$ mice receiving IgG from a healthy control. Comparable studies to determine the atherogenicity of RA associated antibodies have not been reported.

Clinical Relevance

Atherosclerotic vascular disease is a major source of morbidity and mortality among RA patients. The notion that RA patients are subjected to an increased CVD risk from the early stages of their disease process onwards has major consequences with regard to the implementation of preventive strategies in these patients. Despite the fact RA is associated with CVD, atheroprotection is not routinely incorporated in treatment strategies of RA patients. The results of the current study suggest, however, that anti-atherosclerotic therapy should be implemented already during the early stages of RA. The latter is corroborated by two recent studies which demonstrated endothelial dysfunction in patients with recent onset RA. Indeed, as demonstrated by plethysmography, endothelial function was 34%[31] and 59% [32] lower in patients diagnosed with RA within the previous 18 or 12 months respectively when compared to healthy controls. Interestingly, anti-inflammatory and/or DMARD therapy for a period

of six months[31] as well as a single treatment of TNF blockade[32] was shown to reverse endothelial dysfunction. The fact that endothelial dysfunction was readily and fully reversible in newly diagnosed RA patients supports early and aggressive prevention of CVD in all RA patients. Pending the outcome of ongoing statin intervention in RA patients (e.g. trial of atorvastatin for the prevention of cardiovascular events in rheumatoid arthritis (TRACE RA Trial); estimated end date June 2012), the use of CV-preventive compounds in RA patients with persistent inflammatory activity deserves closer attention in the near future.

Study Limitations

Several aspects have to be taken into account when interpreting the results of the present study. A potential limitation of the current cross-sectional study is a diminished capacity to identify additional risk factors due to the fact that atherosclerosis is a continuous process. In addition, only a limited number of patients who were recently diagnosed with RA or autoantibody positive undifferentiated arthritis was included complicating analyses of mean cIMT. Furthermore, we pooled cIMT values of recently diagnosed RA patients with a group of patients with RA specific autoantibodies who did not fulfill the ACR criteria for RA in order to compare them to controls. However, odds ratio of anti-CCP1 and anti-CCP2 for the future development of RA in healthy controls were 64.5 (8.5 to 489) and 28 (8 to 95) respectively in a recent meta-analysis.[33] Considering the group consisting of subjects with RA associated autoantibodies most likely also includes individuals that will not develop RA this potential limitation would lead to an underestimation of accelerated atherosclerosis during the early phases of RA and therefore does not negate our findings.

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Chapter 10

Systemic Inflammation as a Risk Factor for Atherotrombosis

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Abstract

Several chronic inflammatory disorders, such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA) and chronic infections that are associated with a chronic inflammatory state such as Human Immunodeficiency Virus (HIV) infection, are associated with an increased incidence of cardiovascular disease (CVD). Cardiovascular mortality is a major cause of death in patients with these disorders. Direct effects and indirect sequelae of systemic inflammation promote atherothrombotic vascular disease. Pathophysiological processes promoting atherogenesis can initiate years before the diagnosis of a chronic inflammatory disease is made, and since exposure to risk factors in this preclinical phase is widespread, early cardiovascular protection in these patients seems warranted.

Introduction

During the last decades it has become well established that inflammation plays a major role during all stages of atherosclerosis, from fatty streak formation up to plaque destabilization and subsequently atherothrombosis.[1] Recently, it has become clear that systemic inflammation, principally outside the vascular system, can enhance atherogenesis. This is illustrated by the increased incidence of cardiovascular disease (CVD) in patients with chronic inflammatory disorders such as systemic lupus erythematosus (SLE),[2] rheumatoid arthritis (RA),[3] inflammatory bowel disease,[4] human immunodeficiency virus (HIV)[5] or even periodontitis.[6]

Systemic inflammation & CVD: Epidemiology

It has been known for up to thirty years that patients with SLE have an increased risk of developing CVD.[7] This holds particularly true for premenopausal women with SLE, who are 50 times more likely to have a myocardial infarction compared to healthy women.[8] Indeed, subclinical atherosclerosis is present in 40% of all SLE patients,[9] who overall have a 5-10 times increased risk to develop CVD.[10] Similarly, the association between RA and atherosclerotic vascular disease was identified decades ago and these patients have a 4-fold increased risk of CVD.[11] In fact, cardiovascular mortality is currently the main cause of death in RA patients.[12] In the relatively short period of time that HIV is now being diagnosed, it has become clear that also in these patients the atherosclerotic process is accelerated, partly independent of the use of anti-retroviral therapy.[13] In fact, atherogenesis appears to also be enhanced in Sjögren's disease,[14] systemic sclerosis[12,15] and vasculitis.[16] In addition, it has recently been suggested that Crohn's disease is also associated with enhanced atherogenesis.[17,18] Indeed, having an inflammatory disorder is an independent risk factor for vascular co-morbidity[2,11,13] which suggests that the association between systemic inflammation and CVD is universal. In line with this, in several studies disease activity appears to be linked with atherosclerosis progression[19,20] although this could not be confirmed by other studies.[21] It is highly likely that in the near future an association with CVD will be confirmed in other inflammatory disorders as well.

Systemic inflammation and the arterial wall

In general, exacerbations of inflammatory disorders are characterized by activation of leukocytes as well as increased concentrations of cytokines and other inflammatory mediators. This may impose injury on the arterial wall accelerating the atherosclerotic process.[13,22,101] The suggestion that systemic inflammation can enhance atherogenesis has been substantiated by the observation that various anti-inflammatory interventions can protect the vascular wall. Blockade of tumor necrosis factor-α (TNFα) or chemokine receptors for instance, results in attenuated atherosclerosis.[16,23,24]

The atherogenic effects of a systemic inflammatory state can manifest at different levels. First, systemic inflammation can induce endothelial dysfunction. Under physiological circumstances the endothelium produces mediators such as the vasodilator nitric oxide (NO) that protect the arterial wall against atherothrombosis. An inflammatory state results in reduced expression of endothelial NO synthase (eNOS) in conjunction with increased expression of inducible NOS (iNOS). This dysbalance results in the production of excessive amounts of NO and underlies endothelial dysfunction. In addition, a dysbalance between various endothelium derived prostanoids has also been implicated in the origin of endothelial dysfunction.[25] Endothelial functioning can be measured non-invasively by means of Flow-Mediated Dilation (FMD). Following deflation of an occluding forearm cuff, the ensuing reactive hyperemia causes increased shear stress and subsequent production of NO. The ensuing diameter increase of the brachial artery can be measured using ultrasound diameter measurements.[26] Endothelial dysfunction is a sensitive and early marker for atherosclerotic vascular disease and occurs before morphological changes of the arterial wall are present.[27] In several chronic inflammatory disorders such an endothelial dysfunction has been demonstrated.[28,29]

Secondly, systemic inflammation induces secondary dyslipidemia; an atherogenic lipid profile characterized by reduced high-density lipoprotein cholesterol (HDL) and increased triglycerides.[10,30] Indeed dyslipidemia is more common in SLE and RA as exemplified by an increased incidence of the metabolic syndrome.[31,32] In addition to lowering HDL levels, systemic inflammation can also modify the protein and

enzyme composition of the HDL particle.[17,33] Subsequently, HDL can acquire deviant functional characteristics and may even exert pro-atherogenic effects.[34] In a number of studies however, it has been shown that after correction of classical risk factors for CVD such as dyslipidemia, there is still a significantly increased risk of atherosclerotic vascular disease in patients with a chronic inflammatory disorder.[11,35] Hence, although secondary dyslipidemia is likely to contribute to enhanced atherogenesis it does not appear to be the most important mediator.

Third, systemic inflammation can activate the coagulation cascade and vice versa.[36] In addition to tissue factor (TF), platelets play an important role in this bidirectional activation. A systemic inflammatory state results in thrombin generation and activation of platelets, and these processes are closely connected to the development of atherothrombosis.[37] Platelets can adhere to the endothelium well before an atherosclerotic plaque is formed as has been shown in apoE$^{-/-}$ mice, an animal model for atherogenesis.[38] Moreover, following binding to the endothelium, platelets can release a plethora of inflammatory mediators including adhesion molecules, chemokines and coagulation factors that subsequently mediate a pro-inflammatory environment as well as recruitment of leukocytes to the vascular wall and the subendothelial space. Over 300 proteins have been identified that can be released by activated platelets.[39] The atherogenic potential of platelets has been confirmed in a study in which administration of activated platelts to ApoE deficient mice resulted in a 40% increase in atherosclerotic lesion size.[40]

In addition to secretion of mediators of vascular inflammation and thrombosis, activated platelets also express P-selectin, an adhesion factor that facilitates binding to leukocytes (predominantly monocytes) via the receptor P-selectin glycoprotein ligand-1 (PSGL-1) receptor. These so-called platelet-monocyte complexes (PMC) are a sensitive marker for the activation of platelets[41] and it has been suggested that they play a causal role in plaque instability, thrombosis and inflammation.[42] An increased number of circulating PMC has not only been shown in patients with atherosclerotic vascular disease[41,43] but also in patients with a chronic inflammatory disorder such as SLE or RA.[44] Moreover, P-selectin induces tissue factor expression, the most important initiator of the coagulation cascade.

In conclusion, a systemic inflammatory state can lead to endothelial dysfunction, secondary dyslipidemia and activation of coagulation. These mechanisms contribute to the pathogenesis of atherothrombotic vascular disease and represent potential targets for treatment strategies.

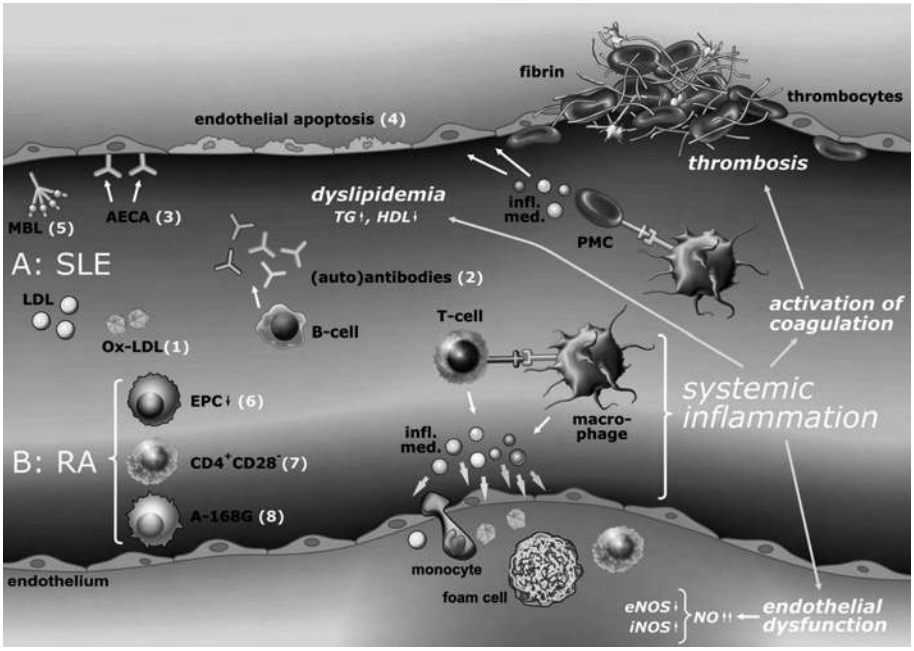

Figure 1. Schematic representation of the atherogenic effects of a systemic inflammatory state. Systemic inflammation induces endothelial dysfunction, dyslipidemia and activation of coagulation which for instance can result in the formation of platelet-monocyt complexes. In individual inflammatory disorders, there are additional mechanisms that can acclerate the accelerate the atherosclerotic process such as in **A.** SLE: increased oxidation of LDL (1), production of atherogenic autoantibodies (2) directed against for instance against the endothelium (3), apoptosis of the endothelium (4) and variant MBL alleles (5) and in **B.** RA: reduced number of EPC (6), increased number of $CD4^{+}CD28^{-}$ cells (7) and a A-168G polymorphism of MHC2TA.

Additional mechanisms, specific for chronic inflammatory disorders

In conjunction with these general effects of systemic inflammation which may occur in various degrees in the different inflammatory disorders, there are additional mechanisms anchored within the pathophysiology of the individual inflammatory disorders that are specific to that particular disorder.

In SLE there is a pro-inflammatory and pro-oxidative state that can accelerate atherosclerosis.[45] This results for instance in enhanced oxidation of low density lipoprotein-cholesterol (LDL) to the very atherogenic ox-LDL.[46] In addition, several antibodies have been reported that can enhance atherogenesis, such as antibodies directed against apolipoprotein H[47] or against the endothelium[48] which may indeed be clinically significant as increased apoptosis of the endothelium has been observed in SLE.[49] Finally, activation of complement has also been suggested to accelerate atherosclerosis in SLE patients. In 91 SLE patients genotyping was performed of mannose-binding lectin (MBL), which activates the lectin pathway of complement. Homozygosity for variant alleles of MBL, resulting in reduced serum levels was associated with a hazard ratio of developing arterial thrombosis of 7.0 (95%CI 1.9 to 25.4).[50] Interestingly, in healthy volunteers increased MBL levels appear to predispose to atherothrombotic disease.[51] It remains unclear whether the role of MBL in atherothrombosis differs between people with or without SLE.

In RA, a reduced number of circulating endothelial progenitor cells (EPC) has been reported. The number of these precursors of mature endothelium is negatively correlated with CVD and a reduction of EPC may be causally related to enhanced atherogenesis in RA.[52] A second cell type that may be involved is a T-lymphocyte subtype, the so-called $CD4^+CD28^-$ T-cell. These cells have a high pro-inflammatory and tissue damaging potential and are increased in patients with unstable atherosclerotic plaques.[53] In RA, the number of circulating $CD4^+CD28^-$ cells has been reported to be increased which was correlated to preclinical atherosclerotic disease including endothelial dysfunction.[54] Finally, a genetic risk factor for both RA and myocardial infarction has been reported.[55] The expression of major histocompatibility complex (MHC) class II is regulated by MHC2TA, the gene that encodes the class II transactivator

(CIITA). An A−168G single nucleotide polymorphism (SNP) in the promoter region of MHC2TA, which leads to decreased MHCII expression, was recently analyzed. Although decreased MHCII expression may lead to reduced antigen presentation and thus attenuation of immune-driven responses within the arthritic joint as well as the atherosclerotic plaque, this mutation was associated with an increased susceptibility to both rheumatoid arthritis and myocardial infarction.[55] The authors hypothesized that the pathogenic mechanism may be related to a less efficient presentation of antigens to protective, regulatory T cells. Indeed, regulatory $CD4^+CD25^+$ T cells appear to play a protective role against both rheumatoid arthritis[56] and atherosclerosis.[57]

Further elucidation of the pathophysiological mechanisms of these chronic inflammatory disorders may result in the identification of the specific processes, within the wide spectrum of immune activation, which accelerate atherosclerosis.

Systemic inflammation & CVD: Clinical consequences

The observation that systemic inflammation can enhance the atherosclerotic process and thereby underlies an increased incidence of CVD has important clinical consequences. It implies that, when composing therapeutic strategies for patients with a chronic inflammatory disorder, cardiovascular protection will have to be part of the management strategy. For several auto-immune disorders this is further strengthened by the observation that the pathophysiological process has started years before manifestation of the disease or the time of diagnosis. In a number of studies it has been shown that in apparently healthy blood donors or soldiers, who were in a later stage diagnosed with RA[58] or SLE,[59] various antibodies were present in the years preceding their diagnosis. In the case of RA it has even been shown that in the years prior to diagnosis, the serum levels of CRP[60] and sPLA2,[61] important markers of cardiovascular morbidity, are increased. Moreover, future RA patients have a significantly more atherogenic lipid profile in comparison to matched blood donors, at least ten years before onset of symptoms.[62] As such, the preclinical phase is not only characterized by the production of autoantibodies but there also appears to be an acute phase response as well as dyslipidemia. This suggests that there may be prolonged exposure to risk

factors that can accelerate the atherosclerotic process and emphasizes the significance of cardiovascular protection in these patients, perhaps even from the time of diagnosis. The characterization of atherosclerosis as an inflammatory disorder has sparked considerable interest in anti-inflammatory or immunosuppressive medication as an anti-atherosclerotic strategy.[23] This holds true for patients with a chronic inflammatory disorder in particular considering these patients are per definition assigned to these classes of drugs. One of the most well known anti-inflammatory intervention is treatment with non-steroid anti-inflammatory drugs (NSAID's). However, in addition to inhibition of inflammation this group of drugs has a much broader range of actions including that on the prostaglandin metabolism in which also atheroprotective mediators (e.g. prostacyclin) are inhibited.[63] Several studies have also evaluated the anti-atherogenic potential of immunosuppression. Blockade of TNF-α in patients with RA[64] or vasculitis[16] for instance has indeed been shown to result in a significant improvement of endothelial function. Hydroxy-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) also fulfill an interesting role. These drugs are effective in lowering LDL-cholesterol and thereby cardiovascular risk. In addition, several immunomodulatory properties have been ascribed to statins.[65] In animal models for SLE[66] and RA[67] it has indeed been shown that statins can inhibit the pathophysiology of these disorders although it should be mentioned these effects were achieved with dosages that exceed human use. Immunomodulatory actions of statins have also been shown in patients with RA[68] and short-term treatment significantly improved endothelial function.[69] In SLE, two randomized, placebo-controlled studies have been initiated to evaluate the potential cardiovascular risk reduction of statins in these patients. One of them was terminated prematurely however, due to difficulties with recruitment and retention of participants.[70] A similar study in children and adolescents with SLE has recently been initiated and results are expected early 2010.[71] Interestingly, mycophenolate mofetil (MMF) has recently also emerged as a potential anti-atherosclerotic strategy.[72] MMF has a strong cytostatic effect on T-lymphocytes by interfering with DNA synthesis in activated T-cells.[73,74] This may significantly attenuate plaque formation since T-cells have a prominent role in atherogenesis illustrated by

the observation that ablation of replicating T-cells, by incorporation of a suicide gene active upon cell division, was shown to result in a 55% reduction of lesion development in mice.[75] In addition, MMF also induces apoptosis in activated T-cells[76] and has beneficial effects on regulatory T-cells[77] which are considered atheroprotective.[57] Furthermore, MMF interferes with the expression of adhesion molecules not only on T-cells[73,78] but also on monocytes/macrophages[79] and the endothelium.[80] MMF can inhibit leukocyte recruitment to the subendothelium and the subsequent reduced activation of leukocytes will translate into attenuation of subendothelial cross-talk between T-cells and macrophages. This cascade of events will interrupt the self-perpetuating pro-inflammatory environment within the arterial wall, the hallmark of atherosclerotic vascular disease.[81] Indeed, in several animal studies MMF has been shown to inhibit the atherosclerotic process[82-84] and human studies are therefore warranted.

Conclusion

Improvements in diagnostic and treatment strategies have led to a significant increase in the life expectancy of patients with chronic inflammatory disorders. This has contributed to the current situation, where now cardiovascular co-morbidity represents a considerable source of mortality and morbidity. Direct and indirect consequences of a systemic inflammatory state mediate atherothrombotic disease. Pathophysiological mechanisms specific to SLE or RA can however also contribute to this. Especially considering the fact that pathophysiological processes involved in these disorders can occur at a subclinical level years before a diagnosis is made, early cardiovascular prevention seems essential in these patients.

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PART 3

Immunomodulation and atherogenesis

Chapter 11

Effect of Torcetrapib on Progression of Carotid Atherosclerosis in Heterozygous Familial Hypercholesterolemia

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Abstract

Background: Torcetrapib, an inhibitor of cholesteryl ester transfer protein (CETP), may reduce atherosclerotic vascular disease by virtue of its capacity to increase high-density lipoprotein-cholesterol (HDL-C).

Methods: Baseline and follow-up B-Mode ultrasound examinations were performed to obtain change in carotid intima-media thickness (CIMT) in 850 subjects with heterozygous familial hypercholesterolemia (FH). These subjects completed an atorvastatin run-in period and were subsequently randomized to blinded atorvastatin monotherapy (A) or atorvastatin combined with 60 mg torcetrapib (T/A) for a period of 2 years.

Results: After 24 months, HDL-C was 52.4 ± 13.5 mg/dL and LDL-C 143.2 ± 42.2 mg/dL in the atorvastatin group versus 81.5 ± 22.6 mg/dL and 115.1 ± 48.5 mg/dL in the T/A group, respectively. Average systolic blood pressure (SBP) during treatment increased 2.8 mmHg in the T/A group. The change in maximal CIMT, the primary efficacy parameter, was 0.0053±0.0028 mm/year in the atorvastatin group and 0.0047±0.0028 mm/year in the T/A group, p=0.87. The secondary efficacy parameter, annualized change in mean CIMT for the common carotid artery indicated increased progression of disease in the T/A group, -0.0014 vs. 0.0038 mm/year, p=0.005.

Conclusions: In FH patients, the use of torcetrapib with atorvastatin did not result in further reduction of progression of atherosclerosis as assessed by a combined measure of carotid arterial wall thickness and, when restricted to the common carotid segment, caused progression of disease. These effects occurred despite an unparalleled increase of HDL-C and a substantial additional decrease of LDL-C and triglyceride levels.

(ClinicalTrials.gov identifier - NCT00136981)

Introduction

The current guidelines for the prevention and management of cardiovascular disease (CVD) focus on reducing low density lipoprotein cholesterol (LDL-C) levels by means of HMG-CoA reductase inhibitors (statins).[1,2] Recent meta-analyses have shown, however, that even with the most aggressive treatment[3,4] these drugs reduce the risk of a major coronary event by only 30%.[5] This combined with the estimation that worldwide cardiovascular mortality will increase by 90% in the year 2020 compared to 1990[6] illustrates the need for novel efficacious drugs. In that context it is important to note that several large prospective epidemiological studies showed that an increase of high-density lipoprotein cholesterol (HDL-C) by 1 mg/dl (0.026 mmol/L) is associated with a 2-3% reduction of CVD risk.[7] Moreover, HDL-C levels remain predictive of recurrent CVD in patients with LDL-C levels below 70 mg/dL (1.8 mmol/L), as reached with intensive statin treatment.[8]

Over the past few years, attempts to raise HDL-C levels have been particularly successful with small molecule inhibitors of cholesteryl ester transfer protein (CETP).[9,10] By blocking the CETP-mediated transfer of cholesteryl ester from HDL-C to apolipoprotein B-containing lipoproteins and the simultaneous transfer of triglycerides in the opposite direction, torcetrapib is very effective in raising HDL-C.[10] Indeed, elevated CETP levels were shown to be associated with an increasing risk of future coronary artery disease in apparently healthy individuals.[11] Furthermore, inhibiting CETP in rabbit models of atherosclerosis dramatically reduces the extent of disease.[12] It is not known, however, whether CETP inhibition attenuates atherosclerosis in humans. Since novel lipid-modulating drugs will be primarily used on top of evidence-based LDL-C lowering, torcetrapib has been developed in combination with atorvastatin. In this setting , torcetrapib not only increases HDL-C and apolipoprotein A-I but also decreases LDL-C levels and apolipoprotein B-100 (the latter especially at higher dosages) and also showed favourable effects on lipoprotein size (larger HDL and LDL size).[10] In the current study, torcetrapib/atorvastatin was used in patients suffering from heterozyguous familial hypercholesterolemia (FH). The rationale for

this target population consists of the fact that mutations in the LDL-receptor gene are associated with decreased levels of HDL-C,[13] smaller HDL particle size[14] as well as increased levels of CETP.[15] Also, the progression of atherosclerosis in FH is related to both HDL-C[16] and CETP concentration[17] and thus it was hypothesized that the use of torcetrapib would have distinct favourable effects in this patient group. Here we present the results of a multi-centre, randomized, double-blind, placebo-controlled trial designed to evaluate the effects of torcetrapib on carotid intima-media thickness (CIMT), a surrogate marker for CVD endpoints in patients suffering from FH.

Methods

Study design

The RADIANCE 1 Trial (**R**ating **A**therosclerotic **D**isease change by **I**maging with a new **CE**TP Inhibitor) was a prospective, double-blind, randomized, multicenter, parallel group study. The study was designed by the trial academic leadership in collaboration with the study sponsor. The Institutional Review Boards of participating centers approved the protocol and subjects provided written informed consent. Subjects were eligible for entry into the study if they had a diagnosis of heterozygous familial hypercholesterolemia either by genotyping or by meeting World Health Organization diagnostic criteria.[18] During a 6-14 week run-in phase, subjects were counseled on therapeutic lifestyle changes[1] and were administered atorvastatin in a dosage of 20, 40 or 80 mg, titrated at 4-week intervals, for up to three visits to reach an LDL-C target according to the National Cholesterol Education Program guidelines[1] or to their maximally tolerated dose. Subjects who at screening, were on cholesterol absorption inhibitors or bile acid sequestrants, were permitted to remain on those medications provided that the dose was not changed during the course of the study. At the conclusion of the run-in period, subjects were randomized to receive either atorvastatin (at the dosage established during the run-in period) with torcetrapib 60 mg or atorvastatin monotherapy with corresponding placebo tablets. Subjects and

study personnel were blinded to treatment assignment, laboratory measurements and the carotid imaging findings.

This manuscript was written by the first author, who vouches for the data and analyses. The study contract specified that a copy of the study database be provided to the coordinating center for independant analysis and granted the academic authors the unrestricted rights to publish the results.

Carotid ultrasound examinations and measurement

Carotid ultrasonography was performed to assess CIMT.[19] Replicate scans were performed within a week of each other at baseline and at 24 months, with interim follow-up scans at the 6, 12, and 18 month visits. At each visit a circumferential scan was performed with image acquisition at four pre-defined angles of the right and left common carotid, bifurcation, and internal carotid artery near and far walls.[20] All imaging centers used the same imaging hardware (Sequoia 512 scanners equipped with 8L5 transducers, Siemens AG, Munich, FRG) and imaging acquisition protocol. Five-second image sequences were saved in Digital Imaging in Communications in Medicine (DICOM^{sm}) format (National Electrical Manufecturers Association, Rosslyn, VA, USA) and written to 640 MB magnetic optical disk for transfer to reading centers. Two reading centers (Vascular Imaging Center, University Medical Center, Utrecht, the Netherlands and Wake Forest University Medical Center, Ultrasound Reading Center, Winston-Salem, NC, USA) used standardized equipment and protocols to process stored images. Semi-automated readings were analyzed using Automated Measurement Software (AMS developed by Image and Data Analysis, Inc., Gothenburg, Sweden).[21] From each image sequence, the reader selected one frame in end diastole from CIMT measurement. The leading edge (far wall) and trailing edge (near wall) of media-adventitia and lumen-intima boundaries were traced within the region of interest specified by the reader. Maximum CIMT was determined from a set of measurements perpendicular to media-adventitia boundary. The readers were blinded to the intervention and to previous CIMT measurements when reading an image. Quality assurance processes included: central training and certification of

all sonographers and readers on each continent; annual international meetings of sonographers and readers to reinforce protocol and standardized implementation; and regular site visits and performance reviews. Intra-class correlation coefficients (ICC) for mean-max CIMT between replicate scans at baseline (n=875) and end of study (n=814) were 0.90 and 0.88, respectively. ICC for the monthly QA scans (n=128) was 0.96. These ICC estimates include within and between visits, within and between sonographers, and within and between reader variability components.

The primary endpoint was annualized change in the maximum CIMT for the 12 carotid artery segments (near and far walls of the right and left CCA, carotid bulb, and the ICA) based on all scans performed over the two-year study period.

Statistical Methods

A sample size of 304 subjects per treatment group was calculated to have 90% power to detect a 0.020 mm/yr difference in the annualized rate of change of CIMT with a two-sided alpha level of 0.05, assuming a common standard deviation of 0.076 mm/yr. A linear mixed-effects model was used to analyze the annualized rate of change in maximum CIMT including 84 maximum CIMT measurements (12 segments x 7 visits) for each subject as the dependent variables with random intercepts and slopes as a function of time and fixed effects for geographic region, atorvastatin dose at run-in, carotid segment, treatment, time, and time by treatment interaction. Testing was two-sided and conducted with a 5% type I error rate.[22] Laboratory parameters were analyzed by analysis of covariance including terms for baseline value, treatment, geographic region, and atorvastatin dose at run-in. Safety data were analyzed using a linear model with terms for baseline value, hypertensive status, age, gender, smoking, diabetes, BMI, creatine clearance, race, and treatment. With multiplicative interaction terms, we studied whether treatment effects differed across subgroups. These pre-specified analyses were performed for age (< and ≥ 65 years), gender, race (white or non-white), HDL-C (< and ≥ 40 mg/dl), LDL-C (above and below the median), triglycerides (< and ≥150mg/dL), smoking, history of diabetes mellitus, history of hypertension, C-reactive protein (< and ≥ 3.0 mg/dl) and baseline maximum CIMT (< and >= the median).

Results

Patient population

Between December 19th, 2003, and November 22nd, 2004, 904 patients were randomized at 37 centers in North America, Europe, and South Africa, 454 in the atorvastatin group and 450 in the torcetrapib-atorvastatin group. 850 patients had remained in the study and had at least one evaluable carotid ultrasound examination at both baseline and follow-up, 427 in the atorvastatin-only group and 423 in the torcetrapib-atorvastatin group (the full analysis set). Demographic characteristics and baseline medications were similar in both treatment groups (Table 1). The titrated dosage of atorvastatin averaged 56.5 mg in both groups.

Laboratory results and blood pressure

Table 1 summarizes laboratory values and blood pressure at baseline and during treatment for the 850 subjects in the full analysis set who had evaluable post-baseline ultrasound studies. After 24 months treatment, HDL-C in the atorvastatin group, increased from 51.8 to 52.4 mg/dL. HDL-C in the torcetrapib group increased from 52.9 to 81.5 mg/dL (Figure 1). In the atorvastatin group, LDL-C levels measured 165.5 mg/dL at screening and fell during the run-in to 138.9 mg/dL at baseline. After 24 months of treatment, LDL-C levels in the atorvastatin group measured 143.2 mg/dL. Comparable LDL-C levels for the same timepoints in the torcetrapib-atorvastatin group measured 168.2, 138.4, and 115.1 mg/dL. The net effect of torcetrapib was a 51.9% relative increase in HDL-C and a 20.6% relative decrease in LDL-C compared with atorvastatin alone. Table 2 shows changes in lipoprotein subclasses between the treatment arms. Baseline blood pressure (BP) was 116/73 in the torcetrapib and 117/74 mmHg in the atorvastatin group. Average post-randomization systolic BP increased 1.3 mmHg in the atorvastatin group and 4.1 mmHg in the torcetrapib group, a LS mean difference of 2.8 mmHg, 95% confidence interval, 1.9-3.7, $p<0.001$.

Table 1. Baseline Characteristics, Blood Pressures and Laboratory Values

All Randomized Subjects – Mean (SD) or Number (Percentage) of Subjects (n=904)			
	Atorvastatin Monotherapy (n=454)	**Atorvastatin plus Torcetrapib (n=450)**	**p-value**
Age in years	45.2 (12.9)	46.8 (12.0)	0.06
Male Gender	232 (51.1%)	214 (47.6%)	0.29
Body mass index (kgs/height^2)	26.7 (4.4)	26.7 (4.3)	1.00
History of Diabetes	19 (4.2%)	12 (2.7%)	0.21
History of Hypertension	114 (25.1%)	110 (24.4%)	0.82
Current Smokers	95 (20.9%)	86 (19.1%)	0.50
Aspirin use at baseline	133 (29.3%)	138 (30.7%)	0.65
B-blocker use at baseline	92 (20.3%)	83 (18.4%)	0.49
ACE/ARB use at baseline	87 (19.2%)	72 (16.0%)	0.21
Ezetimibe use at baseline	50 (11.0%)	47 (10.4%)	0.78
Subjects Completing the Trial (n=850): Mean (SD) or Median (IQR)			
Baseline Values			
	Atorvastatin Monotherapy (n=427)	**Atorvastatin plus Torcetrapib (n=423)**	**p-value**
Total cholesterol (mg/dL)	213.5 (42.1)	213.0 (39.3)	0.86
LDL-C (mg/dL)	138.9 (37.6)	138.4 (35.5)	0.84
HDL-C (mg/dL)	51.8 (12.8)	52.9 (12.7)	0.24
LDL-C/HDL-C ratio	2.7 (2.1, 3.4)	2.5 (2.1, 3.3)	0.29*
Triglycerides (mg/dL)	97.4 (75.2, 141.6)	97.4 (70.8, 132.8)	0.22*
C-reactive protein (mg/L)†	0.8 (0.4, 1.9)	0.8 (0.4, 1.9)	0.70*
Systolic Blood Pressure (mmHg)	116.6 (10.9)	115.9 (11.7)	0.42
Diastolic Blood Pressure (mmHg)	73.5 (7.0)	72.9 (7.5)	0.17
24 Month Follow-up Values: Mean (SD) or Median (IQR)			
Total cholesterol (mg/dL)	218.8 (45.7)	216.9 (51.1)	0.58
LDL-C (mg/dL)	143.2 (42.2)	115.1 (48.5)	<0.001
HDL-C (mg/dL)	52.4 (13.5)	81.5 (22.6)	<0.001
LDL-C/HDL-C ratio	2.7 (2.1, 3.4)	1.3 (1.0, 1.8)	<0.001*
Triglycerides (mg/dL)	97.4 (70.8, 141.6)	88.5 (70.8, 119.0)	0.001*
C-reactive protein (mg/L)	0.8 (0.4, 2.0)	0.9 (0.4, 2.2)	0.85*
Systolic Blood Pressure (mmHg)§	117.9 (9.9) §	120.1 (12.2) §	<0.001
Diastolic Blood Pressure (mmHg)§	74.2 (6.3) §	74.7 (7.0) §	<0.001
Change from Baseline: Mean (SD), Median (IQR) or LS Mean Percentage Change (SE)			
Total cholesterol	5.1 (0.9) †	3.8 (0.9) †	0.28
LDL-C	6.3 (1.3) †	-14.4 (1.3) †	<0.001
HDL-C	2.5 (1.1) †	54.4 (1.1) †	<0.001
Triglycerides	2.1 (-17.6, 25.0)	-7.7 (-25.0, 20.0)	<0.001 ‡
C-reactive protein (mg/L)	0.0 (-0.3, 0.4)	0.0 (-0.3, 0.4)	0.95 ‡
Systolic Blood Pressure (mmHg)	1.3 (6.9) §	4.1 (8.0) §	<0.001
Diastolic Blood Pressure (mmHg)	0.6 (4.4) §	1.8 (4.8) §	<0.001

§ Average of all post-randomization measurements. *p value from Wilcoxon rank sum test. † Least Square mean percentage change (SE) ‡ p value from analysis of covariance on rank transformed data, last observation carried forward. To convert cholesterol levels to millimoles per liter, multiply by 0.02586. To convert triglycerides to millimoles per liter, multiply by 0.01129. ACE = angiotensin converting enzyme inhibitor. ARB = Angiotensin receptor blocker. LDL-C = low density lipoprotein cholesterol. HDL-C = High density lipoprotein cholesterol LS = Least Squares. SE = Standard Error

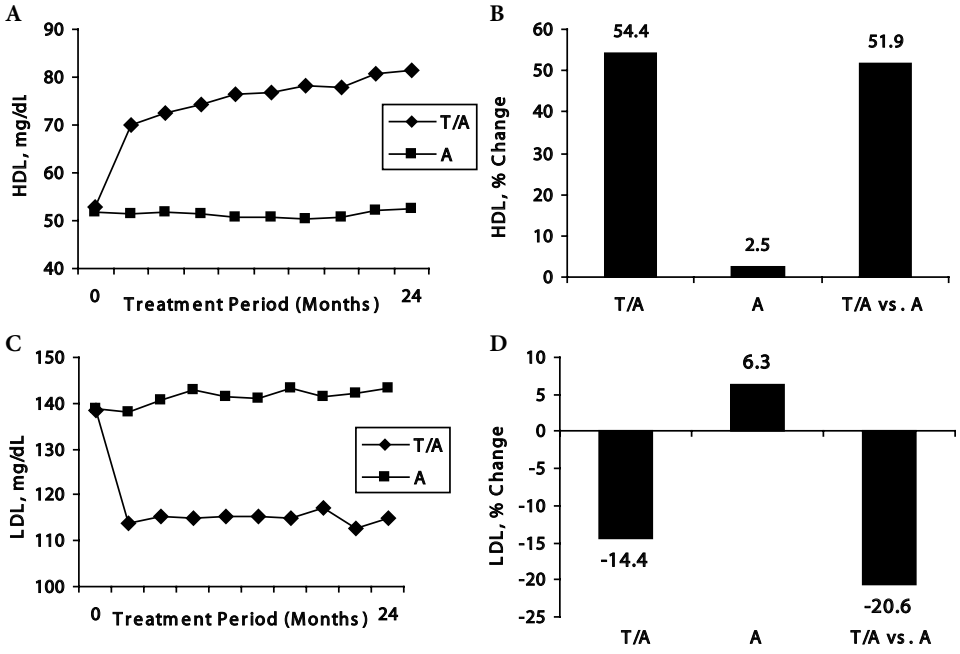

Figure 1. Levels of HDL (mg/dL) (fig. 1A) with the percentage change from baseline (fig. 1B) and levels of LDL (mg/dL) (fig. 1C) with the percentage change from baseline (fig. 1D) in FH patients treated with atorvastatin monotherapy (A) or a combination of torcetrapib and atorvastatin (T/A) for a duration of 24 months.

Table 2. Changes in lipoprotein subclasses

Parameter	Treatment/ comparison	LS Mean	p-value
Apo-A Family			
Apo A-I	T/A vs A	+24.80%	<0.001
HDL-2-C	T/A vs A	+157.12%	<0.001
HDL-3-C	T/A vs A	+45.93%	<0.001
HDL size	T/A vs A	+0.88 nm	<0.001
Apo-B Family			
Apo B-100	T/A vs A	-16.71%	<0.001
Non-HDL-C	T/A vs A	-19.38%	<0.001
LDL particles small	T/A vs A	-376.31 nmol/L	<0.001
LDL size	T/A vs A	+0.45 nm	<0.001

Carotid ultrasound results

Table 3 summarizes the change in the primary and secondary carotid ultrasound efficacy parameters. The primary efficacy measure, annualized rate of change in maximum CIMT, was 0.0053 mm/year in the atorvastatin group and 0.0047 mm/year in the torcetrapib-atorvastatin group, p=0.87 (Figure 2). However, the secondary efficacy parameters, annualized change in the maximum (-0.0042 vs. 0.0040 mm/year, p=0.02) and mean (-0.0014 vs. 0.0038 mm/year, p=0.005) CIMT for the common carotid artery in fact indicated regression of CIMT in the atorvastatin group and progression of CIMT in the T/A group (Table 3). For nearly all prespecified subgroups no heterogeneity in the treatment difference was observed. Annualized change in maximum CIMT in subjects with a history of diabetes was lower in the torcetrapib group (p=0.05) although the number of diabetics was limited (T/A=9 vs. A=17). For subjects with baseline HDL-C<40 mg/dL, the results showed a trend in favor of atorvastatin monotherapy, p=0.09. Both these results, however, are likely chance findings.

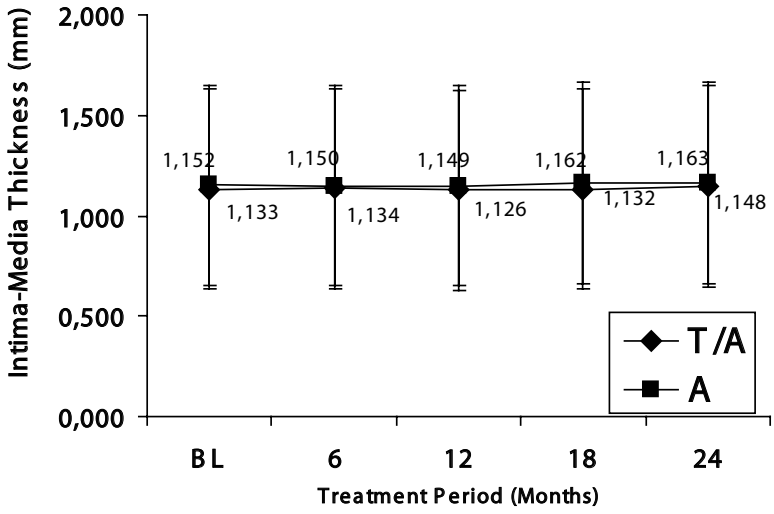

Figure 2. Maximum carotid intima-media thickness (CIMT) (mm) average over twelve carotid segments combined of FH patients treated with atorvastatin monotherapy (A) or a combination of torcetrapib and atorvastatin (T/A) for a duration of 24 months. Error bars represent standard deviation.

Table 3. Baseline, Follow-up, and Change from Baseline in Maximum CIMT Endpoints

	Atorvastatin Monotherapy (n = 427)		Atorvastatin plus Torcetrapib (n = 423)		p-value
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Baseline (mm)					
Maximum CIMT for each of the 12 carotid artery sites	1.15 (0.31)	1.09 (0.93,1.33)	1.13 (0.28)	1.09 (0.94,1.27)	0.38
Maximum CIMT for each of the 4 CCA sites	1.01 (0.23)	0.98 (0.83,1.17)	0.99 (0.22)	0.97 (0.82,1.14)	0.33
Mean CIMT for each of the 4 CCA sites	0.72 (0.15)	0.70 (0.60,0.82)	0.71 (0.15)	0.70 (0.59,0.81)	0.44
24 Month (LOCF) Follow-up (mm)					
Maximum CIMT for each of the 12 carotid artery sites	1.16 (0.33)	1.09 (0.94,1.32)	1.14 (0.29)	1.10 (0.95,1.27)	0.37
Maximum CIMT for each of the 4 CCA sites	1.00 (0.22)	0.97 (0.83,1.13)	1.00 (0.21)	0.99 (0.84,1.13)	0.87
Mean CIMT for each of the 4 CCA sites	0.71 (0.14)	0.70 (0.61,0.80)	0.72 (0.14)	0.71 (0.60,0.81)	0.71
Annualized Change from Longitudinal Model (mm/year)					
	Slope	SE	Slope	SE	**p-value**
Maximum CIMT for each of the 12 carotid artery sites	0.0053	0.0028	0.0047	0.0028	0.87
Maximum CIMT for each of the 4 CCA sites	-0.0042	0.0025	0.0040	0.0025	0.02
Mean CIMT for each of the 4 CCA sites	-0.0014	0.0013	0.0038	0.0013	0.005

IQR=Interquartile range

SD= Standard deviation

SE=Standard error

LOCF= last observation carried forward

Clinical adverse events

The number of patients with at least one serious adverse cardiovascular event was 11 in the A treatment group (1 cardiovascular death, 0 non-fatal myocardial infarctions, 1 stroke, 9 ischemic or other cardiovascular events) and 24 in the T/A group (0 cardiovascular deaths, 3 non-fatal myocardial infarctions, 1 stroke, 23 ischemic or other

cardiovascular events, 1 carotid stenosis and 1 blood pressure elevation considered a serious adverse event). Other cardiovascular events were mostly angina and chest pain not otherwise specified. Investigator-reported hypertensive adverse events were more common in the torcetrapib group, 8.9% vs. 3.7% and BP values >140/90 mmHg were recorded more frequently in the torcetrapib group, 7.8% vs. 3.1%. A sustained increase greater than 15 mmHg in systolic pressure occurred in 2.2% of torcetrapib-treated subjects versus 0.9% of subjects treated with atorvastatin alone.

Discussion

The RADIANCE 1 trial confirms that high dose atorvastatin therapy arrests the progression of atherosclerosis in the carotid arteries of FH patients.[23] Surprisingly, the data also show that addition of torcetrapib to this therapeutic regimen did not provide further protection. If anything, our data suggest a worsening of pathology conferred by this CETP inhibitor, despite an unprecedented 52% increase of HDL-C levels and a robust 21% decrease of LDL-C levels. On the basis of extensive epidemiology and various clinical intervention studies, such lipoprotein changes are anticipated to render significant benefit. Nevertheless, when considered in light of the recent discontinuation of the large mortality and morbidity trial of torcetrapib (ILLUMINATE) that showed an increase in all-cause mortality, our findings are less surprising. Although these results could not have predicted the detrimental outcome of the ILLUMINATE trial, they may have significantly altered the course of further clinical research with this compound.

To study atherosclerosis, we employed ultrasonography to assess carotid intima-media thickness (CIMT), a surrogate marker for CVD.[24] The annualized change in maximum CIMT, the primary end point of this study, did not differ for FH patients treated with atorvastatin alone (A) and those treated with the combination of atorvastatin and torcetrapib (T/A). In fact, CIMT of the common carotid artery, a

secondary endpoint of this study, provided evidence of accelerated atherogenesis in the patients using torcetrapib. It is highly unlikely that the unanticipated outcome of this trial can be attributed to the measurement of CIMT per se. This marker has previously been proven to constitute a strong and accurate predictor of future vascular events in population studies.[25] Furthermore, in studies in which the efficacy of lipid modifying medication,[26,27] anti-oxidants,[28] estrogens[29] and antihypertensive treatment was evaluated[30], CIMT measurements were succesfully applied and were in line with the outcome of subsequent morbidity and mortality trials.

To account for the observed results, the potential benefit of the observed LDL-C decrease needs to be weighed against the detrimental effect of the rise in systolic blood pressure. The divergent effects of torcetrapib on LDL-C (-21%) and SBP (+2.8 mm Hg) are two prominent factors that may have affected CIMT. Focusing on LDL-C, in a 2-year pravastatin study (REGRESS), a comparable 28% decrease in LDL-C levels was associated with a change of CIMT of 0.05 mm.[26] In the ENHANCE trial, the addition of ezetemibe to high dose simvastatin therapy was in fact powered to reduce LDL-C levels by 18-23% and to detect a mean two year absolute CIMT difference of 0.05 mm in a sample size of 650 FH heterozygotes.[31] Extrapolating these findings to RADIANCE 1, the effect on LDL-C would translate into a CIMT difference of 0.03-0.05 mm in favour of the torcetrapib arm.
In contrast, the observed increase of SBP can be expected to adversely affect CIMT. In an attempt to account for this effect, we have used data from a recent meta-analysis on the relationship between SBP and CIMT.[30] That analysis would suggest that the effect of the 2.8 mmHg increase in SBP would favour the atorvastatin arm by 0.014 mm over two years. The net opposing impact of LDL-C and SBP should have left a residual benefit of T/A: the fact that none was observed leaves no room for any beneficial effect of the large HDL-C increase.

In line with the concept that elevation of HDL-C protects against atherosclerosis, small and moderate increases of HDL-C as achieved by the use of nicotinic acid (+21%)[27] or

gemfibrozil (+6%)[32] have previously been reported to yield a significant reduction in the rate of CIMT progression and risk of major cardiovascular events. The absence of an effect of a much greater increase of HDL-C (+52%) in RADIANCE 1 indicates that torcetrapib either has an adverse vascular effect that masked the changes in lipoprotein levels or that CETP inhibition is not an effective therapeutic strategy. While the current analysis does not inform which is the attributable cause, there are several possibilities that merit consideration.With respect to the discrepancy between torcetrapib's remarkable effects on lipid metabolism and CIMT results, a direct vasculotoxic effect of which a rise in blood pressure and peripheral resistance is only a biomarker, appears possible. HDL's natural ability to induce vasorelaxation, an effect that is thought to be mediated via scavenger receptor B1,[33] may be adversely affected by torcetrapib.

An adverse interaction of torcetrapib with atorvastatin is also a remote possibilty, however extensive preclinical and clinical work makes this highly unlikely (unpublished data on file, Pfizer). Another possibility relates to the fact that inhibition of CETP by torcetrapib actually increases CETP plasma levels. At a daily dosage of 60 mg, torcetrapib increases CETP concentration continuously which is ascribed to an enhanced affinity of CETP for HDL.[34] This complex formation (CETP-Torcetrapib-HDL) is in turn associated with extreme elevations of large HDL as exemplified by the substantial increase of HDL2 cholesterol levels (157%). In this context, it is worrisome that HDL-C levels were found to steadily increase over the entire duration of the trial (see figure 1). It can be hypothesized that these effects may interfere with one or more of HDL's activities that include serving as an acceptor of cellular cholesterol, inhibiting oxidation, thrombosis and vascular inflammation, promoting endothelial repair and protecting against endothelial cell apoptosis.[35] That HDL may have lost its anti-inflammatory potential is illustrated by the observation that torcetrapib did not affect CRP levels. In contrast, similar dose atorvastatin monotherapy resulted in a 45% decrease of hsCRP levels in a FH trial of similar duration and size as RADIANCE 1.[36]

The concept of inhibiting CETP to raise HDL-C and provide an anti-atherosclerotic therapy has been discussed in numerous reports and reviews for good reason since studies do not provide an unambiguous answer.[37] Testing the hypothesis of CETP inhibition as a tool to reduce the burden of CVD requires careful further investigation of other small molecule inhibitors of CETP. However, in this quest, alternative strategies to reduce CETP-mediated neutral lipd transfer amongst plasma lipoproteins such as vaccination[38] or strategies aimed at inhibition of the hepatic synthesis of CETP[39] may also provide further insight.

In conclusion, the use of torcetrapib in FH did not result in regression of atherosclerosis as assessed by a combined measure of carotid arterial wall thickness and in fact caused progression of disease in the common carotid segment. These effects occured despite an unparalleled increase of HDL-C (52%) and a substantial additional decrease of LDL-C (21%) levels. The torcetrapib driven increase in SBP alone does not appear to explain these results.

Disclosures

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Listing of the investigators in the appendix.*

APPENDIX

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Chapter 12

The CETP inhibitor Torcetrapib and Off-target Toxicity
A Pooled Analysis of the RADIANCE Trials.

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Abstract

Background: Torcetrapib, an inhibitor of cholesteryl ester transfer protein (CETP), has been shown to increase cardiovascular event rate in spite of conferring a significant high-density lipoprotein cholesterol (HDL-C) increase. Using data from the RADIANCE trials, assessing the impact of torcetrapib on carotid intima-media thickness (cIMT), we set out to explore potential mechanisms underlying this adverse outcome.

Methods and results: Data from the RADIANCE 1 & 2 studies, examining cIMT in 904 subjects with familial hypercholesterolemia and in 752 subjects with mixed dyslipidemia, were pooled. Subjects were randomized to either atorvastatin (A) or torcetrapib combined with atorvastatin (T/A). Mean common cIMT progression was increased in subjects receiving T/A compared to subjects receiving A (0.0076 ± 0.0011 vs. 0.0025 ± 0.0011 mm/year; p=0.0014). Subjects treated with T/A displayed higher post-randomization systolic blood pressure (SBP), plasma sodium and bicarbonate levels, in conjunction with lower potassium levels. The decrease in potassium levels was associated with the blood pressure increase. Markedly, the use of renin-angiotensin-aldosterone-system (RAAS) inhibitors tended to aggravate the blood pressure increase. Subjects receiving T/A with the strongest low-density lipoprotein cholesterol reduction showed the smallest cIMT progression, whereas subjects with the highest SBP increase showed the largest cIMT progression. HDL-C increase was not associated with cIMT change.

Conclusions: These analyses support mineralo-corticoid mediated off-target toxicity in patients receiving torcetrapib as a contributing factor to an adverse outcome. The absence of an inverse relationship between HDL-C change and cIMT progression suggests that torcetrapib-induced HDL-C increase does not mediate atheroprotection. Future studies with CETP inhibitors without off-target toxicity are needed to settle this issue.

Background

A low level of high-density lipoprotein cholesterol (HDL-C) is among the strongest predictors for cardiovascular disease.[1] As a consequence, novel modalities to raise HDL-C as a means to reverse atherosclerosis have raised considerable interest. Small molecule inhibitors of cholesteryl ester transfer protein (CETP) have been shown to increase HDL-C to unparalleled levels.[2-6] Based on these findings it has been suggested that CETP inhibition may further reduce the residual cardiovascular risk that persists during statin therapy. The impact of CETP inhibition by the novel small molecule torcetrapib has subsequently been addressed in intervention studies using atherosclerotic imaging as well as clinical endpoints. In December, 2006 the mortality and morbidity study ILLUMINATE (www.clinicaltrials.gov number NCT00134264) was prematurely terminated upon revealing a significant increase in all-cause mortality in the subjects using torcetrapib.[7] At that time, two of the three studies investigating the efficacy of torcetrapib using vascular imaging techniques had already been completed. In spite of a significant improvement in lipid profiles, none of these imaging studies demonstrated any benefit of adding torcetrapib to atorvastatin therapy in terms of progression of carotid or coronary atherosclerosis.[8-10] In fact, in RADIANCE 1, an increase of the common carotid intima-media thickness (cIMT) of the common carotid artery was observed.[8]

Hypotheses have been put forward to explain these unanticipated results, relating either to the mechanism of CETP inhibition per se, or to off-target adverse effects of the torcetrapib molecule.[11] An important argument favouring a causal role for the torcetrapib molecule rather than the CETP inhibition mechanism pertains to the observed increase in blood pressure. Although safety data regarding other CETP inhibitors is scarce, this appears to be a unique consequence of the torcetrapib molecule as the use of other CETP inhibitors has not been associated with a rise in blood pressure.[12-15] Also, in ILLUMINATE, torcetrapib use has been shown to cause electrolyte changes and elevations in plasma aldosterone, suggestive of a possible link with the unfavourable outcome of the studies with torcetrapib.[7]

In order to gain more insight into the mechanisms underlying this adverse outcome, we merged the databases of the RADIANCE 1 and 2 studies and performed exploratory analyses into the parameters that were associated with cIMT progression and on-trial blood pressure changes. In these analyses we have focused primarily on parameters that are related to CETP inhibition as a mechanism (i.e. on-trial HDL-C and LDL-C changes) as well as on parameters that are presumably connected to off-target toxicity (i.e. on-trial blood pressure and electrolyte changes).

Methods

Data sources

The design and results of the RADIANCE 1 and RADIANCE 2 studies were published previously.[8, 10, 16] Briefly, RADIANCE 1 enrolled subjects with heterozygous familial hypercholesterolemia and RADIANCE 2 enrolled subjects with mixed dyslipidemia. In both studies, the atorvastatin dose was titrated to a target LDL-C level according to the patient's cardiovascular risk based on the NCEP ATP III guidelines[17], or to the maximally tolerated dose (20, 40, 80 mg/day in RADIANCE 1; 10, 20 40 or 80 mg/day in RADIANCE 2). Next, subjects were randomly assigned to receive either atorvastatin monotherapy (A) or atorvastatin combined with 60 mg of torcetrapib (T/A) for 2 years. All subjects underwent B-mode ultrasonography at baseline, during follow-up and at the end of the study to assess changes in cIMT. The ultrasound protocol and the procedures for off-line cIMT reading were identical for RADIANCE 1 and RADIANCE 2.[16] The primary endpoint of the original RADIANCE studies, the mean cIMT of 12 carotid segments, showed no significant difference between treatment arms and was therefore unlikely to provide insight into the potential mechanisms relating to the adverse outcome caused by torcetrapib. We therefore selected the annualized change in the mean common cIMT as the endpoint for the present paper, since previous results indicated harm of torcetrapib on this outcome.[8] This measure has been used as a primary efficacy outcome in a number of recent trials.[18-23]

Risk factor information

At baseline, information was collected on cardiovascular risk factors, body mass index, medication use, lipid levels (total cholesterol, HDL-C, low-density lipoprotein-cholesterol (LDL-C), triglycerides), C-reactive protein, systolic and diastolic blood pressure, plasma electrolyte levels and cIMT. After randomization, information was collected on lipid levels, blood pressure, electrolyte levels, concomitant medication and investigator-reported adverse events. Information on cIMT was collected every 6 months after baseline and in duplicate at the end of the study.

In order to study the changes from baseline in the different laboratory and blood pressure values and to smooth out intra-individual and measurement variation, eight post-randomization values were averaged, after which baseline values were subtracted. Thus, a positive data point represents an increase in the average post-randomization values.

Data analyses

Baseline and on-trial characteristics of the subjects are presented by treatment arm in means and proportions. Student t tests and chi square tests were applied to examine differences between treatment arms in means and proportions, respectively. Mann-Whitney U tests were used for between-group comparisons when data were not normally distributed. Analyses of cIMT were performed in the full analysis set, consisting of 1533 subjects. For these subjects, at least one follow-up cIMT measurement was available. A linear mixed-effects model was used to analyze the annualized rate of change in mean common cIMT including the near and far wall measures of the right and left common carotid artery (4 segments x 7 visits) for each participant as the dependent variables with random intercepts and slopes as a function of time and fixed effects for study, geographic region, atorvastatin dose at run-in, carotid segment, treatment, and time. A term for treatment by time interaction was included when appropriate. Testing was two-sided and conducted with a 5% type I error rate.[24, 25]

First we evaluated which known risk factors were related to mean common cIMT progression to allow for adjustments in later analyses. These analyses were performed in strata of assigned treatment. Linear mixed-effects models were run with the risk

factor and the interaction term of time multiplied by risk factor. The factors evaluated were baseline risk factors (age, gender, body mass index, smoking, history of diabetes, history of hypertension, HDL-C levels, LDL-C levels, triglyceride levels) and on-trial change in risk factors (HDL-C change, LDL-C change, TG change, SBP change, DBP change). Those factors of which the interaction terms were statistically significant, were entered into a multivariate model (factor and interaction term) to determine the independent relationship with cIMT progression.
The influences of CETP inhibition related (HDL change and LDL change) and putative off-target (sodium change, potassium change, bicarbonate change and SBP change) effects of torcetrapib on cIMT progression were further examined in a different set of analyses. To examine whether the increased cIMT progression in the T/A arm could be (partly) explained by off-target effects, we calculated the difference in cIMT progression between treatment groups, and then made additional adjustments for off-target effects to see whether the difference in cIMT progression would be attenuated. A similar analysis was performed for effects related to CETP inhibition.

To assess whether electrolyte changes were related to on-trial systolic blood pressure change, a linear regression analysis was performed, using on-trial sodium, bicarbonate and potassium change in one model. A similar analysis was performed for baseline use of different types of antihypertensive medication to assess their effect on systolic blood pressure change. In this analysis, the use of angiotensin receptor blockers (ARB's), angiotensin converting enzyme (ACE) inhibitors, diuretics, beta blockers and calcium blockers were entered into one model. All linear regression analyses were done for the atorvastatin monotherapy arm and the T/A arm separately and were weighted for cohort size.

Role of the funding source

All analyses were performed academically by the lead authors. The study data were analysed independently by the study sponsor, Pfizer. The sponsor reviewed the manuscript and provided editorial comments to the lead authors. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written

Results

Table 1 shows the baseline characteristics of all 1656 randomized subjects. The annualized change in mean common cIMT was 0.0025 mm/yr [95% CI 0.0003; 0.0047 mm/yr] in the atorvastatin-only group and 0.0076 mm/yr [95% CI 0.0054; 0.0097 mm/yr] in the T/A group, reflecting a highly statistically significant increased progression rate in the T/A treated individuals (p=0.0014).

Table 1: Baseline characteristics of all randomized subjects (n=1656).

	Atorvastatin monotherapy N=829			Atorvastatin plus torcetrapib N=827		
Age (years)	50.4	±	12.4	51.8	±	11.9
Sex (male)	477 (58%)			451 (55%)		
BMI (kg/m²)	28.27	±	4.73	28.19	±	4.61
History of diabetes	111 (13%)			80 (10%)		
Current smokers	153 (18%)			149 (18%)		
Blood pressure (mm Hg)						
Systolic	117.9	±	10.6	118.0	±	11.4
Diastolic	74.1	±	6.8	73.5	±	7.2
Medication use						
Aspirin	337			347		
β blocker	182			194		
ACE inhibitor	158			137		
ARB	46			67		
Diuretic	133			136		
Cholesterol (mg/dL)						
Total	200.5	±	39.4	200.2	±	38.5
LDL	121.6	±	36.3	121.5	±	36.1
HDL	49.9	±	12.0	50.6	±	12.4
Triglycerides (mg/dL)						
Median	127.0			129.0		
Interquartile range	88.5	to	185.9	88.5	to	179.0
C-reactive protein (mg/dL)						
Median	0.3			0.4		
Interquartile range	0.1	to	0.9	0.1	to	1.0
Electrolytes (mEq/dL)						
Sodium	140.0	±	2.2	140.3	±	2.1
Potassium	4.2	±	0.4	4.2	±	0.4
Bicarbonate	24.6	±	2.7	24.7	±	2.6
cIMT (mm)						
Maximum cIMT	1.22	±	0.31	1.22	±	0.32
Mean common cIMT	0.77	±	0.2	0.77	±	0.16

Data are given as means ± SD or number (%). BMI=body mass index. ACE=angiotensin converting enzyme. ARB=angiotensin receptor blocker. LDL=low-density lipoprotein. HDL=high-density lipoprotein. cIMT=carotid intima-media thickness. Maximum cIMT is the average of the maximum intima-media thickness for each of the 12 carotid artery sites. Mean common cIMT is the average of the mean intima-media thickness for each of the 4 common carotid artery sites.

On-trial characteristics

Compared to atorvastatin monotherapy, addition of torcetrapib treatment resulted in increased levels of HDL-C (+24.5 mg/dL vs. -1.0 mg/dL, p<0.0001) and lower levels of LDL-C (-19.2 mg/dL vs. +4.3 mg/dL, p < 0.0001) as well as triglycerides (-7.7 mg/dL vs. 3.3 mg/dL, p<0.0001). Diastolic (75.5 mmHg vs. 74.7 mmHg; p=0.021) and systolic blood pressure readings (122.7 mmHg vs. 119.3 mmHg; p<0.0001) were higher in the group receiving torcetrapib-atorvastatin. (table 2, panel A and B). The distribution of systolic blood pressure changes in both treatment groups is depicted in figure 1.

Table 2: On-trial characteristics and adverse events in separate treatment arms

	Atorvastatin monotherapy			Atorvastatin plus torcetrapib			P value
A. Average post-randomization values							
Cholesterol (mg/dL)							
Total	204.6	±	1.4	203.8	±	1.4	<0.68
LDL	126.1	±	1.3	102.1	±	1.3	<0.0001
HDL	49.0	±	0.4	75.1	±	0.7	<0.0001
Triglycerides (mg/dL)							<0.0001
Median	136.1			118.0			
Interquartile range	96.2	to	184.6	86.3	to	159.3	
Electrolytes (mEq/dL)							
Sodium	139.9	±	0.1	140.4	±	0.1	<0.0001
Potassium	4.26	±	0.01	4.17	±	0.01	<0.0001
Bicarbonate	24.8	±	0.1	25.1	±	0.1	<0.016
Blood pressure (mm Hg)							
systolic	119.3	±	0.4	122.7	±	0.4	<0.0001
diastolic	74.7	±	0.2	75.5	±	0.3	<0.021
B. Change from baseline							
Cholesterol (mg/dL)							
Total	3.9	±	0.9	3.7	±	1.1	<0.85
LDL	4.3	±	0.7	-19.2	±	1.1	<0.0001
HDL	-1.0	±	0.2	24.5	±	0.4	<0.0001
Triglycerides (mg/dL)							<0.0001
Median	3.3			-7.7			
Interquartile range	-17.85	to	27.53	-35.38	to	14.38	
Electrolytes (mEq/dL)							
Sodium	-0.111	±	0.063	0.058	±	0.067	<0.067
Potassium	0.062	±	0.010	-0.014	±	0.011	<0.0001
Bicarbonate	-0.055	±	0.088	0.131	±	0.086	<0.130
Blood pressure (mm Hg)							
systolic	1.5	±	0.2	5.2	±	0.3	<0.0001
diastolic	0.6	±	0.2	2.1	±	0.2	<0.0001

Data are mean ± SE unless otherwise specified. LDL=low-density lipoprotein. HDL=high-density lipoprotein.

The average post-randomization values in the T/A group, compared to those for A alone, were significantly lower for potassium and higher for sodium and bicarbonate (table 2, panel A and B). When analyses were restricted to those not receiving blood pressure lowering medication, similar findings were obtained: the change in potassium from baseline in the T/A and A groups (-0.095 mEq/dL vs. -0.055mEq/dL, respectively, p = 0.0004) as well as post-randomization sodium and potassium levels (140.2 mEq/dL vs. 139.9 mEq/dL, respectively, p = 0.0003 and 4.20 mEq/dL vs. 4.27 mEq/dL, respectively, p = 0.0004) remained significantly different. These electrolyte changes were not associated with the torcetrapib-induced increase in HDL-C levels.

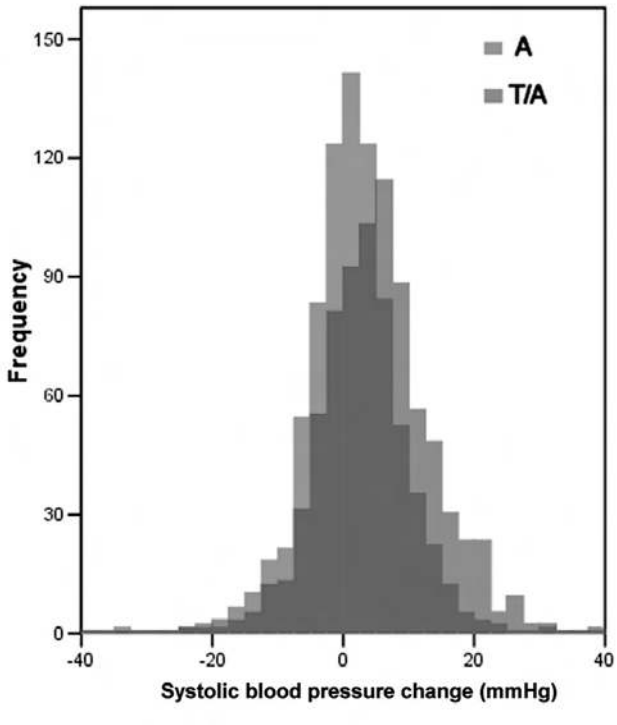

Figure 1. Distribution of systolic blood pressure changes in both treatment groups of RADIANCE 1 & 2 combined. A denotes the atorvastatin monotherapy group and T/A the torcetrapib/atorvastatin group. The differences (in mmHg) between baseline and mean post-randomization systolic blood pressure measurements are depicted. The overlap between both distributions is shown in dark green.

(Changes in) risk factors related to common cIMT progression

We evaluated the effects of baseline risk factors and on-trial changes in risk factors on carotid carotid atherosclerosis progression. In the atorvastatin-only group, apart from a history of diabetes mellitus (p value of interaction term 0.004), none of the baseline risk factors contributed to common cIMT progression. Furthermore, post-randomization changes in lipid levels (LDL-C (p=0.78), HDL-C (p=0.50), TG (p=0.65)) did not relate to mean common cIMT progression. In this group, those with low post-randomization cIMT progression rates had higher baseline cIMT values and those with larger post-randomization cIMT changes had lower baseline values, indicating regression to the mean. This is in line with the fact that all subjects were titrated to reach target LDL cholesterol levels, or to maximum tolerated statin dose during the run-in period.

In the T/A group, age at baseline (p = 0.045), history of hypertension (p=0.06), post-randomization changes in LDL-C (p=0.010), in TG (p=0.06), in systolic blood pressure (p=0.001) as well as in diastolic blood pressure (p=0.007) contributed to mean common cIMT progression. Notably, post-randomization changes in HDL-C did not predict mean common cIMT progression (p=0.63). In a multivariate linear mixed-effects model, baseline age, history of hypertension, post-randomization change in LDL-C and in SBP remained significantly related to common cIMT progression. Relationships between post-randomization variables and common cIMT progression, as well as interactions between these variables, are depicted in figure 2. The effects of LDL-C change and SBP change on cIMT progression oppose each other, whereas no interaction between HDL change and any of the other variables was observed.

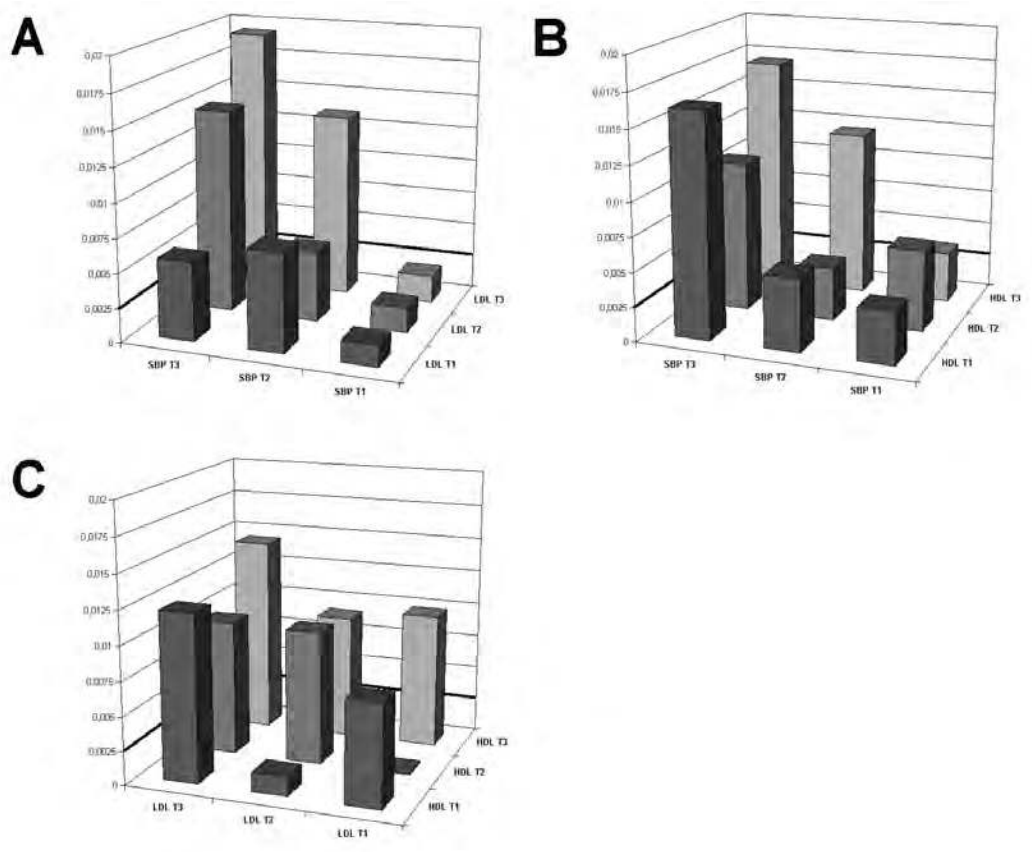

Figure 2. Relationships and interactions between mean common cIMT progression rates and tertiles of post-randomization changes in systolic blood pressure (SBP), HDL cholesterol and LDL cholesterol in the torcetrapib/atorvastatin group of RADIANCE 1&2 combined. Effects of SBP change and LDL change on cIMT progression oppose each other (A). There are no apparent interactions between SBP change and HDL change (B) or HDL change and LDL change (C). No relationship was found between these variables and cIMT progression rate in the atorvastatin-only arm. The bold line represents the average mean common cIMT progression rate in the atorvastatin-only group.

CETP inhibition related and off-target effects on cIMT progression

Next, we looked specifically at the influence on cIMT progression of effects related to CETP inhibition (LDL change and HDL change) and of putative off-target effects (SBP change, sodium change, potatssium change and bicarbonate change). The mean difference in common cIMT progression between the atorvastatin/torcetrapib arm and the atorvastatin arm alone in the combined RADIANCE studies was 0.0050 mm (SE 0.0016), $p = 0.0016$. The difference in cIMT progression was attenuated with 20% after adjustment for off-target effects (on-trial changes in blood pressure, bicarbonate, sodium, potassium): 0.0040 mm (SE 0.0016), p=0.013. In contrast, the difference in cIMT progression became 28% more pronounced after adjustment for effects related to CETP inhibition (LDL change and HDL change): 0.0063 mm (SE 0.0028), p=0.024.

Factors related to blood pressure increase

Next, we examined whether the observed electrolyte changes induced by torcetrapib were associated with systolic blood pressure change. Of the studied variables - potassium change, sodium change and bicarbonate change – only lower potassium levels were associated with an increase in systolic blood pressure in subjects using torcetrapib and atorvastatin (figure 3, upper panel): a 1 mEq/dL difference in potassium change was associated with a -2.57 mmHg change in SBP [95% CI -4.54; -0.60], p=0.01) in subjects in the T/A group. In the atorvastatin monotherapy group, no such associations were found.

Effects of antihypertensive medication

To verify whether the use of anti-hypertensive medication at baseline influenced systolic blood pressure readings in the T/A group, we performed a linear regression analysis. The use of ACE inhibitors or ARB's at baseline was associated with a greater systolic blood pressure increase in the T/A group (2.81 mmHg [95% CI 0.43; 5.18], p=0.02 and 1.81 mmHg [95% CI 0.05; 3.57]; p=0.04, respectively). Other antihypertensive drugs did not show this effect (figure 3, lower panel). Similarly, follow-up antihypertensive treatment with ACE inhibitors or ARB's did not result in lower end-of-study blood

pressure readings in the T/A group, whereas the initiation of diuretics for the same indication did reduce blood pressure readings (data not shown).

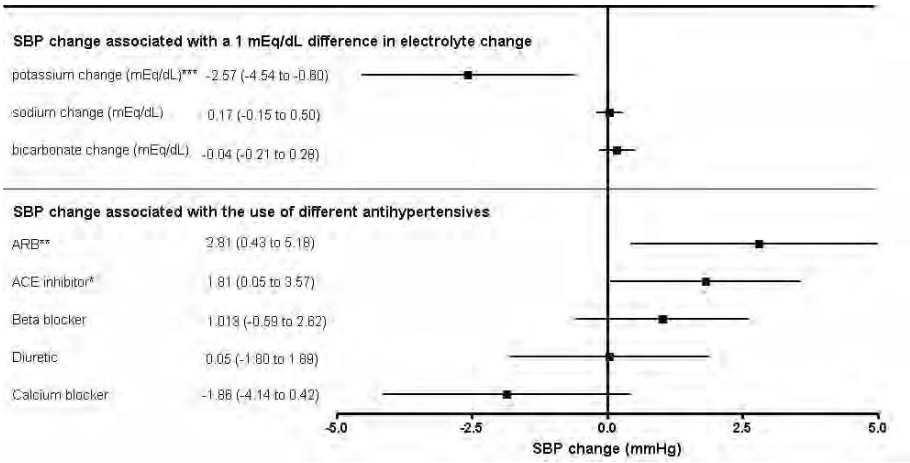

Figure 3. Linear regression analyses for determinants of SBP change in the T/A group. The results from two separate linear regression analyses are depicted in the upper and lower panel. In the upper panel, the difference in SBP in mmHg associated with a 1 mEq/dL on-trial electrolyte change is indicated. In the lower panel the difference in SBP in mmHg associated with baseline use of specific antihypertensive drugs is indicated.Error bars represent 95% confidence intervals. *** p = 0.01. ** p=0.02. * p=0.04

Discussion

In the present study we confirmed that the use of torcetrapib induces electrolyte changes, a blood pressure increase and an increased cIMT progression. The blood pressure changes were related to both the electrolyte changes and the increased cIMT progression. In contrast, torcetrapib-induced HDL increase was unrelated to either electrolyte changes or cIMT progression. The difference in cIMT progression between treatment groups was attenuated after adjustment for off-target effects (blood pressure and electrolyte changes) but not after adjustment for effects related to CETP inhibition

(LDL-C and HDL-C changes). These findings suggest that off-target toxicity of the torcetrapib molecule has contributed to the adverse outcome in these patients.

Mineralocorticoid excess

There are several arguments in favour of a mineralocorticoid excess, elicited by torcetrapib, as a contributing factor to the observed adverse outcome. First, a mineralocorticoid excess can explain both the electrolyte changes as well as the blood pressure increase. Aldosterone, produced in the adrenal gland, activates mineralocorticoid receptors on the principal cells of the distal tubule of the nephron, leading to increased sodium and bicarbonate reabsorbtion in exchange for potassium excretion, which is accompanied by a concomitant increase in plasma volume. Higher plasma aldosterone levels, even within the physiological range, already predispose to hypertension.[26]. The finding that potassium decrease was a significant predictor of the SBP increase following torcetrapib further substantiates the mineralocorticoid hypoothesis.

Second, we observed that the use of ACE inhibitors or ARB's at baseline was associated with an even greater SBP increase, whereas the use of diuretics, calcium blockers or beta blockers was not. Treatment with RAAS inhibitors leads to decreased aldosterone levels. Under these circumstances, a mineralocorticoid stimulus can be expected to have a more pronounced impact on blood pressure. To corroborate this, we observed that the initiation of RAAS inhibitors for blood pressure increase following the start of torcetrapib did not contribute to lower end-of-study blood pressure readings. In contrast, the initiation of diuretics for the same indication did result in reduced blood pressure levels. These findings are consistent with a renin- and angiotensin-independent, direct mineralocorticoid effect by torcetrapib contributing to a volume overload. Our results agree with findings from the ILLUMINATE study, in which torcetrapib use was associated with similar electrolyte changes, as well as with increased circulating aldosterone levels.[7] In fact, the observed interrelation between the parameters mentioned in the present study strengthen the notion that an increased mineralocorticoid activity may be causally related to the adverse outcome associated with torcetrapib use.

Mineralocorticoid hormones have a pro-atherogenic effect on the vasculature that can be only partly attributed to blood pressure.[27] Thus, aldosterone induces arterial stiffening through collagen deposition in the extracellular matrix, and subjects with hyperaldosteronism have been shown to have a thicker common cIMT compared to subjects with essential hypertension.[28, 29] These findings may explain why cardiovascular events in the T/A group of the RADIANCE studies occurred across the entire spectrum of blood pressure change, and were not restricted to subjects characterized by the largest blood pressure increases. Conversely, the mineralocorticoid excess may also be a marker of torcetrapib's effects on the adrenals, rather than the direct cause of the adverse outcomes associated with its use. Recently, Forrest et al. reported that torcetrapib exerts direct pressor effects in animal models, depending on the presence of the adrenal glands, but not completely inhibitable by the aldosterone receptor blocker eplenerone.[30] In addition, torcetrapib can upregulate the expression of RAAS genes in endothelial cells of rat aorta.[31] In view of the pro-atherogenic effects of tissue RAAS in the vessel wall, which may operate independently of the circulating RAAS,[32] this could further add to adverse effects, independent from blood pressure.

The exact mechanism by which torcetrapib elicits the release of substances with mineralocorticoid effects remains to be established. Torcetrapib is known to form a stable complex with CETP, firmly attached to HDL particles.[33] In this respect, it is interesting to note that HDL cholesterol also serves as a cholesterol-donating substrate for the adrenal, requiring cholesterol for the production of steroid hormones.[34] Based on these data it is tempting to speculate that torcetrapib enters the adrenals as part of the HDL particle, which may provide the basis of the mineralocorticoid release. Further studies are needed to test this hypothesis.

No evidence for anti-atherosclerotic effect of HDL increase mediated by CETP inhibition

In the ILLUMINATE study, a post-hoc analysis showed a trend towards fewer major cardiovascular events in those subjects who experienced an above-median HDL-C increase under torcetrapib treatment, suggestive of a positive impact of the lipid

changes mediated by CETP inhibition.[7] Similarly, in the present study, the mild LDL-lowering effect of torcetrapib was associated with a decreased cIMT progression. However, the absence of a relationship between HDL-C increase and decreased cIMT progression in this study could imply that CETP-inhibitor conferred HDL increase lacks an atheroprotective effect. Indeed, there has been concern that CETP inhibition might slow the recycling of HDL particles and reduce hepatic cholesterol uptake through the LDL receptor, thereby diminishing the flux of cholesterol through the reverse cholesterol transport pathway.[35] Furthermore, studies regarding the impact of CETP inhibition on inflammatory as well as oxidation markers have revealed mixed results.[35] However, against the background of off-target toxicity it is difficult to draw final conclusions on HDL's atheroprotective capacity following CETP inhibition in the present study.

Study limitations

Some aspects of this study merit caution. In the present study, the data from the RADIANCE 1 & 2 trials were merged, yielding a large sample size of more than 1600 subjects. This carries a potential limitation, because the studied populations (i.e. familial hypercholesterolemia and mixed dyslipidemia, respectively) are heterogeneous. Furthermore, while we gathered indirect evidence from a number of analyses, we did not directly quantify RAAS activity or mineralocorticoid levels in the RADIANCE studies. It should be stated explicitly that phase III studies are not intended to reveal mechanistic insights. Therefore, our results are hypothesis generating and should be followed-up by appropriate analyses in humans as well as in animal models.

Molecule or mechanism?

Despite a large increase in HDL-C, and not related to this increase of HDL-C, torcetrapib use was strongly associated with adverse arterial wall changes. Although it cannot be excluded that the mechanism of CETP inhibition and its HDL effect were adverse by itself, torcetrapib-induced electrolyte changes and their association with blood pressure elevations suggest that torcetrapib, as a molecule, resulted in an

off-target mineralocorticoid excess. In turn, the association of the blood pressure elevations with the cIMT changes suggests that the adverse intimal changes may be the result of this off-target effect. Other CETP inhibitors currently in clinical development (anacetrapib, dalcetrapib) do not appear to raise blood pressure.[12, 14, 15, 36] Whether the use of these agents will yield clinical benefit will have to be awaited until the results of a number of phase III trials are available in the future. Until then, the final verdict for CETP inhibition is still out.

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Chapter 13

Sustained changes in lipid profile and macrophage migration inhibitory factor (MIF) levels after anti-TNF therapy in rheumatoid arthritis.

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Abstract

Background: Macrophage migration inhibitory factor (MIF) has recently emerged as an important cytokine possibly linking rheumatoid arthritis (RA) and atherogenesis. Because atherogenesis is accelerated in RA we investigated whether anti-TNF therapy could lead to sustained downregulation of systemic MIF levels and improvement of lipid profiles.

Methods: Fifty RA patients with active disease (disease activity score 28 (DAS28 ≥ 3.2)), who started adalimumab therapy 40 mg every other week, were included. At baseline, week 16 and 52 serum levels of MIF and lipids were assessed. In addition, the DAS28 and serum C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR) were determined.

Results: After 16 weeks of adalimumab therapy, both DAS28 and MIF levels were significantly decreased ($P < 0.001$ and $P = 0.020$, respectively). This was sustained up to week 52 ($P < 0.001$ and $P = 0.012$, respectively). CRP levels and ESR were significantly reduced after 16 and 52 weeks of adalimumab therapy ($P < 0.001$). HDL cholesterol levels increased at week 16 ($P < 0.001$), but returned to baseline at week 52. Apo A-I levels increased at week 16 ($P <0.001$) and remained stable ($P = 0.005$). This resulted in an improved apo B/ A-I ratio.

Conclusions: Our results underline sustained downregulation of MIF as a potential new mechanism by which anti-TNF therapy might reduce vascular inflammation, and as such perhaps cardiovascular morbidity in RA patients. This hypothesis is supported by an improved apo B/ A-I ratio as well as reduced CRP levels in our patients.

Introduction

The atherosclerotic process is accelerated in patients with rheumatoid arthritis (RA), resulting in increased cardiovascular mortality when compared to the general population. It has been suggested that the chronic systemic inflammatory state in RA enhances atherogenesis[1] over and above the presence of traditional risk factors (e.g. diabetes, smoking, obesity, dyslipidemia). Inflammatory mediators from the synovium and perhaps other sites can be released into the circulation where they can alter the function of various tissues, such as skeletal muscle, liver, and vascular endothelium. This in turn may induce an array of pro-atherogenic changes, including insulin resistance, characteristic dyslipidemia, and endothelial dysfunction.[2] Moreover, circulating inflammatory mediators may also stimulate leukocytes and smooth muscle cells within the atherosclerotic plaque thereby promoting plaque growth or rupture.[3] Macrophage migration inhibitory factor (MIF) has emerged as a cytokine linking RA and atherogenesis.[4] The association of coronary heart disease with a haplotype containing the rs755622C allele, which has been reported before to increase the susceptibility for various inflammatory conditions, supports the notion that MIF plays a role in inflammation and atherogenesis, although there was no difference in MIF serum levels between patients with incident coronary heart disease and individuals without such disease during follow-up in a population-based case-cohort study.[5] However, in another prospective population study in apparently healthy volunteers elevated levels of MIF were associated with an increased risk of future coronary artery disease.[6] The receptors CXCR2 on monocytes and CXCR4 on T cells have been identified as the functional receptors for MIF.[7] Interaction of CXCR2 with MIF on aortic endothelial cells was shown to induce monocyte arrest. Similarly, the interaction of CXCR4 with MIF resulted in the arrest of T cells. MIF can also induce the secretion of tumor necrosis factor (TNF) by macrophages and, conversely, TNF is able to augment MIF production.[8] In an animal model of atherosclerosis MIF blockade reduced plaque infiltration by monocytes and T cells, and even led to plaque regression.[7] Recent studies have demonstrated that MIF secretion by dendritic cells

can be regulated by Toll-like receptors (TLR).[9] In the atherosclerotic lesion, especially TLR4 has been shown to be expressed by residing macrophages and dendritic cells.[10,11] When TLR4 is triggered by its ligands (for instance lipopolysaccharide (LPS)), various cytokines, including TNF, IL-12, IL-23 and MIF, can be secreted, hereby further enhancing the inflammatory response.[9,10] Together, the available data indicate that MIF exerts chemokine-like functions and is an important regulator of inflammatory cell recruitment and atherogenesis. Thus, it is conceivable that reducing MIF might be a potential therapeutic target for patients with atherosclerosis.

The notion that inflammation in RA and atherogenesis are linked is supported by data suggesting that reducing disease activity by adequate disease modifying anti-rheumatic therapy may result in a decrease in cardiovascular mortality.[12,13] TNF blockade could diminish the increased cardiovascular risk associated with RA by attenuating not only local but also systemic inflammation associated with atherogenesis.[14,15]

To explore the relationship between inflammation and factors involved in atherogenesis, we investigated the early and long term effects of anti-TNF therapy on serum MIF levels and known risk factors such as C-reactive protein levels and lipid profile in RA patients.

Patients and methods

Patients

Fifty RA patients with active disease (Disease Activity Score in 28 joints (DAS28)) ≥ 3.2 were included in the study. All patients received adalimumab 40 mg subcutaneously every other week in combination with methotrexate (MTX) in a stable dose for at least 8 weeks. The concomitant use of prednisone (≤10 mg/day) and non-steroidal anti-inflammatory drugs (NSAIDs) was allowed if stable for at least one month. Approval for this study was obtained from the institutional ethics review committee at the Academic Medical Center/ University of Amsterdam. All participants gave written informed consent.

Clinical assessments

RA disease activity was assessed at baseline, week 16 and 52 after start of adalimumab treatment using the DAS28. Clinical response was evaluated by the EULAR response criteria. For comparison of data between responders (good and moderate) and non-responders we used response measured at week 16. In addition, the presence of extra-articular manifestations (such as vasculitis, nodules, and pleuritis) was noted before entry in the study.

Cardiovascular risk factor profiles

In the assessment of cardiovascular risk factors the following data were recorded: medical history including cardiovascular events, smoking (current smoker, ever smoker), and current medication; hypertension; dyslipidemia; diabetes; and body mass index (kg/m^2).

Lipid profiles

Serum total cholesterol (TC), high density lipoprotein (HDL), and low density lipoprotein (LDL) cholesterol, triglyceride (TG), and lipoprotein (a) (Lp(a)) levels were assessed by standard laboratory techniques. Apo A-I and apo B levels were measured by an automated nephelometric assay using an array protein system nephelometer (Beckman, Mijdrecht, The Netherlands). In addition, erythrocyte sedimentation rate (ESR) (mm/hour) and C-reactive protein (CRP) levels (mg/L) were determined. Blood was drawn from patients while fasting at baseline and at 16 and 52 weeks after initiation of adalimumab therapy. All values were determined by the GLP certified routine clinical chemistry laboratory at the Academic Medical Center in Amsterdam.

MIF ELISA

Natural serum MIF levels (pg/ml) were determined with a commercial quantitative sandwich-enzyme-immunoassay (human MIF, DY289, R&D systems Inc, Minneapolis, MN). The assay was performed according to manufacturer's instructions. Fasting serum samples were stored at -80°C and analyzed all at once.

Statistical analysis

A paired t- test or the Wilcoxon signed ranks test, whichever was appropriate, was used to determine significant changes from baseline. Probability values <0.05 were considered statistically significant in a 2-tailed test. This exploratory study was not powered to correct for multiple comparisons by Bonferroni correction. Independent samples t-tests were used for sub-analysis to detect differences in baseline values or changes after treatment between groups. Correlations were assessed with the Pearson product-moment or Spearman rank-order correlation coefficients, whichever was appropriate. Stepwise backward multivariable linear regression analysis was used to identify possible baseline predictors of change in MIF level at week 16 and 52. Because delta MIF levels had a skewed distribution, values were rank-transformed before linear regression analysis. Baseline variables included in the analysis were sex, BMI, MIF, CRP, triglyceride, total cholesterol and HDL levels. Values are expressed as the mean ± standard deviation (SD) or median and interquartile range (IQR), whichever was appropriate. SPSS 12.0.2 for Windows (SPSS, UK) was used.

Results

Patients and clinical response

The baseline patient characteristics of 50 patients are shown in Table 1. The DAS28 score decreased significantly after 16 weeks (DAS28 3.7 ± 1.2) and 52 weeks (3.4 ± 1.4) of adalimumab therapy compared to baseline (5.6 ± 1.1) (both $P < 0.001$). At week 16 all patients were evaluable for clinical response: 11 (22%) patients were EULAR non-responders, 25 (50%) moderate responders and 14 (28%) good responders. At week 52 there were 44 patients with an evaluable response of whom 6 (14%) were non-responders, 18 (41%) moderate responders, and 20 (45%) good responders. Six patients dropped out of the study between 16 and 52 weeks follow-up due to lack of efficacy in 5 patients and a serious adverse event in 1 patient.

Table 1. Baseline patient characteristics.

	All n = 50	Responders n = 39	Non-responders n = 11	P-value
Age, years	51 ± 13	51 ± 12	47 ± 17	0.331
Female (%)	38 (76)	28 (72)	10 (91)	0.190
Disease duration, months	59 (33-145)	61 (29-149)	54 (34-142)	0.935
Erosive disease (%)	33 (66)	26 (67)	7 (64)	0.851
RF positive (%)	36 (72)	29 (74)	7 (64)	0.484
Anti-CCP positive (%)	35 (70)	28 (72)	7 (64)	0.602
Extra articular manifestations	14 (28)	12 (31)	2 (18)	0.412
DAS28	5.6 ± 1.1	5.7 ± 1.1	5.3 ± 0.8	0.225
BMI (kg/m^2)	27 ± 6.3	27 ± 6.3	26 ± 6.7	0.557
Smokers, current (%)	12 (24)	10 (26)	2 (18)	0.609
Smokers, ever (%)	31 (62)	27 (69)	4 (36)	*0.047*
SBP (mmHg)	132 ± 15	133 ± 16	129 ± 12	0.606
DBP (mmHg)	80 ± 9	80 ± 9	83 ± 8	0.241
ESR (mm/hour)	20 (11-35)	20 (11-35)	20 (15-36)	0.824
CRP (mg/L)	17 (5-20)	10 (4-22)	8 (5-16)	0.779
DM 2 (%)	4(8)	4 (10)	0 (0)	0.268
Prior CV event (%)	4(8)	3 (8)	1 (9)	0.687
Statin use (%)	6 (12)	5 (13)	1 (9)	0.717
Anti-HT drugs use (%)	16 (32)	13 (41)	3 (27)	0.704
MTX (mg/wk)	18.4 ± 7.6	18.0 ± 7.7	19.5 ± 7.5	0.508
Use of corticosteroids (%)	16 (32)	11 (28)	5 (45)	0.279
Prednisone dose (mg/day)	7.8 ± 2.4	7.8 ± 2.4	8.0 ± 2.7	0.268
NSAID use (%)	36 (72)	28 (78)	8 (73)	0.951

* mean values ± standard deviation (SD), median and interquartile range (IQR) or percentages are shown. P-values < 0.05 (2 sided) are significant and shown in *Italic*. BMI = body mass index, BP = blood pressure, ESR = erythrocyte sedimentation rate, NSAID = non-steroidal anti-inflammatory drug.

Pre-treatment serum MIF levels

Large variability in MIF levels was observed between patients varying from the lowest detectable concentration of 60 pg/ml up to 6571 pg/ml. There was no significant relationship with use of low dose corticosteroids or dosage of MTX, nor with clinical measures of disease activity at baseline (data not shown).

Pre-treatment apo A-I and HDL levels are inversely correlated with systemic inflammation

There was a negative correlation between pre-treatment apo A-I levels and CRP levels (r -0.338, P = 0.017) as well as ESR (r -0.347, P = 0.014). Similarly, pre-treatment HDL cholesterol correlated inversely with CRP levels and ESR (r -0.290, P = 0.041 and r -0.340, P = 0.016, respectively).

Interestingly, higher pre-treatment HDL levels correlated with lower MIF levels before initiation of adalimumab therapy (r -0.294, P = 0.040). As expected, baseline LDL cholesterol was significantly lower in the 6 patients who used statins compared to the other 44 who did not use a statin (P = 0.007); other lipoproteins did not differ between these groups. All patients who used statins were known with a history of hypercholesterolaemia. Four of the 6 patients had a prior cardiovascular event, and all 6 were on concomitant anti-hypertensive drugs. One patient also had type 1 diabetes.

Sustained downregulation of MIF and inflammatory parameters, but not of HDL cholesterol after adalimumab therapy

Serum MIF levels were significantly decreased 16 weeks after initiation of adalimumab therapy (median 171 pg/ml, IQR 60-444) compared to baseline (median 333 pg/ml, IQR 93-1544, P = 0.020). This effect was sustained up to week 52 (median 145, IQR 60-335, P = 0.012) (Figure 1). CRP and ESR levels decreased significantly after 16 and 52 weeks of adalimumab therapy (both P < 0.001, Table 2).

The mean HDL cholesterol levels increased at week 16 compared to baseline (P < 0.001). However, HDL levels returned to nearly baseline at week 52 (Table 2). LDL cholesterol levels did not change after adalimumab treatment. Furthermore, Lp(a) levels decreased significantly at week 16 up to week 52 after treatment (both P = 0.001, Table 2).

Table 2. Changes in MIF levels and lipid profile over time.

	Week 0 (n=50)	Week 16 (n=50)	P- value	Week 52 (n=44)	P-value
MIF (pg/ml)	333 (90-1544)	171 (60-444)	*0.020*	145 (60-335)	*0.012*
TC (mmol/L)	4.86 ± 1.07	5.06 ± 1.16	0.053	4.98 ± 1.13	0.301
HDL (mmol/L)	1.52 ± 0.38	1.66 ± 0.38	*<0.001*	1.62 ± 0.39	0.061
LDL (mmol/L)	2.88 ± 0.95	2.96 ± 1.01	0.392	2.93 ± 1.02	0.577
TG (mmol/L)	1.01 ± 0.51	0.99 ± 0.54	0.513	0.94 ± 0.43	0.350
Apo A-I (mmol/L)	1.46 ± 0.25	1.56 ± 0.22	*<0.001*	1.56 ± 0.21	*0.005*
Apo B (mmol/L)	0.99 ± 0.25	1.00 ± 0.27	0.465	0.99 ± 0.28	0.816
Lp (a) (mmol/L)	198 (65-356)	175 (65-377)	*<0.001*	171 (47-375)	*<0.001*
TC/HDL	3.33 ± 0.93	3.15 ± 0.85	*0.034*	3.19 ± 0.84	0.272
Apo B/Apo A-I	0.70 ± 0.21	0.65 ± 0.20	*0.014*	0.65 ± 0.20	0.050
Glucose (mmol/L)	5.13 ± 1.97	4.95 ± 1.14	0.847	5.04 ± 1.34	0.930
CRP (mg/L)	8.8 (4.6-19.6)	3.8 (1.6-9.1)	*<0.001*	2.7 (1.1-5.6)	*<0.001*

* Values are represented as mean ± standard deviation, or median and interquartile range. P values < 0.05 (two-sided) are significant and shown in *Italic*.

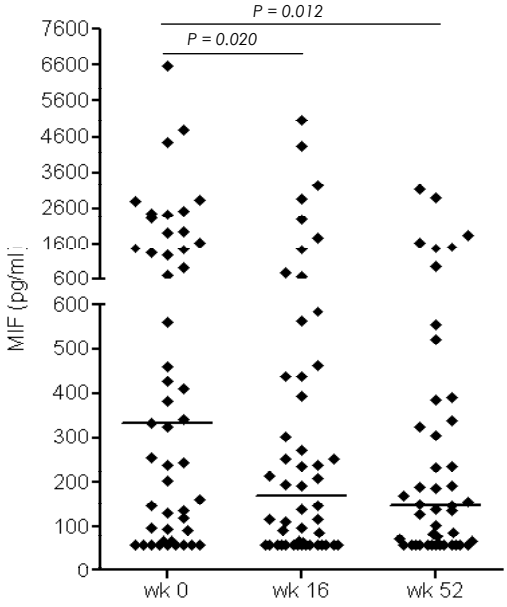

Figure 1. Serum MIF levels before, and 16 and 52 weeks after adalimumab therapy.

The median values and range are shown for each time point. A large variability in MIF concentration was observed between patients. Some high pre-treatment MIF concentrations may be due to the presence of MIF promoter polymorphisms in certain patients. The presence of such polymorphisms was not analyzed in this study, the data however show that even high baseline MIF concentrations diminish significantly after anti-TNFα therapy.

Improvement of the atherogenic index after adalimumab therapy

The mean apo A-I levels (high levels are thought to be cardioprotective) were significantly increased to 1.56 ± 0.22 mmol/L at week 16 compared to 1.46 ± 0.25 mmol/L at baseline ($P < 0.001$) and remained significantly elevated up to week 52 (1.56 ± 0.22 mmol/L) ($P = 0.005$). Of interest, apo B levels (an indicator of the total number of atherogenic particles) did not change over the course of 52 weeks adalimumab therapy. Thus, there was a significant decrease in the apo B/A-I ratio at week 16 ($P = 0.014$), which remained lowered up to week 52 ($P = 0.050$). The total cholesterol/HDL ratio showed temporary improvement ($P = 0.034$), due to the increase in HDL levels at week 16, which had diminished again one year after start of treatment (Table 2).

Baseline MIF concentrations and gender predict changes in MIF after adalimumab treatment. With stepwise backward multivariable linear regression analysis we identified baseline predictors for change in MIF levels after treatment. Included in the analysis were the following baseline variables: sex, BMI, MIF, CRP, triglyceride, total cholesterol and HDL levels. No baseline predictors for change in MIF concentration over time were identified other than patient gender and pre-treatment MIF concentrations. Baseline MIF concentration in combination with gender predicted 45 % of the variance in change of MIF concentration at week 16 (adjusted $R^2 = 0.453$). MIF concentration at baseline alone predicted 38% of the variance in change of MIF concentration at week 52 (adjusted $R^2 = 0.383$). A significantly larger decrease in MIF concentration was seen in female compared to male patients at week 16 ($P = 0.011$), but no gender difference was observed at week 52. We observed no association between baseline levels of inflammation or lipid profile and change in MIF levels after treatment.

Changes in MIF levels and lipid profile in relationship to clinical response. We analyzed whether changes in MIF concentration differ between EULAR responders versus non-responders. We found no relationship between clinical response and changes in MIF concentration at week 16 nor at week 52. However, the decrease in Lp(a) concentration was greater in responders than in non-responders at week 16 (P

= 0.018), with a similar trend at week 52 (P = 0.087). Furthermore, in accordance with previous data, an increase in HDL levels at week 16 was associated with a decrease in DAS28 score at the same time point (r -0.308, P = 0.030).[16] Similarly, HDL levels increased more in EULAR responders than in non-responders, although this difference did not reach statistical significance (P = 0.068). The increase in apo A-I was not different between response groups.

Discussion

Both RA and atherosclerosis are related to chronic inflammation. There is increasing evidence that TNF and MIF are involved in these conditions and that the role of these cytokines is linked.[4,8,17] In both RA and atherosclerosis enhanced MIF levels have been observed at the site of inflammation[17,18] and MIF was shown to mediate leukocyte recruitment into the inflamed joint and vessel wall.[19,20] Furthermore, MIF can mediate integrin activation and induce expression of other inflammatory cytokines, such as IL-6, and TNF, and matrix metalloproteinases (MMP) associated with joint damage in RA and plaque instability in atherosclerosis.[20-22] The role of TNF is supported by the observation that anti-TNF therapy may reduce the increased cardiovascular risk associated with RA by decreasing systemic inflammation. Previous work has shown that TNF blockade may influence lipid levels, insulin resistance, vascular adhesion molecule expression, and endothelial function.[16,23-26] We performed the present study to provide more insight into the mechanisms that could be involved in the effects of anti-TNF therapy on cardiovascular risk. The results confirm our hypothesis that adalimumab treatment leads to downregulation of MIF with potential beneficial consequences for vascular inflammation. Moreover, we show for the first time that long term TNF blocking therapy with adalimumab has a favorable influence on the lipid profile of RA patients.

It has previously been suggested that chronic systemic inflammation in RA and subsequent atherogenesis are in part the result of chronic cytokine overflow from the inflamed joints into the circulation.[2] Anti-TNF therapy has been shown to diminish

local inflammation in the joint by decreasing synovial cell infiltration and expression of adhesion molecules, chemokines, and cytokines which coincides with a reduction of acute phase reactants.[24,27-29] A decrease in CRP levels was previously shown to be accompanied by a reduction in synovial MIF and TNF expression in the same patient when disease activity was reduced by conventional disease-modifying antirheumatic drug (DMARD) therapy.[30] In light of these data we hypothesized that pro-inflammatory cytokine release from the inflamed joint could be diminished after adalimumab treatment, resulting in a decrease in systemic levels of cytokines, including MIF (Figure 2). Consistent with this notion we found serum MIF levels to be significantly downregulated within 16 weeks after adalimumab therapy, an effect that was sustained up to one year after initiation of treatment. Whether anti-TNF therapy reduces MIF in the atherosclerotic lesion in patients is as yet unknown, but beneficial effects of TNF inhibition on atherosclerotic lesions have been demonstrated in animal atherosclerosis models.[31] A decrease in MIF expression could lead to reduced monocyte and T cell influx into the inflamed vessel wall, hence arresting plaque formation.[7]

HDL levels increased temporarily after treatment, resulting in an improved atherogenic index (total cholesterol/ HDL cholesterol) at week 16, which was no longer present after 1 year of adalimumab treatment. A brief rise in HDL cholesterol, also known as an inverse phase reaction, is to be expected after reversing the inflammatory state and was previously reported in other studies with infliximab.[16,32] In addition we observed a sustained increase in apo A-I levels and thus improvement of the apo B/A-I ratio. Based on these findings one can speculate that the apo B/A-I ratio may better reflect the cardiovascular risk profile after TNF blocking therapy than the traditional atherogenic index (total cholesterol/ HDL), as differential effects of adalimumab therapy can be observed for apo A-I and HDL levels. Of interest, serum apo-A1 has previously been shown to inhibit T-cell contact induced monocyte activation.[33] As a result cytokine (TNFα and IL-1β) production by monocytes was inhibited while monocyte proliferation remained unaltered. These data indicate a novel anti-inflammatory mechanism of this apolipoprotein. Conceivably, the sustained increase of apo-A1 levels in our study might lead to inhibition of T-cell induced monocyte activation, both in the inflamed synovium and in the vessel wall.

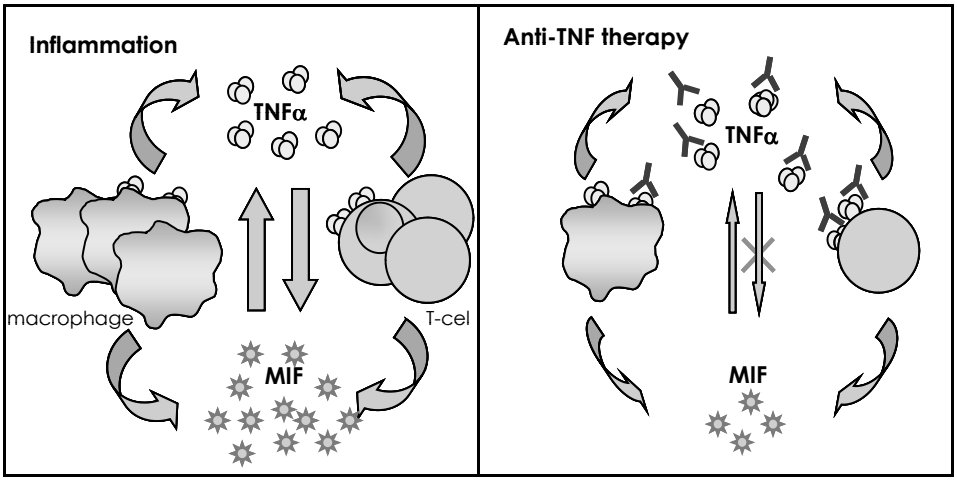

Figure 2. Schematic view of the interaction between MIF and TNF.

Both macrophages and T cells as well as dendritic cells and fibroblast-like synoviocytes produce MIF and TNF. TNF induces the production of MIF, and vice versa TNF production can be induced by MIF.[8, 17] In RA increased levels of MIF and TNF have been found locally in the synovial fluid and synovial tissue, which perpetuate the inflammatory process not only by inducing further cytokine secretion, but also by enhancing leukocyte migration towards the site of inflammation.[19] With anti-TNF antibody therapy available bioactive TNF is neutralized. Furthermore, the infiltration of the inflamed synovium by macrophages (main producers of TNF and MIF) was shown to diminish early after treatment.[27] Hence, both the number of MIF producing cells as well as the concentration of bioactive TNF decreases after anti-TNF therapy potentially leading to a decrease in systemic MIF levels.

Lipoprotein (a) has been demonstrated to have a spectrum of pathogenic activities among which increased vascular adhesion molecule expression, chemotaxis of monocytes, foam cell formation, smooth muscle cell proliferation, and increased platelet aggregation. Different clinical studies have shown Lp(a) levels to be an independent risk factor for developing CHD.[34,35] Hence, the significant decrease in levels op Lp(a) both 16 and 52 weeks after adalimumab therapy could contribute to a decrease in the pro-atherogenic state. This could be a direct effect of anti-TNF therapy but may also be an indirect of the overall diminishment in inflammation.

The open label rather than placebo-controlled design is obviously a limitation of this study, as we cannot conclude with complete certainty that the decease in MIF levels resulting from decreased inflammation was a direct effect of TNF blockade or merely a result of regression to the mean. However, the patients had persistent disease activity in spite of at least 2 conventional DMARDs before inclusion in the study, suggesting that the reduction of inflammation was the result of TNF blockade. Thus, the data presented in this exploratory study provide the rationale for future studies with a controlled design to confirm the effects on lipid profiles and MIF levels in relationship to cardiovascular endpoints.

In conclusion, TNF blocking therapy reduced systemic MIF levels, possibly reflecting a reduction in atherogenic state. Apart from reduced systemic inflammation as shown by reduced CRP and ESR levels, the sustained decrease in apo B/apo A-I ratio suggests a favorable effect of adalimumab treatment on markers associated with atherogenesis.

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Disclosures

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Chapter 14

MMF as an Immunomodulatory Silver Bullet in Atherogenesis?

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Abstract

Atherosclerotic vascular disease is a chronic disorder of the vasculature with a substantial impact on society. Although the availability of statins has represented an unparalleled improvement in the treatment of patients with such cardiovascular disease, even more effective measures are required to reverse this disorder with a continuously growing incidence. The classification of atherosclerosis as an inflammatory disorder has prompted the hypothesis that immunomodulation could comprise a novel anti-atherosclerotic strategy. Mycophenolate mofetil (MMF) has various anti-atherogenic effects on major components of the atherosclerotic plaque such as T-lymphocytes, monocytes/macrophages and the endothelium. MMF can inhibit leukocyte recruitment to the subendothelium and the subsequent reduced activation of leukocytes will translate into attenuation of subendothelial cross-talk between T-cells and macrophages. This cascade of events will interrupt the self-perpetuating pro-inflammatory environment within the arterial wall, the hallmark of atherosclerotic vascular disease.

Introduction

Atherosclerosis is a lifelong disorder of the arterial wall possibly initiated even before birth.[1] The impact of the disease continues to grow as it is estimated that, worldwide, in the year 2020 there will be almost 33.000 deaths daily due to ischemic heart disease. Compared to the year 1990 this would be equivalent to an increase of 90%.[2] These alarming numbers illustrate the urgent need for effective therapeutic strategies. In this regard, the introduction of statins to our armentarium has represented a major step forward in both the prevention and treatment of atherosclerotic vascular disease. Indeed, in a large meta-analysis, statin treatment was shown to reduce the risk of major coronary events by 26–36%.[3] This efficacy, however, emphasizes the need for additional drugs that will lead to end point reduction of more than 50%. Continuing elucidation of the precise pathophysiology of atherothrombosis may bring forth such drugs with alternative modes of action. Novel insights into the metabolism of high density lipoprotein (HDL) for instance, has led to the development of cholesterol ester transfer protein (CETP) inhibitors.[4] Furthermore, the role of inflammation during all phases of atherosclerosis, from plaque initiation up to plaque rupture, has been widely acknowledged. Subsequently, a potential role of anti-inflammatory regimens in the treatment of atherosclerosis has sparked much interest. Such drugs would ideally be able to exert anti-atherogenic effects on several major components of the plaque and may in fact already be available. In addition to its primary mode of action, a cytostatic effect on lymphocytes, mycophenolate mofetil (MMF) has several immunosuppressant actions that may attenuate atherosclerotic lesion formation and these will now be reviewed.

T-lymphocytes in atherogenesis

Naïve T-cells, as part of immune surveillance, traffic from the systemic circulation, through peripheral tissues. Upon encountering antigen(s), they become activated and may subsequently differentiate and proliferate. Indeed, a T-cell infiltrate is always present in atherosclerotic lesions and consists predominantly of $CD4^+$ cells that

recognize protein antigens presented by MHC II molecules.[5] Several potential antigens have been hypothesized to underlie the initiation and propagation of atherosclerotic vascular disease such as oxidized LDL, heat shock protein 60 (HSP 60) and Chlamydia antigens. Upon activation, helper T cells can polarize into pro-inflammatory T helper-1 (Th1) cells or anti-inflammatory T helper-2 (Th2) cells, of which the first subtype predominates in the atherosclerotic plaque. Th1 cytokines, such as interferon-γ and IL-2, contribute to an inflammatory environment in the subendothelium by activating macrophages but can also activate endothelial and smooth muscle cells (SMC). In addition, T-cells themselves can contribute to atherogenesis by acquiring direct cytotoxic abilities allowing them to kill endothelial cells and SMC.[6] It is currently believed that the outcome of the atherosclerotic process is determined in part by the balance between Th1 and Th2 driven responses. This concept and the crucial role of T-cells in atherogenesis in general has been substantiated in several animal studies which showed that downregulation,[7] depletion[8] or signal inhibition of Th1-cells[9,10] results in significant reduction of atherosclerosis. Recently, another T-cell subtype has also been suggested to play an important role in these processes. $CD4^+CD25^+$ regulatory T-cells (T_{reg}) suppress both Th1 and Th2 pathogenic immune responses against self or foreign antigens, and control T-cell homeostasis. Indeed in different mouse models, such regulatory T-cells have been shown to reduce the development of atherosclerosis.[11,12]

T-lymphocytes and MMF

MMF is an inhibitor of the enzyme inosine monophosphate dehydroxygenase (IMPDH) and thereby depletes the pool of dGTP required for DNA synthesis (figure 1).[13] Most cell types can synthesize guanosine nucleotides by the *de novo* pathway as well as by a salvage pathway. Lymphocytes however, are dependent upon *de novo* synthesis. In addition, MMF is fivefold more potent as an inhibitor of the type II isoform of IMPDH which is expressed in activated lymphocytes, than of the housekeeping type I isoform, which is expressed in most cell types.[13] Taken together, MMF has a potent cytostatic effect on T-lymphocytes and has indeed been shown to induce a G1

arrest in activated T-cells.[14] This is particularly relevant when considering MMF as an anti-atherogenic agent since it has been shown that the proatherogenic role of T-cells depends on cell division. In a recent study, a transgenic mouse model was generated, specifically expressing a suicide gene in both $CD4^+$ and $CD8^+$ T cells, killing these cells upon division. Indeed, ablation of replicating T-cells resulted in a 55% reduction of lesion development with fewer macrophages and $CD4^+$ T cells detectable in the atherosclerotic lesions.[15]

These findings corroborate the crucial role of T-cells in atherogenesis, for which cell division is apparently essential, and further contribute to the contention that blockade of T-cell division induced by MMF represents a novel anti-atherosclerotic strategy. Even more so considering the fact that MMF may also affect T_{reg} function. Indeed, it has been shown that in diabetic mice receiving islet allografts, treatment with a combination of MMF and the active form of vitamin D_3 resulted in increased frequency of $CD4^+CD25^+$ regulatory cells.[16] Although this study suggests MMF may enhance T_{reg} generation, there was no evidence this was solely due to MMF. Moreover, the effect of MMF on the level of FoxP3 expression, which is a key regulatory gene for the development of T_{reg},[17] was not evaluated. Recently however, MMF was shown to protect murine T_{reg} function, expansion as well as FoxP3 expression when administered after allogeneic bone marrow transplantation.[18] In addition, MMF may also attenuate recruitment of T-lymphocytes to the subendothelial area. Mechanistically, MMF-mediated depletion of GTP inhibits the transfer of fucose and mannose to glycoproteins.[13] Some of these are adhesion molecules that facilitate the attachment of T-cells to the endothelium and are thus involved in atherogenesis. Indeed, MMF has been shown to interfere with the expression of LFA-1[19] and VLA-4.[20] Finally, MMF can also attenuate T-cell driven progression of the atherosclerotic plaque by inducing apoptosis of T-cells.[21,22]

Thus, in addition to a cytostatic effect on T-lymphocytes, MMF has been shown to protect T_{reg} functioning, reduce expression of adhesion molecules and induce apoptosis of T-cells and may thereby exert potent atheroprotective functions.

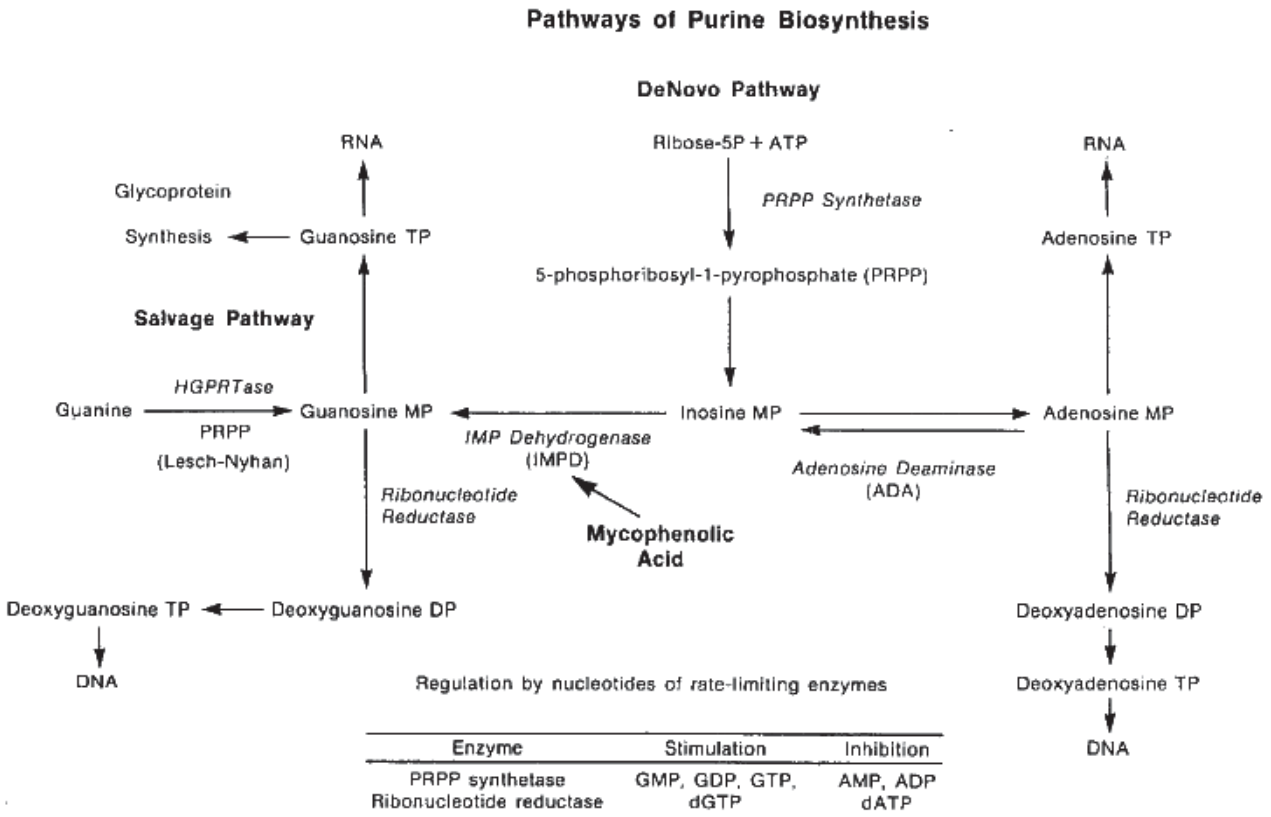

Figure 1. Pathways of purine biosynthesis.
Mycophenolic acid, the active metabolite of MMF, inhibits dehydrogenase, thereby depleting GMP, GTP, and dGTP. Two rate-limiting enzymes in lymphocytes are activated by guanosine ribonucleotides and dGTP, but inhibited by AMP, ADP and by dATP, respectively.

Monocytes/macrophages in atherogenesis

Monocytes/macrophages play a major role in all phases of atherothrombotic disease, ranging from fatty streak formation to rupturing of the vulnerable plaque and subsequent arterial thrombosis. Subendothelial retention of atherogenic apoB-containing lipoproteins is one of the earliest events in atherogenesis.[23] This is followed by oxidative and enzymatic modifications of the LDL particle which results in activation of the endothelium and the expression of leukocyte adhesion molecules. Monocytes attach to the endothelium and the subsequent migration to the subendothelial area is facilitated by chemokines such as monocyte chemoattractant protein-1 (MCP-1). Indeed, hypercholesterolemic mice genetically deficient for either MCP-1[24]or its receptor CCR2,[25] showed a reduction of atherosclerotic lesion size by approximately 60%–70% and 50%, respectively. In the course of atherogenesis, monocytes differentiate into macrophages and this is characterized by changes in gene expression, such as upregulation of several scavenger receptors. Via these receptors, macrophages internalize modified LDL which ultimately transforms them into foam cells. Macrophages produce a wide variety of mediators that contribute to the pro-inflammatory milieu of the subendothelium, plaque destabilization and thrombus formation. For instance, macrophage derived myeloperoxidase (MPO) modifies LDL into an atherogenic form, compromises the anti-atherogenic function of HDL and, by catalytic consumption of nitric oxide (NO), promotes endothelial dysfunction. Moreover, MPO generates numerous reactive oxidants and diffusible radical species that increase oxidative stress and promote plaque instability.[26]

Monocytes/macrophages and MMF

Similar to the effect seen in T-lymphocytes, MMF lowers the level of GTP in monocytes[13] and has also been shown to affect monocyte/macrophage functioning. MMF also modulates the expression of adhesion molecules[13,27] that are involved in the transmigration of monocytes/macrophages to the subendothelium. Indeed, treatment of monocytes with MMF (10 μg/ml) reduced the binding of monocytes to human umbilical vein endothelial cells (HUVEC) by 30 %. In addition, MMF was shown to

attenuate upregulation of ICAM-1- and MHC-II-expression on monocytes stimulated with interferon-γ, a Th1 cytokine with a central role in atherogenesis.[28] MMF may further reduce recruitment of monocytes/macrophages to inflammatory sites such as the atherosclerotic plaque by attenuating their subsequent chemokine-mediated migration from the circulation.

Indeed, in a study with streptozotocin-induced diabetic rats, MMF treatment was able to prevent increased expression of MCP-1 when compared to untreated diabetic rats.[29] These findings were corroborated by other studies where MMF treatment attenuated pulmonary MCP-1 overexpression in Long-Evan rats after ischemia-reperfusion injury of the lung[30] and in MRL/lpr mice.[31]

Interestingly, in various tumor cell lines, MMF was also able to down regulate the expression profile of several chemokine receptors.[32] In line with this, animal studies have confirmed that MMF can reduce the recruitment of monocytes/macrophages to sites of inflammation. In rats that underwent subtotal nephrectomy for instance, MMF treatment showed a four-fould reduction in macrophage infiltration in glomeruli and interstitium.[33] In this study, MMF also significantly attenuated lipid peroxidation, which also plays a pivotal role in atherogenesis. This is corroborated by other studies which showed that MMF treatment reduced macrophage infiltration as well as oxidative stress in hypertensive[34,35] or diabetic rats.[29] Moreover, MMF treatment reduced MPO production in the lungs of rats that were subjected to ischemia-reperfusion injury.[30] Finally, MMF may further downgrade the pro-inflammatory effects of macrophages by inducing apoptosis of these cells.[21]

The additive effect of all of these individual anti-inflammatory actions of MMF on T-cells as well as on macrophages can be expected to exert potent anti-atherosclerotic effects.

The Endothelium in atherogenesis

In the course of atherogenesis, the endothelial barrier between circulation and arterial wall constitutes the interface that several major components of the atherosclerotic plaque need to cross. As mentioned above, entrapment of LDL-cholesterol and the

subsequent oxidation thereof activates endothelial cells which then upregulate leukocyte adhesion molecules. The following step, tethering and rolling of leukocytes along the endothelium, is mediated predominantly by the transient interaction of selectin molecules present on both leukocytes and endothelial cells.[36] Firm adhesion to the endothelial monolayer is induced by intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAM-1), as well as some of the integrins.[37] Subsequent transmigration to the subendothelium is facilitated by various chemokines. Under physiological circumstances, the endothelium protects against plaque formation not only by reduced or absent expression of adhesion molecules but also by producing mediators of vasodilation such as nitric oxide (NO). A reduction in NO production results in impaired vasodilation and can be considered as one of the earliest signs of atherosclerosis. NO is formed from the amino acid L-arginine by one of the three isoforms of nitric oxide synthase (endothelial NOS, inducible NOS and neuronal NOS). Tetrahydrobiopterin (BH_4) is a cofactor for the catalytic conversion of L-arginine to NO by NOS. For iNOS, a direct relation between BH_4 concentration and NO production has been demonstrated. Most cellular components of the atherosclerotic plaque such as SMC, endothelial cells and macrophages have the capacity to express iNOS upon inflammatory or radical stimuli.[38] Upon expression, iNOS produces NO in the nanomolar range, whereas the constitutive eNOS is active only at the picomolar range. At high concentrations and particularly in the presence of increased radical stress, increased NO concentration avidly react with superoxide to form peroxynitrite. The latter is one of the most potent radicals, which has the capacity to nitrosylate a wide number of enzymes and proteins.[38,39]

Endothelium and MMF

Similar to the anti-atherogenic effects described for T-cells and monocytes, MMF can also alter the expression profile of adhesion molecules in endothelial cells. Indeed, MMF treatment was shown to prevent upregulation of P-selectin,[40,41] E-selectin,[40,41] VCAM-1[41,42] and ICAM-1[,42,43] in HUVEC *in vitro* although some studies report conflicting results.[44] In addition, MMF treatment of a HUVEC monolayer attenuated

the number of penetrating T cells.[41] Interestingly, the capacity of MMF to protect the endothelium against atherogenesis may also lead to beneficial effects on vascular tone. By reducing intracellular GTP levels, MMF can reduce intracellular levels of BH_4.[45] Indeed, treatment of endothelial cells with MMF attenuated iNOS activity. Moreover, basal NO production, mediated by eNOS, was not affected.[46] In line with this, MMF treatment of endothelial cells induced a significant decrease in mRNA expression and release of the vasoconstrictor endothelin-1 (ET-1)[47] as well as a significant increase in the release of the vasodilator prostacyclin (PGI_2).[48]

MMF may thus exert atheroprotective effects on the endothelium by inhibiting the expression of adhesion molecules and by promoting vasodilation.

Conclusion

Clinical experience with MMF stems primarily from auto-immune disorders and transplantation medicine. Interestingly, MMF has already been shown to reduce transplantation arteriosclerosis.[49,50] Although this is considered a distinct disease entity, certain pathophysiological aspects correspond with those of non-transplant atherosclerosis. Indeed, MMF may have various anti-atherogenic effects on major components of the atherosclerotic plaque such as T-cells, monocytes/macrophages and endothelial cells (figure 2). The beneficial potential of MMF is supported by additional atheroprotective effects of MMF on other components of the atherosclerotic plaque. For instance, MMF has already been shown to inhibit proliferation of smooth muscle cells,[50] the maturation of dendritic cells as well as their ability to activate T-cells.[51] These anti-atherogenic effects of MMF are discussed elsewhere.[52] In addition to this, MMF has also been reported to inhibit the aggregation of platelets,[53] although the effect of MMF on coagulation responses requires further elucidation. The net result of all these actions may translate into attenuation of subendothelial cross-talk between leukocytes thereby interrupting the self-perpetuating pro-inflammatory environment within the arterial wall. Indeed, in several animal studies MMF treatment inhibited the atherosclerotic process[54,55] as discussed more elaborately elsewhere in this edition of *Lupus*.

Clearly, when considering MMF as a candidate for anti-atherogenic therapy, the safety profile needs to be taken into account. Although gastrointestinal side effects are present in 20-30% of patients taking MMF doses of two grams, MMF is generally well tolerated.[56,57] In fact, in transplantation medicine gastrointestinal side effects are usually limited to higher doses of MMF and are significantly reduced at lower doses of MMF.[58] In addition, MMF has no effect on classical risk factors of atherosclerosis (i.e. lipids, blood pressure),[59] is not associated with wound-healing complications[60] nor with an increased risk of lymphoma or other malignancies.[61]

Whether MMF truly constitutes a silver bullet in atherogenesis remains to be demonstrated. However, there is an abundance of *in vitro* and *in vivo* studies that demonstrate anti-atherogenic effects of MMF. Studies that evaluate the anti-atherogenic potential of MMF in humans are needed.

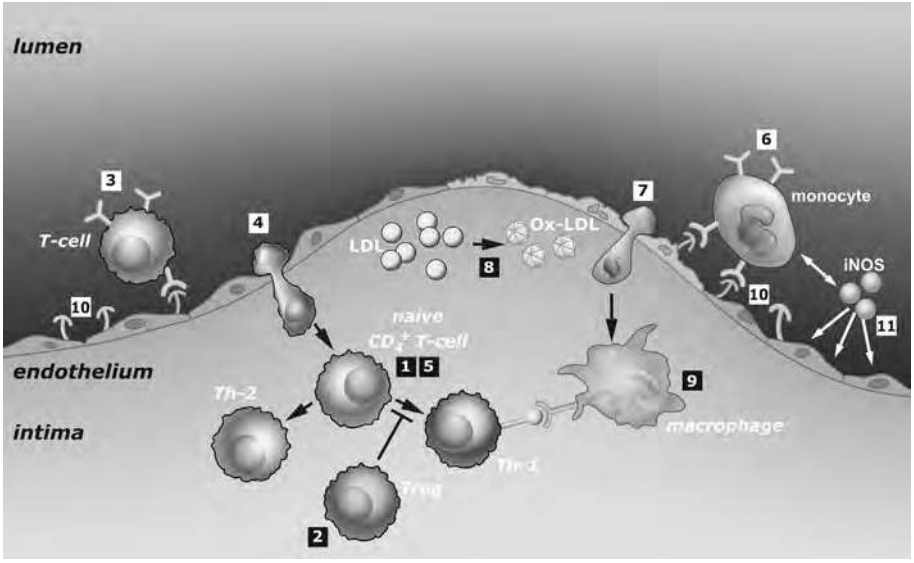

Figure2. Schematic representation of the anti-atherogenic effects of MMF.

MMF exerts anti-atherogenic effects on *T-cells*; inhibition of proliferation (1), preservation of T_{reg} function (2), downregulation of adhesion molecules (3), inhibition of recruitment (4), induction of apoptosis (5), *monocytes/macrophages*; downregulation of adhesion molecules (6), inhibition of recruitment (7), reduction of oxidative stress (8), induction of apoptosis (9), *endothelium*; downregulation of adhesion molecules (10), downregulation of iNOS expression (11).

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Chapter 15

Mycophenolate mofetil attenuates plaque inflammation in patients with symptomatic carotid artery stenosis

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Abstract

Background: Atherosclerotic vascular disease is increasingly considered a manifestation of a chronic inflammatory process. Therefore, immunomodulation holds promise as an anti-atherosclerotic strategy. Hence, we decided to evaluate the impact of immunosuppression on plaque characteristics in patients with symptomatic carotid artery stenosis.

Methods and Results: Twenty-one patients were randomized to receive treatment with either 1000 mg. mycophenolate mofetil (MMF) or placebo for at least 2 weeks prior to undergoing carotid endarterectomy (CEA). The serial sections of the CEA specimens were immunostained for activated T-cells ($CD3^+CD69^+$), regulatory T-cells ($CD3^+FOXP3^+$) and macrophages (CD68). In addition, gene-expression profiling was performed by Illumina gene-array.

Immunostaining revealed a reduction of activated T-cells in nine MMF-treated patients compared to twelve placebo treated control patients (19.7% vs 28.1%;$p<0.05$) as well as an increase of regulatory T-cells (3.8% vs 1.8%; $p=0.05$). Micro-array analyses confirmed beneficial changes to plaque phenotype, showing a strongly reduced expression of pro-inflammatory genes. Also profound reduction in expression of metalloproteinases and osteopontin was present in three out of nine MMF-treated patients compared to nil in the placebo group.

Conclusions: In the present study we show that immunosuppressive treatment for only two-and-a-half weeks pre-CEA elicits changes in the plaque phenotype of symptomatic patients. These changes include reduced inflammatory cell presence with a concomitant decrease in pro-inflammatory gene expression.

Introduction

Atherosclerotic vascular disease might be regarded as an unchecked inflammatory response directed against the deposition of oxidized lipids in the arterial wall.[1, 2] As a consequence, the cornerstone of prevention and treatment of atherosclerosis consists of reducing low density lipoprotein cholesterol (LDL) levels by means of statin therapy.[3, 4] Large clinical trials, however, have revealed that statin use reduces the risk of major coronary events by only 30%[5], in spite of intensive therapeutic regimens.[6, 7] Attempts to further improve the prevention and treatment of atherosclerosis by elevating high-density lipoprotein cholesterol (HDL) levels have thus far led to disappointing results.[8-10] In contrast, the additive impact of C-reactive protein (CRP)-lowering on top of LDL lowering[6, 11] has emphasized the promise of dedicated anti-inflammatory strategies as a means to further reduce cardiovascular disease burden. In support, a variety of experimental studies has corroborated that attenuation of leukocyte invasion into the arterial wall reduces the formation of atherosclerotic lesions.[12, 13] In humans, data is also accumulating to show that accelerated atherosclerosis is a hallmark of chronic inflammatory disease states[14, 15], whereas suppression of inflammatory activity attenuates this association. To date, however, no data are available demonstrating the direct anti-atherosclerotic effect of immune-modulating interventions in humans.

Mycophenolate mofetil (MMF) is an inhibitor of the enzyme inosine monophosphate dehydroxygenase (IMPDH) and has a strong cytostatic effect on T-cells by interfering with DNA synthesis in activated T-cells.[16] Since T-cells play a prominent role in the pathophysiology of atherosclerosis[1], MMF has been suggested to exert beneficial effects on the progression of atherosclerosis.[17] This is corroborated by the observation that exclusive ablation of dividing T-cells, by virtue of an activated suicide gene, was shown to result in a 55% reduction of lesion development in mice.[18] In addition to its effects on T-cells, MMF exerts beneficial effects on other cellular components of the atherosclerotic plaque, such as macrophages and endothelial cells.[17] As a proof-of-concept, we evaluated whether short-term treatment with MMF in symptomatic

patients scheduled for carotid endarterectomy was associated with altered cellular infiltration and/or changes in plaque inflammatory activity, as assessed with mRNA expression profiling.

Methods

Study design

Consecutive patients with a carotid artery stenosis (>70% diameter stenosis on angiography) as defined by the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria combined with an ipsilateral vascular event (transient ischemic attack and/or cerebrovascular attack) were prospectively included at the outpatient department of Vascular Surgery at the Sint Antonius Hospital in Nieuwegein, the Netherlands. Patients were randomized to receive either MMF 1000 mg or a placebo and were treated until the day before surgery. Patients and investigators were blinded to study group assignments. Serial sections of the obtained atherosclerotic plaques were stained for collagen, cholesterol and smooth muscle cell content as well as immunostaining for macrophages (CD68), activated T-cells ($CD3^+CD69^+$) and regulatory T-cells ($CD3^+FOXP3^+$). In addition, gene-expression profiling was performed by Illumina gene-array. Data of immuno-histochemical analyses are presented as mean ± SD. An unpaired, two-tailed *t* test was used to compare the 2 groups. The anterior for statistical significance was set at alpha ≤ 0.05. Statistical analysis was performed using the SPSS statistical analyses software package (version 15.0 for Windows).

Statistical analysis of the gene array data was carried out by using scripts in R/Bioconductor for quantile normalization followed by the one-way Anova test with a Bayesian algorithm, including a Tukey posthoc group comparison.[19, 20] Anova criteria for significant differences were a False Discovery Rate (FDR) < 25% (Benjamini/Hochberg multiple-testing correction) and a posthoc p-value <0.05 with a >2-fold difference in average gene expression level per group. Gene set enrichment analysis

(GSEA) (MSigDB version 2.5) was used for pathway and functional analysis of differentially expressed genes as described.[21] Microarray data have been submitted to the Gene Expression Omnibus GEO under accession no. GSE13922.

The Institutional Review Board of the Sint Antonius Hospital Nieuwegein approved the study, and written informed consent was obtained prior to enrollment. This study is registered as an International Standardised Randomised Controlled Trial, number ISRCTN84092396 and was conducted in compliance with Good Clinical Practise guidelines. Details of biochemical analysis, tissue processing, histology, immunohistochemistry, quantification methods and mRNA gene expression analyses are available in the supplemental methods.

Results

Baseline characteristics and safety profile of MMF treatment

A total of 21 patients participated in this study, 9 of whom were randomly assigned to MMF treatment and 12 to placebo treatment. One patient of the placebo group was excluded because of technical difficulties obtaining a specimen of the atherosclerotic plaque. Table 1 summarizes the demographic characteristics and lipid profiles of both groups. MMF, although, generally well tolerated, may lead to side effects, consisting of gastrointestinal (GI) discomfort and, sporadically, bone marrow suppression. Therefore, we monitored the onset of any such side effects. During the short-term treatment period hematological parameters remained stable and none of the patients developed opportunistic infections (data not shown). One patient treated with MMF suffered from mild abdominal discomfort which, however, did not require dose adjustment.

Table 1. Baseline characteristics

	Placebo (n=12) Mean ± (SD)	MMF (n=9) Mean ± (SD)
Treatment duration, days	16.7 (2.7)	17.1 (2.9)
Age, y	67.1 (10.0)	59.8 (8.0)
Male/Female	8 / 4	7 / 2
BMI, kg/m^2	27.3 (1.4)	27.5 (1.8)
Systolic BP, mmHg	138.8 (12.6)	135.6 (18.3)
Diastolic BP, mmHg	80.8 (6.3)	82.8 (9.4)
Glucose, mmol/L	5.6 (1.0)	5.1 (0.4)
TC, mmol/L	4.7 (1.1)	4.9 (1.0)
LDL, mmol/L	2.7 (0.7)	2.6 (1.0)
HDL, mmol/L	1.5 (0.4)	1.5 (0.3)
TG, mmol/L	1.0 (0.3)	1.6 (0.7)
Diabetes Mellitus, %	25.0	11.1
Current smoker, %	33.3	44.4
Statin use, %	66.7	55.6
Aspirin use, %	75.0	88.9
Antihypertensives use, %	58.3	55.6

Abbreviations: BMI, body mass index; TC, total cholesterol; LDL, low-density cholesterol; HDL, high-density cholesterol; TG, triglycerides.

Effect of MMF on plaque phenotype

The percentage lipid staining of the intimal surface area of the atherosclerotic plaque was 36.6% ± 11.7% in patients treated with the placebo and 34.6% ± 10.5% in the patients treated with MMF (p=0.69, Figure 1A-B). Similarly, treatment of MMF did not induce significant changes in the percentage collagen surface area of atherosclerotic plaques compared to patients receiving placebo treatment (9.8% ± 6.1 % versus 11.5% ± 5.0%, respectively; p=0.49, Figure 1C-D). Finally, the percentage surface area of intimal smooth muscle cells in plaques of patients receiving the placebo was comparable to that of MMF-treated patients (0.029% ± 0.018% versus 0.024% ± 0.010%), respectively; p=0.417, Figure 1E-F).

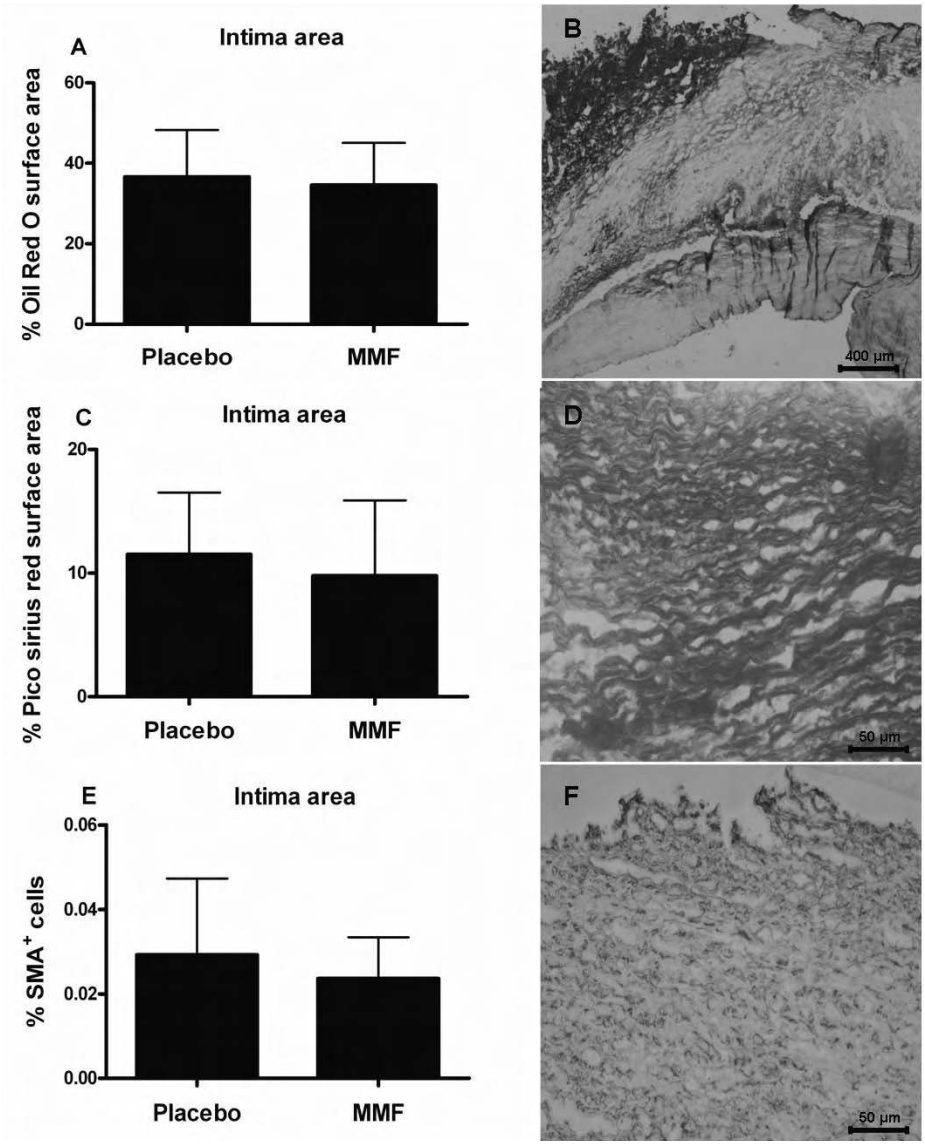

Figure 1. Percentage oil red O stained surface area for lipids (A), percentage Picosirius Red stained surface area for collagen (C) and percentage SMA-1 stained surface area for vascular smooth muscle cells (E) of total intima area in atherosclerotic tissue of patients treated with either placebo or MMF. Representative sections showing an oil red O staining (B), a Picosirius red staining (D) and a SMA-1 staining (F).

Effect of MMF on leukocytes

The percentage surface area of macrophages in atherosclerotic plaques from patients receiving placebo (0.040% ± 0.026%) was similar to that found in the plaques from patients randomized to MMF treatment (0.026 % ± 0.015%, p=0.17, Figure 2A-B). In contrast, the percentage of $CD3^{+}$T-cells expressing the activation marker CD69 was lower in plaques from subjects receiving MMF (19.7% ± 7.9%) as opposed to plaques from subjects receiving placebo (28.1% ± 6.2%, p=0.01, Figure 2C-D). Concomitantly, the percentage of regulatory T-cells (FOXP3 / CD3 x 100) was increased in plaques obtained from the group receiving MMF. (3.79% ± 2.51% versus 1.83% ± 1.76%; p = 0.05, Figure 2E-F).

Effect of MMF on microarray mRNA expression profiling

To gain insight into the processes affected by MMF treatment in the carotid lesions, we performed global genome-wide mRNA expression profiling on complete lesions. Although transcriptome changes in individual cell types will be masked by the multicellular nature of these lesions, this profiling will yield information on the global effects of MMF on the lesions' inflammatory and remodeling processes. We performed a statistical comparison of all the MMF-treated patients with the placebo-treated patients, and found genes with statistically significant differences between these groups. However, the genes showing a trend towards differential expression between MMF and the placebo displayed an altered mRNA expression level in three out of the nine MMF-treated patients (Figure I, available online at http://circinterventions.ahajournals.org). Therefore, within the subsequent statistical transcriptome analyses the 'high responders' (n=3) and 'low responders' (n=6) were compared separately to the placebo. These separate comparisons showed a differential response between the MMF-treated subgroups. Representative expression profiles of these separate statistical comparisons are given in figure 3. MMF predominantly represses gene expression in the high responders whereas global gene expression is unaltered in low-responders compared to placebo. The genes which showed an altered expression level in the low-responders did not overlap with the high-responders (Table IA-B, available online at http://circinterventions.ahajournals.org).

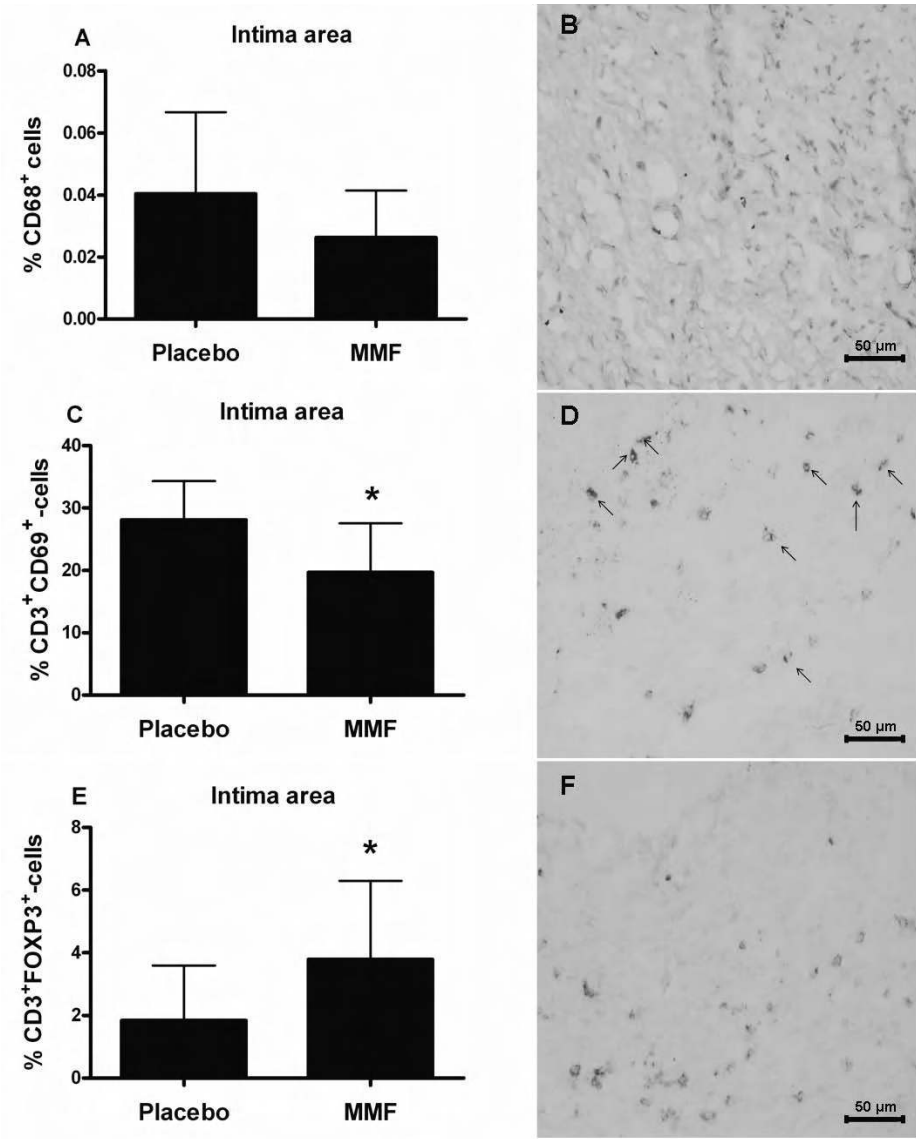

Figure 2. Percentage $CD68^+$ cells for macrophages of total intima area (A), percentage of $CD3^+CD69^+$ cells for early activated T-cells (C) and percentage $CD3^+Foxp3^+$ cells for regulatory T-cells(E) of total $CD3^+$ cells in intima of atherosclerotic tissue of patients treated with either placebo or MMF. * $p \leq 0.05$ vs placebo. Representative sections showing a $CD68^+$ staining (B), a $CD3^+CD69^+$ staining (D), and a $CD3^+Foxp3^+$ staining (F).

Effects of MMF on plaque composition and cellular / functional pathways

The microarray transcriptome data show that the intra-plaque expression levels of marker genes for several inflammatory cell types, being macrophages, dendritic cells and T-cells, $CD4^+$ and $CD56^+$ NK, are all decreased in the plaques of high responders to MMF treatment, as compared to those in the plaques of patients who received the placebo (Table 2). In low responders, however, these overall levels did not deviate significantly from the placebo group. Several vascular smooth muscle cell (VSMC) marker genes have increased expression levels in the high-responders to MMF treatment (Table 2). These data imply that MMF treatment altered the mRNA profile of inflammatory cell types and VSMC in the plaque, but only in the patients showing a pronounced response to MMF treatment. Analysis of the functional pathways affected by MMF treatment, revealed a number of dominant effects. First, genes involved in extracellular matrix (ECM) and its remodeling were severely repressed in high responders. The mRNA expression level of 11 important atherosclerotic plaque destabilizing ECM degrading enzymes, including matrix metalloproteinases (MMPs) and cathepsins had a decreased expression, as shown in Table 2. The most dominant change was > 20-fold decrease in osteopontin (OPN), whereas spondin-1 (SPON1) increased > 4-fold, consistently in the high-responder group only (Fig 3C-D). Second, many genes involved in lipid metabolism where affected, in accordance with a decreased content of foam-cell macrophages. These included atherosclerosis-associated genes like ApoE, PPARγ, ABC-G1 and ABC-A1 (Table 2).

Table 2: Effect of MMF treatment on functions and cell-types in the high responders

Function / celltype	Gene Symbols and fold-difference MMF versus Placebo
Lipid metabolism N=10 genes	CD36 -11.0, ApoC1 -7.0, ApoE -5.5, PPARG -5.2, ABCG1 -4.7, LPL -4.2, VNN1 -4.1, ABCA1 -2.9, GLA -2.8, SCD -2.3
Extracellular matrix N=11 genes	SPP1 -22.5, MMP9 -17.6, CD36 -11.0, MMP7 -9.5, MMP12 -9.4, CTSB -9.2, CTSC -7.0, CTSS -5.9, LAMC3 +2.2, NPNT +3.2, SPON1 +4.2
CD14+ monocyte / macrophage N=82 genes	SPP1 -22.5, CCL3 -19.9, MMP9 -17.6, FPRL2 -14.5, CD74 -11.6, FCER1G -10.8, AQP9 -9.6, MMP12 -9.4, CTSB -9.2, VSIG4 -8.1, TYROBP -7.7, UCP2 -7.4, CD163 -7.2, CTSC -7.0, CD14 -6.9, MARCO -6.5, EVI2B -6.4, LIPA -6.4, FBP1 -6.4, LY86 -6.3, SNX10 -6.3, IGSF6 -6.3, AIF1 -6.3, PLAUR -6.1, CTSS -5.9, CD84 -5.7, SGK -5.2, RNASE6 -5.1, MAFB -5.1, SLC31A2 -5.0, SMPDL3A -4.8, SLAMF8 -4.8, PLEK -4.7, KYNU -4.7, TBXAS1 -4.6, CD68 -4.6, CD53 -4.3, EMR2 -4.3, SLC1A3 -4.2, VNN1 -4.1, CTSZ -4.1, GM2A -4.0, LCP2 -3.9, HCLS1 -3.9, LPXN -3.9, P2RX7 -3.8, WAS -3.8, HLA-DOA -3.7, STXBP2 -3.6, GK -3.6, GLRX -3.4, MAN2B1 -3.4, SLC7A7 -3.3, MFSD1 -3.3, PGD -3.2, GRN -3.2, MAPKAPK3 -3.1, TREM1 -3.1, FPR1 -3.1, SLC29A3 -3.1, CSF1R -3.0, MSR1 -2.9, S100A9 -2.8, CAPG -2.8, MPP1 -2.8, RNASET2 -2.8, SLC38A6 -2.8, MYO1F -2.7, MNDA -2.6, C3AR1 -2.6, BATF -2.6, GMIP -2.5, EFHD2 -2.5, TPP1 -2.5, DOK2 -2.5, BID -2.4, LAIR2 -2.4, SLC16A3 -2.3, CSF2RA -2.3, TM7SF4 -2.3, ADAMDEC1 -2.2, ACP2 -2.1
BDCA4+ Dendritic Cells N=28 genes	RGS1 -17.0, HLA-DPA1 -14.7, CYBA -8.1, SLC15A3 -4.9, CD37 -4.6, IRF8 -4.5, TNFRSF21 -4.3, ALOX5 -4.3, DOCK2 -4.1, CECR1 -4.0, MS4A6A -3.9, HLA-DMB -3.5, PSCD4 -3.2, SYK -2.9, HLA-DMA -2.9, CYBB -2.9, ALOX5AP -2.7, C13orf18 -2.7, LILRA2 -2.5, NCF4 -2.5, OAS1 -2.5, BLNK -2.3, TLR7 -2.3, IRF7 -2.2, PRKCB1 -2.2, OPN3 -2.1, KIAA0746 -2.1, GPM6B +2.5
CD56+ NK T-cells N=20 genes	SERPINA1 -9.3, RAC2 -4.8, FGR -4.3, ARHGAP2 -4.1, TNFRSF1B -3.9, ITGAM -3.8, LCP1 -3.5, C5AR1 -3.1, CXorf9 -3.0, LST1 -2.8, TMC6 -2.7, FLJ14213 -2.6, STK10 -2.5, RASSF4 -2.5, CSK -2.4, INPP5D -2.2, SIPA1 -2.1, PLCG2 -2.1, NCALD +2.0, PPP2R2B +2.4
VSMC N=6 genes	MAP1B +2.1, CNN1 +2.1, SGCE +2.3, SFRP1 +2.5, DPFZP586H2123 +2.7, ENAH +3.1
Leukocytes N=7 genes	CMFG -4.5, SLA -4.4, HLA-B -4.4, ADAM8 -4.4, TREM2 -4.3, HLA-DRB3 -4.3, S100A8 -3.9
T cells N=5 genes	CXCR4 -5.4, FYB -4.1, GIMAP6 -3.0, CD4 -2.7, OAS2 -2.5

Marker genes for the different cell types are based on the Novartis human transcriptome Atlas.[50] See supplement Table IA for the complete set of 541 genes with expression levels altered by MMF treatment.

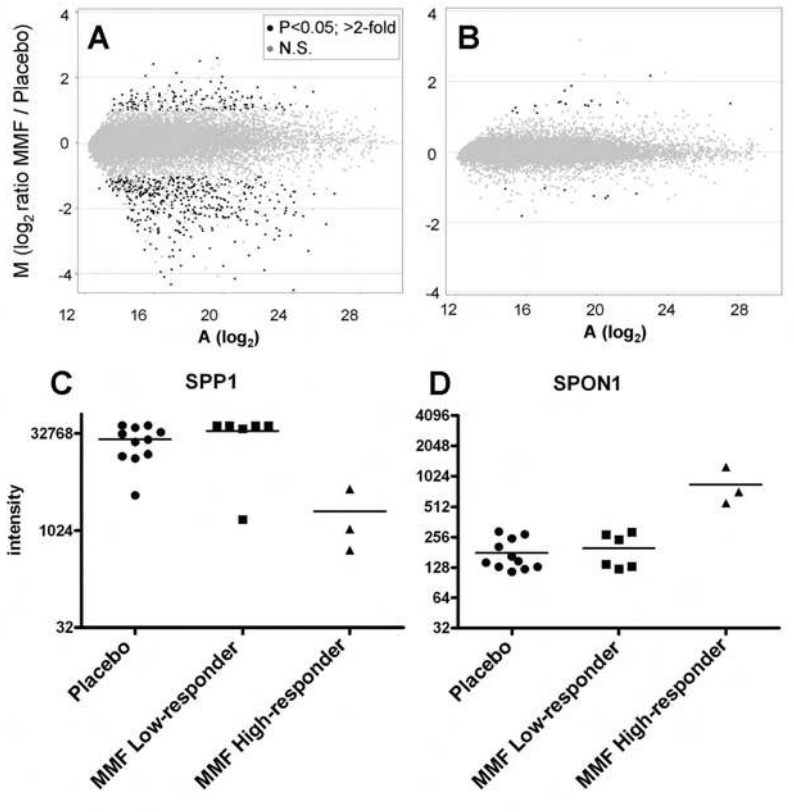

Figure 3. Microarray transcriptome analysis led to the identification of two patient subgroups with either a high or a low response to MMF treatment.

Within these M versus A plots the black dots indicate genes with differential expression levels between MMF and Placebo treatment. A) In the three patients that showed a high response to MMF, treatment altered the mRNA expression level of N=541 genes. Within these patients MMF treatment decreased the expression level of the majority of these genes (N=403 decreased; N=138 elevated), compared to placebo. In contrast, none of these N=541 genes had a similarly changed expression level in the six patients with a low response to MMF. B) N=25 genes were differentially expressed between these low responders and the placebo treated patients (N=17 genes up in MMF; N=8 genes down in MMF). The individual genes with differential expression between MMF and placebo are given in Table IA for High responders and in Table 1B for Low responders (supplemental data). C-D) Representative dot-plots of SPP1 and SPON1 in placebo, high and low-responders to MMF treatment.

Discussion

In the present study we show that immunosuppressive therapy with MMF in patients with manifest cardiovascular disease leads to a reduced number of activated T-cells with a concomitant increase of regulatory T-cells in the carotid plaques. This anti-inflammatory profile was further supported by the reduced expression of inflammatory cell type marker genes as well as reductions of metalloproteinase genes, including osteopontin and MMP9. These observations imply that immunomodulation per se holds promise in the treatment of symptomatic atherothrombotic disease.

Activated T-cells are reduced in the atherosclerotic plaques of MMF treated patients. CD69 is the earliest inducible cell surface glycoprotein that is expressed upon lymphocyte activation. It is triggered by various pathways, including the T-cell receptor and IL-2 stimulation. Whereas there is no CD69 expression on resting lymphocytes, it is abundantly expressed in activated inflammatory cells and during active immune responses *in vivo*. MMF has a strong cytostatic effect on activated T-cells[16], which concurs with the lower CD69 expression observed in plaques of MMF-treated patients in the present study. However MMF has also been shown to reduce many pro-inflammatory cytokines, including IL-2, a potent CD69 expression molecule, providing a secondary pathway to explain our observations. Interestingly, a recent study in apoE$^{-/-}$mice suggested that upregulation of CD69 induces the activation of a large pool of T-cells during atheroma development.[18] Moreover, CD3^{+}CD69^{+} percentage in atherosclerotic plaques increases with the severity of the coronary syndrome in humans.[22] The reduced CD69 expression following MMF treatment may therefore indicate an interruption of the inflammatory activation cycle within the arterial wall. In fact, antibodies against CD69 significantly inhibited the ability of T-cells to activate macrophages[23], indicating that downregulation of CD69 expression impairs the activation-signaling pathway for macrophages. Although we did not observe a reduction in the overall number of tissue macrophages in the present study, a beneficial impact on both numbers as well as activation status of macrophages can be expected following prolonged treatment with MMF.

Higher percentage of regulatory T-cells in MMF treated patient group

The importance of regulatory T-cells in the development and progression of the atherosclerotic plaque has now been firmly established in mice and man alike.[24] Regulatory T-cell deficiency was shown to enhance atherosclerotic lesion development in $LdLr^{-/-}$ mice, and regulatory T-cell depletion using an anti-CD25 antibody also enhanced atherosclerosis in $apoE^{-/-}$ mice.[25] In contrast, adaptive transfer of $CD4^{+}CD25^{+}$ regulatory T-cells reduces atherosclerotic lesions in the $apoE^{-/-}$ model.[25, 26] Recently, a debate has arisen concerning the potential of stimulating regulatory T-cells as a therapeutic approach for atherosclerosis and other auto-immune diseases.[13] In diabetic mice receiving islet allografts, treatment with a combination of MMF and the active form of vitamin D_3 resulted in increased frequency of $CD4^{+}CD25^{+}$ regulatory cells.[27] Although this study suggested that MMF may enhance T_{reg} generation, there was no evidence that this was solely due to MMF. Recently however, MMF was shown to protect murine T_{reg} function, expansion as well as FoxP3 expression when administered after allogenic bone marrow transplantation.[28] In the current study we show increased arterial wall content of T_{regs} in MMF treated patients which may also be associated with attenuated atherogenesis during prolonged follow-up.

mRNA expression profiling indicates a less inflammatory plaque phenotype in MMF responders

When the endarterectomy transcriptomes from treated and untreated patients were compared employing Baysian ANOVA with multiple testing corrections, no genes with differential expression were found that had within-group consistency. This was caused by the fact that transcriptomes of 6/9 MMF-treated patients were almost identical to the transcriptomes of the placebo group. An unsupervised clustering heat map of the top 50 genes with most-differential expression levels between the treated and untreated groups, visualizes the resemblances between the expression patterns of the placebo and the 6 MMF-treated transcriptomes (Figure I). However, transcriptomes of 3/9 MMF-treated atherectomy samples were specifically and reproducibly different from the other placebo and the non-responsive treated samples (Figure 3C-D, Figure

I). Differences between the three high-responders versus the six low-responders could not be attributed to demographic or plaque characteristics. The probability that the difference in these expression profiles is not related to MMF treatment is extremly small for several reasons: First, the response patterns in high-responders are in line with changes in inflammatory cluster marker genes observed following immunosuppressive therapy in endothelial cells following MMF therapy.[29] Second, the expression profiles in the high-responders were strikingly homologous, whereas none of the patients in the control group displayed such an expression profile. Following randomization, it is unlikely that the latter can be attributed to pre-existent differences at baseline. In fact, the relatively small percentage of high-responders is comparable to the response rates reported for MMF treatment in other chronic auto-immune disease states such as inflammatory bowel disease and thrombotic thrombocytopenic purpura.[30, 31] Part of this differential response may pertain to the large variation in bio-availability of MMF between individuals.[32-34] In the present study, we therefore classified high-responders and low-responders in the MMF-treated patients[35] and compared the expression profiles of these groups separately with the expression profiles of placebo-treated patients.

Further microarray transcriptome analysis showed that the intra-plaque levels of combined marker genes for several inflammatory cell types were reduced in the high-responder group. Inflammatory cell infiltration in the atherosclerotic plaque is a hallmark of unstable plaque phenotype[36], which are present in high concentrations following plaque rupture.[37] Together with an upregulation of marker genes of VSMC this indicates a shift towards a more stable, less inflammatory plaque phenotype, supporting the immunohistochemical findings discussed above. Other clusters of marker genes that were significantly altered by MMF treatment were 11 genes coding for extracelluar matrix degrading enzymes. The most potently suppressed gene was OPN, classified as a T-helper 1 cytokine and thus believed to play a role in the exacerbation of chronic inflammatory diseases, including atherosclerosis. In fact, high OPN expression levels have been observed in human atherosclerotic lesions[38]

as well as in animal models.[39] Transgenic OPN overexpression was associated with increased atherosclerotic lesion size[40], whereas OPN deficiency was shown to attenuate atherogenesis.[41] Interestingly, supported by the observation that apoE$^{-/-}$OPN$^{-/-}$ mice had less AII-induced aortic aneurysm formation and decreased MMP-2 and MMP-9 activity compared to apoE$^{-/-}$OPN$^{+/+}$ mice,[42] it has been suggested that OPN plays an important role in MMP regulation and vessel rupture.[43] Interestingly, the high-responder group showed an 18-fold lower expression of matrix metalloproteinase 9 in conjunction with OPN downregulation. MMP-9 is found in high concentrations in atherosclerotic areas and considered to play a major role in the proteolytic weakening of the fibrous cap[44], making it prone to plaque rupture.

Various genes of lipid metabolism clusters were also affected in high-responders. Profound changes were observed in genes associated with atherosclerosis like ApoE, PPARγ, ABC-G1, ABC-A1 and a decreased amount of marker gene expression of foam-cell macrophages was found (Table 2).[1, 45, 46, 47] Thus, in high-responders MMF treatment facilitated changes which can be qualified as reduced unstable matrix characteristics, as well as attenuation of a lipid-rich, inflammatory plaque phenotype, which combined define an unstable atherosclerotic plaque phenotype.

Study limitations

Several limitations of the present study merit consideration. First, our results are based on a limited number of observations. However, in spite of the limited sample size, we did observe significant changes in plaque composition lending further support to the beneficial effect of immunosuppressive regimens, such as MMF. Second, given the nature of international guidelines for performing a CEA in symptomatic patients, they could only receive MMF for a limited time span (< 3 weeks). The latter may have contributed to an underestimation of the true anti-inflammatory effect of MMF at longer exposure, since its ability to reduce transplant arteriosclerosis in humans could only be detected after considerably longer treatment periods.[48] Conversely, it could also lead to an underestimation of the occurrence of side effects.

Clinical Implications

This study provides a first proof of concept for the fact that the inflammatory process in the arterial wall that characterizes the atherosclerotic process in humans can be modified by immunomodulatory therapy. We have to state here that this is based on a small number of observations and needs confirmation in larger studies. A decrease of early T cell activation and an increase in regulatory T-cells after short-term treatment with MMF are compatible with a dampening of the inflammatory process, which may lead to stabilization and/or regression of the atherosclerotic plaque following longer-term MMF exposure. This is in line with mRNA expression profiles of the plaques of the high-responders in whom the plaque phenotype shifted towards a more stable phenotype. Interestingly, Shaw et al. recently showed that infusion of rHDL in 10 patients with claudication 5-7 days prior to atherectomy led to acute changes of lipid content of atherosclerotic plaques[49]. Together with the current changes following short-term treatment with MMF, this re-emphasizes the dynamic state of atherosclerotic plaques in humans and the possibilities of immunotherapy in atherosclerotic disease. In summary, the present observation indicates a potential for immunomodulation in the future prevention of atherothrombotic complications. However, much more robust studies are warranted to evaluate the role of long-term MMF treatment on cardiovascular event rates.

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Disclosures

None.

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Supplement Materials

Supplemental methods

Biochemical Analysis

Blood was collected in EDTA, citrate, heparin anti-coagulated aliquots and serum tubes, which were kept on ice and centrifuged at 1600*g* for 15 minutes at 4°C, snap-frozen, and stored at −80°C until analysis. Plasma total cholesterol was measured with an enzymatic colorimetric procedure (CHOD-PAP; Boehringer Mannheim, Mannheim, Germany). HDL was determined after precipitation of apoB-containing lipoproteins by $MnCl_2$. LDL was calculated using the Friedewald formula. Triglycerides were measured using an enzymatic colorimetric method using lipase, glycerol kinase, and glycerol-3-phosphate 3 oxidase. Hematology parameters were assessed by standard laboratory techniques.

Tissue Processing

The CEA specimens were prepared as previously described.[1] In short, the atherosclerotic plaque obtained during CEA was processed immediately and dissected in parts of 5 mm. All specimens were snap-frozen in liquid nitrogen and stored at −80 °C. Of each lesion, adjacent serial sections were dissected at 5 μm and fixed with acetone; every first section was stained with hematoxylin–eosin for histomorphology, the second and third section with Pico Sirius red stain and Oil red O for visualization of total collagen content and total fat respectively, while the subsequent sections were used for immunohistochemistry.

Immunohistochemistry

For immunohistochemistry, the following monoclonal antibodies were used: polyclonal CD3 for all T-cells (SP7, LabVision, Freemont, CA), CD68 for macrophages (clone EBM11, Dako), CD69 for activated T-cells (clone FN40, Dako), Foxp3 for Treg cells (clone 236A/E7; Abcam, Cambridge, UK) and SMA-1 for vascular smooth muscle cells

(clone 1A4, Dako). As second step reagents we used horseradish peroxidase (HRP) conjugated Powervision anti-mouse (PV a-Ms/HRP), alkaline phosphatase (AP) conjugated Powervision anti-Rabbit (Pv a-Rb/AP), HRP conjugated goat anti-mouse Ig (Gt a-Ms/HRP), Tyramide-fluorescein (Dako, CSA-II kit): Rb anti-fluorescein/HRP (Dako, CSA-II kit). As chromogens, Vector Blue and Nova Red Immunohistochemical single stainings were performed using a two-step technique. After fixation in acetone, endogenous HRP activity was blocked using 0.3% H2O2 and 0.1% sodium azide in TBS. After washing in TBS, ultraV protein block (Labvision), the primary antibodies (CD68 or SMA-1) were sequentially applied to the sections followed by PV a-Ms/HRP. Enzyme activity was detected using VNova Red (Vector Labs, Peterborough, England). Doublestaining with CD3/CD69 was performed by sequentially performing the following steps: acetone fixation, blocking of endogenous peroxidase as described above, UltraV Block, mixture of the primary antibodies (CD3/CD69), PV a-Rb-AP, normal rabbit serum (10%); Gt a-Ms/HRP, tyramide-fluorescein, and Rb anti-fluorescein/HRP. Finally, AP activity was visualized first using Vector Blue, followed by the visualization of HRP activity using Vector Nova Red. Immunodoublestains with CD3 and FOXP3 were performed using a different protocol, in which the following sequence of incubations was performed: fixation in acetone, fixation with neutral buffered formalin (necessary for nuclear antigens), Ultra V block, mixture of the monoclonal (a-FOXP3) and polyclonal (CD3) antibody, mixture of PV a-Ms/HRP and PV a-Rb/AP. Finally, AP activity was visualized using Vector Blue, followed by HRP activity using Vecor Nova Red.

Quantification

All quantification procedures were performed by an observer blinded to treatment allocation.

Frequency of double-stained cells ($CD3^+CD69^+$ and $CD3^+FOXP3^+$) were determined by taking a digital overview image (with a Leica DFC500 camera mounted on a Leica DM5000 microscope (Leica, Germany) of each of the individual sections at low power magnification (2x). In these overview images, 5 areas of interest with the highest

density of CD3 positive cells were selected, of which digital images were acquired at high power magnification (20x) The total numbers of single and positive cells in interrelated images were counted using the "manual tag" option of Image Pro Plus (IPP) image analysis software (vs 5.01, Media Cybernetics, Silver Spring, MD), and the numbers of FOXP3- and CD69-positive cells were expressed as percentages of CD3 positive cells. Images stained with CD68, Picosirius red, Oil Red O and SMA were acquired by taking a digital overview of each of the individual sections at a low power magnification (2x). Immuno-positive areas were segmented with IPP and expressed as a percentage of the total plaque area.

mRNA gene expression analyses

Total RNA was isolated from 5 consecutive 10 μm thick cross-sections of carotid endarterectomy from 20 individual patients (N=9 MMF-treated; N=11 Placebo) by using Trizol (Invitrogen), followed by column purification including DNase-I treatment using RNeasy Micro Isolation (Qiagen). RNAs were amplified to biotinylated cRNAs using Illumina Total Prep Amplication (Ambion). Amplified cRNAs were randomly hybridized to HumanRef-8 Expression bead chip arrays (Illumina), followed by scanning, feature extraction and normalization at ServiceXS (Leiden, The Netherlands), as previously described.[2]

Supplemental references

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Supplement 1A. N=541 genes with differential mRNA expression between good responders to MMF treatment and Placebo

Symbol	Description	celltype signature	Function	Accession	fold difference	FDR (%)	p-value*
SPP1	secreted phosphoprotein 1	CD14+	ECM_regulation	NM_000582.2	-22.50	0.6	4.0E-06
CCL3	chemokine ligand 3	CD14+		NM_002983.1	-19.86	14.9	3.7E-04
MMP9	matrix metallopeptidase 9	CD14+	ECM_regulation	NM_004994.2	-17.59	0.2	2.2E-05
RGS1	regulator of G-protein signalling 1	BDCA4+ DC		NM_002922.3	-16.97	6.1	3.9E-05
CCL3L3	chemokine ligand 3-like 3			NM_001001437.2	-15.31	14.8	6.4E-04
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	BDCA4+ DC		NM_033554.2	-14.71	15.1	1.6E-04
FPRL2	formyl peptide receptor-like 2	CD14+		NM_002030.3	-14.55	15.8	1.6E-04
LYZ	lysozyme			NM_000239.1	-13.46	9.8	5.7E-04
CD74	CD74 antigen, transcript variant 1,CD74 antigen, transcript variant 3	CD14+		NM_001025158.1,NM_001025159.1	-11.63	14.3	7.5E-05
CD36	CD36 antigen, transcript variant 1		ECM_regulation,lipid metabolism	NM_001001548.1	-11.03	6.5	2.9E-03
MS4A7	membrane-spanning 4-domains, subfamily A, member 7, transcript variant 3			NM_206939.1	-10.94	12.0	3.7E-04
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	CD14+		NM_004106.1	-10.75	2.0	1.0E-05
AQP9	aquaporin 9	CD14+		NM_020980.2	-9.63	1.0	2.0E-04
MMP7	matrix metallopeptidase 7		ECM_regulation	NM_002423.3	-9.54	2.0	6.1E-04
MMP12	matrix metallopeptidase 12	CD14+	ECM_regulation	NM_002426.2	-9.39	6.6	9.5E-04
SERPINA1	serpin peptidase inhibitor, clade A, member 1, transcript variant 2	CD56+ NK		NM_001002236.1	-9.26	13.5	1.9E-04
CTSB	cathepsin B, transcript variant 1	CD14+	ECM_regulation	NM_001908.3	-9.18	14.3	1.4E-03
VSIG4	V-set and immunoglobulin domain containing 4	CD14+		NM_007268.1	-8.14	13.6	3.3E-04
CYBA	cytochrome b-245, alpha polypeptide	BDCA4+ DC		NM_000101.2	-8.06	23.7	1.7E-04
TYROBP	TYRO protein tyrosine kinase binding protein, transcript variant 1	CD14+		NM_003332.2	-7.70	2.0	1.5E-05
UCP2	uncoupling protein 2, nuclear gene encoding mitochondrial protein	CD14+		NM_003355.2	-7.37	16.0	1.4E-03
NPL	N-acetylneuraminate pyruvate lyase			NM_030769.1	-7.26	0.6	3.1E-05
CD163	CD163 antigen, transcript variant 1,CD163 antigen, transcript variant 2	CD14+		NM_004244.3,NM_203416.1	-7.15	11.5	1.0E-03
IFI30	interferon, gamma-inducible protein 30			NM_006332.3	-7.11	0.6	3.0E-05
APOC1	apolipoprotein C-I		lipid metabolism	NM_001645.3	-7.02	1.9	1.8E-04
CTSC	cathepsin C, transcript variant 1	CD14+	ECM_regulation	NM_001814.2	-7.01	18.6	3.9E-04
CD14	CD14 antigen	CD14+		NM_000591.1	-6.93	12.2	5.4E-04
SLC11A1	solute carrier family 11, member 1, transcript variant 2			NM_001032220.1	-6.58	1.8	2.8E-04
SDS	serine dehydratase			NM_006843.2	-6.55	11.6	3.5E-03
MARCO	macrophage receptor with collagenous structure	CD14+		NM_006770.3	-6.51	5.6	2.0E-04
EVI2B	ecotropic viral integration site 2B	CD14+		NM_006495.2	-6.45	13.7	5.4E-04
HS3ST2	heparan sulfate 3-O-sulfotransferase 2			NM_006043.1	-6.43	24.5	1.4E-02
LIPA	lipase A, lysosomal acid, cholesterol esterase	CD14+		NM_000235.2	-6.38	15.3	1.5E-04
FBP1	fructose-1,6-bisphosphatase 1	CD14+		NM_000507.2	-6.35	1.0	2.4E-05
HAMP	hepcidin antimicrobial peptide			NM_021175.2	-6.35	5.6	1.7E-04
LY86	lymphocyte antigen 86	CD14+		NM_004271.3	-6.34	0.9	7.0E-05
SNX10	sorting nexin 10	CD14+		NM_013322.2	-6.30	0.6	9.0E-06
IGSF6	immunoglobulin superfamily, member 6	CD14+		NM_005849.1	-6.29	23.4	7.5E-03
AIF1	allograft inflammatory factor 1, transcript variant 3	CD14+		NM_001623.3	-6.29	9.0	3.3E-04
CD300A	CD300a antigen			NM_007261.2	-6.21	6.8	2.9E-04
PLAUR	plasminogen activator, urokinase receptor, transcript variant 2	CD14+		NM_001005376.1	-6.10	3.8	2.6E-04
FLJ22662	hypothetical protein FLJ22662			NM_024829.4	-6.10	2.9	5.2E-05
ITGB2	integrin, beta 2, lymphocyte function-associated antigen 1; macrophage antigen 1 beta subunit)			NM_000211.1	-6.05	0.2	9.0E-06
C15orf48	chromosome 15 open reading frame 48, transcript variant 2			NM_032413.2	-6.02	2.4	9.3E-04
EMILIN2	elastin microfibril interfacer 2			NM_032048.2	-6.01	1.3	1.5E-04
CTSS	cathepsin S	CD14+	ECM_regulation	NM_004079.3	-5.94	2.3	8.9E-05
CD84	CD84 antigen	CD14+		NM_003874.1	-5.71	7.8	4.0E-04
CFD	complement factor D			NM_001928.2	-5.59	12.7	3.4E-03
MAPK13	mitogen-activated protein kinase 13			NM_002754.3	-5.55	1.5	3.0E-05
HAVCR2	hepatitis A virus cellular receptor 2			NM_032782.3	-5.54	0.2	1.0E-05
APOE	apolipoprotein E		lipid metabolism	NM_000041.2	-5.54	1.5	1.6E-04
CXCR4	chemokine receptor 4, transcript variant 1,chemokine receptor 4, transcript variant 2	T cells		NM_001008540.1,NM_003467.2	-5.38	5.5	3.9E-04
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog, transcript variant 1			NM_005360.3	-5.36	12.6	4.2E-04
CD83	CD83 antigen			NM_004233.2	-5.31	2.3	3.2E-04
DSC2	desmocollin 2, transcript variant Dsc2b			NM_004949.2	-5.28	6.6	1.6E-04
PPARG	peroxisome proliferative activated receptor, gamma, transcript variant 1		lipid metabolism	NM_138712.2	-5.20	15.2	4.5E-03
SGK	serum/glucocorticoid regulated kinase	CD14+		NM_005627.2	-5.17	2.7	1.1E-04
OSCAR	osteoclast-associated receptor, transcript variant 3			NM_130771.2	-5.15	5.6	4.1E-04
RNASE6	ribonuclease, RNase A family, k6	CD14+		NM_005615.2	-5.12	12.4	4.6E-04
PKD2L1	polycystic kidney disease 2-like 1			NM_016112.2	-5.12	21.5	5.9E-03
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B	CD14+		NM_005461.3	-5.10	1.2	3.5E-05
DOCK8	dedicator of cytokinesis 8			NM_203447.1	-5.09	3.6	2.3E-05
SLC31A2	solute carrier family 31, member 2	CD14+		NM_001860.1	-5.03	10.6	9.3E-04
CA2	carbonic anhydrase II			NM_000067.1	-5.00	12.1	1.1E-03
TNFSF13B	tumor necrosis factor superfamily, member 13b			NM_006573.3	-4.97	17.1	4.4E-04
IL1RN	interleukin 1 receptor antagonist, transcript variant 1			NM_173842.1	-4.93	1.8	6.1E-03
CPVL	carboxypeptidase, vitellogenic-like, transcript variant 1			NM_031311.2	-4.91	4.2	2.2E-04
SLC15A3	solute carrier family 15, member 3	BDCA4+ DC		NM_016582.1	-4.86	4.2	6.8E-04
CENTA2	centaurin, alpha 2			NM_018404.1	-4.85	1.0	5.8E-05
SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	CD14+		NM_006714.2	-4.84	13.5	1.6E-03
ADFP	adipose differentiation-related protein			NM_001122.2	-4.81	7.4	1.0E-03
RAC2	ras-related C3 botulinum toxin substrate 2	CD56+ NK		NM_002872.3	-4.80	1.1	1.0E-04
SLAMF8	SLAM family member 8	CD14+		NM_020125.1	-4.79	2.0	6.0E-04
SLC2A5	solute carrier family 2, member 5			NM_003039.1	-4.77	23.7	5.5E-03
BIN2	bridging integrator 2			NM_016293.2	-4.77	10.4	4.1E-04
CCL8	chemokine ligand 8			NM_005623.2	-4.74	12.5	9.3E-03
ABCG1	ATP-binding cassette, sub-family G, member 1, transcript variant 4		lipid metabolism	NM_004915.3	-4.73	7.3	7.6E-04
PLEK	pleckstrin	CD14+		NM_002664.1	-4.72	1.7	3.3E-04
GPX1	glutathione peroxidase 1, transcript variant 2			NM_201397.1	-4.70	12.6	1.3E-04
RGC32	response gene to complement 32			NM_014059.1	-4.66	4.2	1.1E-04
KYNU	kynureninase, transcript variant 1	CD14+		NM_003937.2	-4.66	8.3	3.5E-04
CD37	CD37 antigen	BDCA4+ DC		NM_001774.1	-4.64	3.0	1.3E-04
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog			NM_002350.1	-4.63	15.2	3.1E-04
TBXAS1	thromboxane A synthase 1, transcript variant TXS-I	CD14+		NM_001061.2	-4.62	4.9	3.0E-04
CXCL16	chemokine ligand 16			NM_022059.1	-4.60	0.8	5.6E-05
CD68	CD68 antigen	CD14+		NM_001251.1	-4.58	1.3	5.4E-05
CD48	CD48 antigen			NM_001778.2	-4.56	2.9	2.6E-04
GMFG	glia maturation factor, gamma	leukocyte		NM_004877.1	-4.53	1.5	5.9E-05
IRF8	interferon regulatory factor 8	BDCA4+ DC		NM_002163.2	-4.52	2.3	1.2E-04
SPI1	spleen focus forming virus proviral integration oncogene spi1			NM_003120.1	-4.46	0.6	8.0E-06
LILRB3	leukocyte immunoglobulin-like receptor, subfamily B, member 3			NM_006864.1	-4.46	1.6	1.7E-04
IFI27	interferon, alpha-inducible protein 27			NM_005532.3	-4.45	23.4	3.4E-03
HMOX1	heme oxygenase 1			NM_002133.1	-4.45	3.0	1.2E-04
SLA	Src-like-adaptor	leukocyte		NM_006748.1	-4.42	0.2	6.0E-06
HLA-B	major histocompatibility complex, class I, B	leukocyte		NM_005514.5	-4.40	21.7	2.3E-04
RASGRP3	RAS guanyl releasing protein 3			NM_170672.1	-4.39	2.3	1.2E-04
EPB41L3	erythrocyte membrane protein band 4.1-like 3			NM_012307.2	-4.37	1.3	4.0E-05
ADAM8	ADAM metallopeptidase domain 8	leukocyte		NM_001109.2	-4.36	1.7	8.0E-04

TREM2	triggering receptor expressed on myeloid cells 2	leukocyte		NM_018965.1	-4.35	3.8	3.1E-04
HLA-DRB3	major histocompatibility complex, class II, DR beta 3	leukocyte		NM_022555.3	-4.34	3.7	1.3E-04
FGR	Gardner-Rasheed feline sarcoma viral oncogene homolog	CD56+ NK		NM_005248.1	-4.33	0.9	4.3E-05
SAT	spermidine/spermine N1-acetyltransferase			NM_002970.1	-4.32	1.3	3.8E-05
CD53	CD53 antigen	CD14+		NM_000560.2	-4.31	1.0	1.9E-05
EMR2	egf-like module containing, mucin-like, hormone receptor-like 2, transcript variant 1	CD14+		NM_013447.2	-4.31	3.3	1.8E-04
GPR116	G protein-coupled receptor 116			NM_015234.3	-4.29	10.9	1.1E-03
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	BDCA4+ DC		NM_014452.3	-4.27	6.5	1.6E-03
ALOX5	arachidonate 5-lipoxygenase	BDCA4+ DC		NM_000698.2	-4.26	1.3	1.0E-04
LPL	lipoprotein lipase		lipid metabolism	NM_000237.1	-4.23	2.7	2.9E-04
CD72	CD72 antigen			NM_001782.1	-4.23	13.2	4.0E-03
RGS19	regulator of G-protein signalling 19			NM_005873.1	-4.20	13.5	1.4E-04
SLCO2B1	solute carrier organic anion transporter family, member 2B1			NM_007256.2	-4.18	17.9	3.4E-03
CTSL	cathepsin L, transcript variant 2			NM_145918.1	-4.17	10.7	4.5E-03
CD86	CD86 antigen, transcript variant 1			NM_175862.2	-4.16	1.3	2.4E-05
SLC1A3	solute carrier family 1, member 3	CD14+		NM_004172.3	-4.15	6.4	9.6E-04
VNN1	vanin 1	CD14+	lipid metabolism	NM_004666.1	-4.13	15.8	3.2E-03
CTSZ	cathepsin Z	CD14+		NM_001336.2	-4.13	7.1	1.0E-03
SCAP2	src family associated phosphoprotein 2			NM_003930.3	-4.13	6.9	2.4E-04
LAPTM5	lysosomal associated multispanning membrane protein 5			NM_006762.1	-4.12	1.3	1.1E-04
HK3	hexokinase 3, nuclear gene encoding mitochondrial protein			NM_002115.1	-4.12	5.6	3.6E-04
CCL3L1	chemokine ligand 3-like 1			NM_021006.4	-4.11	19.1	7.3E-03
DOCK2	dedicator of cytokinesis 2	BDCA4+ DC		NM_004946.1	-4.11	1.3	7.9E-05
ARHGAP25	Rho GTPase activating protein 25, transcript variant 2	CD56+ NK		NM_014882.2	-4.11	24.6	2.0E-03
FYB	FYN binding protein, transcript variant 2	CD4+ T cells		NM_199335.2	-4.10	1.0	1.4E-05
ARHGAP30	Rho GTPase activating protein 30, transcript variant 2			NM_181720.2	-4.09	1.7	1.3E-04
SLC16A10	solute carrier family 16, member 10			NM_018593.3	-4.03	1.3	2.2E-03
OLR1	oxidised low density lipoprotein receptor 1			NM_002543.2	-4.02	0.2	8.0E-06
CECR1	cat eye syndrome chromosome region, candidate 1, transcript variant 1	BDCA4+ DC		NM_017424.2	-4.02	2.2	2.5E-04
GNA15	guanine nucleotide binding protein, alpha 15			NM_002068.1	-4.01	1.9	1.2E-04
VMO1	vitelline membrane outer layer 1 homolog			NM_182566.1	-4.00	4.2	1.6E-03
GM2A	GM2 ganglioside activator	CD14+		NM_000405.3	-3.99	15.2	2.7E-03
FLJ11259	hypothetical protein FLJ11259			NM_018370.1	-3.96	12.5	5.4E-04
TBC1D2	TBC1 domain family, member 2			NM_018421.2	-3.96	9.4	3.5E-04
VAMP8	vesicle-associated membrane protein 8			NM_003761.2	-3.95	1.7	2.4E-04
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	CD56+ NK		NM_001066.2	-3.94	2.3	1.3E-04
LCP2	lymphocyte cytosolic protein 2	CD14+		NM_005565.3	-3.94	1.3	1.2E-04
SERPINF1	serpin peptidase inhibitor, clade F, member 1			NM_002615.4	-3.93	6.1	1.2E-03
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A, transcript variant 2,membrane-spanning 4-domains, subfamily A, member 6A, transcript variant 3	BDCA4+ DC		NM_022349.2,NM_152851.1	-3.93	4.9	6.5E-04
AMICA1	adhesion molecule, interacts with CXADR antigen 1			NM_153206.1	-3.92	5.6	1.3E-03
HCLS1	hematopoietic cell-specific Lyn substrate 1	CD14+		NM_005335.3	-3.89	0.6	2.9E-05
LPXN	leupaxin	CD14+		NM_004811.1	-3.88	2.5	5.7E-05
C1orf162	chromosome 1 open reading frame 162			NM_174896.2	-3.88	2.4	3.0E-04
PTPN6	protein tyrosine phosphatase, non-receptor type 6, transcript variant 3			NM_080549.2	-3.87	3.3	4.2E-04
S100A8	S100 calcium binding protein A8	leukocytes		NM_002964.3	-3.85	17.1	5.1E-03
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7, transcript variant 1	CD14+		NM_002562.4	-3.82	15.3	2.0E-03
WAS	Wiskott-Aldrich syndrome	CD14+		NM_000377.1	-3.81	0.8	2.8E-05
ITGAM	integrin, alpha M, macrophage antigen alpha polypeptide)	CD56+ NK		NM_000632.2	-3.78	1.3	2.7E-05
CMTM7	CKLF-like MARVEL transmembrane domain containing 7, transcript variant 1			NM_138410.2	-3.75	7.6	4.4E-04
TMEM51	transmembrane protein 51			NM_018022.1	-3.72	2.0	9.5E-05
HLA-DOA	major histocompatibility complex, class II, DO alpha	CD14+		NM_002119.3	-3.72	2.9	4.0E-04
IL10RA	interleukin 10 receptor, alpha			NM_001558.2	-3.70	5.8	9.5E-04
PLAU	plasminogen activator, urokinase			NM_002658.2	-3.69	6.0	2.4E-04
TTYH3	tweety homolog 3			NM_025250.2	-3.68	7.0	1.0E-03
STXBP2	syntaxin binding protein 2	CD14+		NM_006949.1	-3.63	2.0	1.3E-04
DPEP2	dipeptidase 2			NM_022355.1	-3.60	2.5	1.8E-04
GK	glycerol kinase, transcript variant 2	CD14+		NM_000167.3	-3.59	6.2	2.2E-04
GAS7	growth arrest-specific 7, transcript variant b			NM_201432.1	-3.54	5.0	2.0E-04
LCP1	lymphocyte cytosolic protein 1, transcript variant 5	CD56+ NK		NM_002298.2	-3.52	1.5	1.4E-04
HLA-DMB	major histocompatibility complex, class II, DM beta	BDCA4+ DC		NM_002118.3	-3.51	1.7	1.2E-04
C14orf58	chromosome 14 open reading frame 58			NM_017791.1	-3.49	4.5	1.5E-04
LY96	lymphocyte antigen 96			NM_015364.2	-3.48	3.8	1.3E-04
HCK	hemopoietic cell kinase			NM_002110.2	-3.47	4.9	6.2E-04
C1QB	complement component 1, q subcomponent, beta polypeptide			NM_000491.2	-3.45	4.2	6.0E-04
CD33	CD33 antigen			NM_001772.2	-3.43	2.0	5.8E-05
GLRX	glutaredoxin	CD14+		NM_002064.1	-3.43	8.0	8.6E-05
APBB1IP	amyloid beta precursor protein-binding, family B, member 1 interacting protein			NM_019043.3	-3.41	2.7	1.2E-04
URP2	UNC-112 related protein 2, transcript variant URP2SF			NM_031471.4	-3.37	7.7	8.6E-04
SH2D3C	SH2 domain containing 3C, transcript variant 2			NM_170600.1	-3.36	18.5	2.6E-03
CORO1A	coronin, actin binding protein, 1A			NM_007074.2	-3.36	3.4	1.7E-04
MAN2B1	mannosidase, alpha, class 2B, member 1	CD14+		NM_000528.2	-3.36	22.6	1.6E-04
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1			NM_152309.2	-3.35	3.6	6.3E-04
PDPN	podoplanin, transcript variant 4			NM_001006625.1	-3.35	6.6	9.4E-03
SLC7A7	solute carrier family 7, member 7	CD14+		NM_003982.2	-3.35	1.3	4.5E-05
ANKRD33	ankyrin repeat domain 33			NM_182608.2	-3.32	15.9	3.8E-03
PILRA	paired immunoglobin-like type 2 receptor alpha, transcript variant 1,paired immunoglobin-like type 2 receptor alpha, transcript variant 2			NM_013439.2,NM_178272.1	-3.31	3.9	2.4E-04
RAB20	RAB20, member RAS oncogene family			NM_017817.1	-3.26	10.0	1.3E-03
MFSD1	major facilitator superfamily domain containing 1	CD14+		NM_022736.1	-3.26	2.5	1.4E-04
PSCD4	pleckstrin homology, Sec7 and coiled-coil domains 4	BDCA4+ DC		NM_013385.2	-3.22	1.4	5.9E-05
TRPV4	transient receptor potential cation channel, subfamily V, member 4, transcript variant 2			NM_147204.1	-3.21	2.4	3.5E-04
EPSTI1	epithelial stromal interaction 1, transcript variant 2			NM_033255.2	-3.21	2.3	2.3E-04
PYCARD	PYD and CARD domain containing, transcript variant 3			NM_145183.1	-3.19	1.3	6.9E-05
PGD	phosphogluconate dehydrogenase	CD14+		NM_002631.2	-3.19	13.6	2.4E-04
NCF2	neutrophil cytosolic factor 2			NM_000433.2	-3.19	4.9	1.4E-04
GRN	granulin, transcript variant 1,granulin, transcript variant 2	CD14+		NM_001012479.1,NM_002087.2	-3.18	16.4	8.0E-04
NR1H3	nuclear receptor subfamily 1, group H, member 3			NM_005693.1	-3.16	16.0	3.5E-03
ZMYND15	zinc finger, MYND-type containing 15			NM_032265.1	-3.14	2.3	4.6E-05
HCST	hematopoietic cell signal transducer, transcript variant 1			NM_014266.3	-3.14	3.3	1.2E-03
LNK	lymphocyte adaptor protein			NM_005475.1	-3.13	5.7	2.9E-04
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3	CD14+		NM_004635.3	-3.13	4.2	3.9E-04
GCHFR	GTP cyclohydrolase I feedback regulator			NM_005258.2	-3.12	24.9	7.2E-03
TREM1	triggering receptor expressed on myeloid cells 1	CD14+		NM_018643.2	-3.11	3.3	1.9E-03
FPR1	formyl peptide receptor 1	CD14+		NM_002029.3	-3.11	8.4	3.8E-03
GPSM3	G-protein signalling modulator 3			NM_022107.1	-3.11	1.3	4.7E-05
PLEKHQ1	pleckstrin homology domain containing, family Q member 1			NM_025201.3	-3.11	5.7	5.7E-04
C1QA	complement component 1, q subcomponent, alpha polypeptide			NM_015991.1	-3.10	3.5	5.4E-04
SLC29A3	solute carrier family 29, member 3	CD14+		NM_018344.3	-3.10	5.8	4.2E-04
TM6SF1	transmembrane 6 superfamily member 1			NM_023003.1	-3.09	13.5	1.3E-04

C5AR1	complement component 5a receptor 1	CD56+ NK		NM_001736.2	-3.09	3.7	2.3E-04
EVI2A	ecotropic viral integration site 2A, transcript variant 2			NM_014210.2	-3.09	11.6	7.3E-04
RPS6KA1	ribosomal protein S6 kinase, 90kDa, polypeptide 1, transcript variant 2			NM_001006665.1	-3.07	6.4	5.6E-04
CNDP2	CNDP dipeptidase 2			NM_018235.1	-3.06	2.0	1.3E-04
FLJ20647	hypothetical protein FLJ20647			NM_017918.3	-3.05	3.0	1.1E-03
KLHL6	kelch-like 6			NM_130446.1	-3.03	10.7	6.2E-04
DENND2D	DENN/MADD domain containing 2D			NM_024901.3	-3.02	5.2	8.8E-04
CXorf9	chromosome X open reading frame 9	CD56+ NK		NM_018990.2	-3.02	5.6	9.3E-04
ARHGAP9	Rho GTPase activating protein 9			NM_032496.1	-3.00	1.3	7.2E-05
CARD9	caspase recruitment domain family, member 9			NM_052813.2	-2.99	3.3	3.6E-04
IGSF4	immunoglobulin superfamily, member 4			NM_014333.2	-2.98	1.8	2.8E-04
CLEC5A	C-type lectin domain family 5, member A			NM_013252.2	-2.98	4.9	6.1E-03
GIMAP6	GTPase, IMAP family member 6, transcript variant 1	CD4+ T		NM_024711.3	-2.97	15.4	9.3E-04
CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral oncogene homolog	CD14+		NM_005211.2	-2.97	1.2	8.9E-05
SLC37A2	solute carrier family 37, member 2			NM_198277.1	-2.94	9.0	2.3E-03
SYK	spleen tyrosine kinase	BDCA4+ DC		NM_003177.3	-2.94	4.2	2.3E-04
HLA-DMA	major histocompatibility complex, class II, DM alpha	BDCA4+ DC		NM_006120.2	-2.94	2.2	2.2E-04
PTGER4	prostaglandin E receptor 4			NM_000958.2	-2.93	9.0	1.3E-03
CTSD	cathepsin D			NM_001909.3	-2.92	13.6	4.4E-03
PSCDBP	pleckstrin homology, Sec7 and coiled-coil domains, binding protein			NM_004288.3	-2.92	4.2	3.4E-03
HCP5	HLA complex P5			NM_006674.2	-2.92	6.4	1.8E-03
LRRC33	leucine rich repeat containing 33			NM_198565.1	-2.92	4.2	2.7E-04
SUSD1	sushi domain containing 1			NM_022486.3	-2.92	17.6	3.6E-03
EBI2	Epstein-Barr virus induced gene 2			NM_004951.3	-2.91	3.5	1.5E-03
ASGR1	asialoglycoprotein receptor 1			NM_001671.2	-2.88	5.0	2.4E-04
SOD2	superoxide dismutase 2, mitochondrial, nuclear gene encoding mitochondriat protein, transcript variant 2			NM_001024465.1	-2.88	24.9	1.4E-02
CYBB	cytochrome b-245, beta polypeptide	BDCA4+ DC		NM_000397.2	-2.88	7.0	9.2E-04
ABCA1	ATP-binding cassette, sub-family A, member 1		lipid metabolism	NM_005502.2	-2.88	2.9	6.9E-04
MSR1	macrophage scavenger receptor 1, transcript variant SR-AI	CD14+		NM_138715.1	-2.85	13.1	2.8E-03
S100A9	S100 calcium binding protein A9	CD14+		NM_002965.2	-2.85	8.3	1.8E-03
GLA	galactosidase, alpha		lipid metabolism	NM_000169.1	-2.85	4.2	1.9E-04
GPR137B	G protein-coupled receptor 137B			NM_003272.1	-2.84	3.5	5.8E-04
C6orf105	chromosome 6 open reading frame 105			NM_032744.1	-2.84	20.7	1.1E-02
SAMSN1	SAM domain, SH3 domain and nuclear localisation signals, 1			NM_022136.3	-2.81	15.9	8.1E-04
STS-1	Cbl-interacting protein Sts-1			NM_032873.3	-2.81	4.2	1.2E-03
CAPG	capping protein, gelsolin-like	CD14+		NM_001747.2	-2.80	6.0	9.4E-04
ADORA3	adenosine A3 receptor, transcript variant 1,adenosine A3 receptor, transcript variant 2			NM_000677.2,NM_020683.5	-2.80	11.4	2.4E-03
BMP2K	BMP2 inducible kinase, transcript variant 2			NM_017593.3	-2.80	19.8	9.4E-04
AGTRL1	angiotensin II receptor-like 1			NM_005161.2	-2.80	22.8	7.0E-02
MPP1	membrane protein, palmitoylated 1, 55kDa	CD14+		NM_002436.2	-2.80	6.1	5.0E-04
RNASET2	ribonuclease T2	CD14+		NM_003730.3	-2.80	4.9	2.1E-04
SLC38A6	solute carrier family 38, member 6	CD14+		NM_153811.1	-2.79	16.0	2.6E-03
LST1	leukocyte specific transcript 1, transcript variant 1,leukocyte specific transcript 1, transcript variant 2	CD56+ NK		NM_007161.2,NM_205837.1	-2.79	8.7	2.7E-03
PTPNS1	protein tyrosine phosphatase, non-receptor type substrate 1			NM_080792.1	-2.79	1.3	7.5E-05
PARP12	poly polymerase family, member 12			NM_022750.2	-2.79	12.6	3.3E-03
PLCB2	phospholipase C, beta 2			NM_004573.1	-2.78	3.1	4.0E-04
ITGAL	integrin, alpha L, lymphocyte function-associated antigen 1; alpha polypeptide)			NM_002209.1	-2.76	17.5	3.5E-03
ICA1	islet cell autoantigen 1, 69kDa, transcript variant 3			NM_022308.1	-2.76	16.1	1.1E-03
ARHGAP27	Rho GTPase activating protein 27			NM_199282.1	-2.76	5.2	1.6E-03
MTP18	mitochondrial protein 18 kDa, nuclear gene encoding mitochondrial protein, transcript variant 1			NM_016498.3	-2.75	5.6	4.4E-03
RHBDF2	rhomboid 5 homolog 2, transcript variant 1			NM_024599.2	-2.75	3.6	2.9E-04
LILRA3	leukocyte immunoglobulin-like receptor, subfamily A, member 3			NM_006865.2	-2.74	18.6	7.4E-03
LHFPL2	lipoma HMGIC fusion partner-like 2			NM_005779.1	-2.74	2.9	2.4E-04
RENBP	renin binding protein			NM_002910.4	-2.72	12.9	2.3E-03
MYO1F	myosin IF	CD14+		NM_012335.2	-2.72	11.6	1.6E-03
SLC36A1	solute carrier family 36, member 1			NM_078483.2	-2.71	17.1	9.9E-04
SLC25A19	solute carrier family 25, member 19			NM_021734.3	-2.71	6.4	7.5E-04
OAF	OAF homolog			NM_178507.2	-2.70	4.2	1.4E-03
TMC6	transmembrane channel-like 6	CD56+ NK		NM_007267.5	-2.69	10.8	1.7E-03
HS3ST3A1	heparan sulfate 3-O-sulfotransferase 3A1			NM_006042.1	-2.69	3.9	3.7E-04
PLEK2	pleckstrin 2			NM_016445.1	-2.69	5.2	6.7E-04
ARHGAP4	Rho GTPase activating protein 4			NM_001666.2	-2.68	5.7	1.4E-03
ALOX5AP	arachidonate 5-lipoxygenase-activating protein	BDCA4+ DC		NM_001629.2	-2.67	5.8	2.9E-04
COTL1	coactosin-like 1			NM_021149.2	-2.67	10.4	9.9E-04
CD4	CD4 antigen	CD4+ T cells		NM_000616.2	-2.67	4.2	3.7E-04
C13orf18	chromosome 13 open reading frame 18	BDCA4+ DC		NM_025113.1	-2.66	3.5	2.9E-04
CLDN23	claudin 23			NM_194284.1	-2.65	10.0	1.4E-03
SCIN	scinderin			NM_033128.1	-2.64	18.5	8.3E-03
LGALS9	lectin, galactoside-binding, soluble, 9, transcript variant long			NM_009587.1	-2.64	9.3	7.6E-04
TM4SF19	transmembrane 4 L six family member 19			NM_138461.1	-2.63	6.8	3.3E-03
LFNG	lunatic fringe homolog			NM_002304.1	-2.62	2.8	7.7E-04
POU2F2	POU domain, class 2, transcription factor 2			NM_002698.1	-2.62	10.5	2.7E-03
FABP5	fatty acid binding protein 5			NM_001444.1	-2.61	21.7	4.7E-03
FAM20A	family with sequence similarity 20, member A			NM_017565.2	-2.61	7.3	3.6E-04
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A, transcript variant 2			NM_022837.1	-2.60	4.7	4.5E-04
TMEM71	transmembrane protein 71			NM_144649.1	-2.60	4.9	8.7E-04
LRRC8D	leucine rich repeat containing 8 family, member D			NM_018103.3	-2.60	12.2	2.0E-04
PCK2	phosphoenolpyruvate carboxykinase 2, nuclear gene encoding mitochondrial protein, transcript variant 1			NM_004563.2	-2.59	4.0	9.8E-05
C19orf28	chromosome 19 open reading frame 28			NM_174983.2	-2.59	24.5	1.5E-03
MNDA	myeloid cell nuclear differentiation antigen	CD14+		NM_002432.1	-2.58	8.2	4.0E-04
RGS10	regulator of G-protein signalling 10, transcript variant 2			NM_002925.3	-2.58	8.7	1.1E-03
C3AR1	complement component 3a receptor 1	CD14+		NM_004054.2	-2.58	4.8	3.0E-04
HSPA6	heat shock 70kDa protein 6			NM_002155.3	-2.58	12.3	3.8E-03
FOLR2	folate receptor 2			NM_000803.2	-2.57	16.8	5.5E-03
CIAS1	cold autoinflammatory syndrome 1, transcript variant 1			NM_004895.3	-2.57	3.7	4.0E-04
CYGB	cytoglobin			NM_134268.3	-2.56	21.2	1.6E-02
BATF	basic leucine zipper transcription factor, ATF-like	CD14+		NM_006399.2	-2.56	7.1	6.1E-04
FLJ14213	hypothetical protein FLJ14213	CD56+ NK		NM_024841.2	-2.56	11.2	6.2E-04
GMIP	GEM interacting protein	CD14+		NM_016573.1	-2.55	3.5	1.6E-04
CMIP	c-Maf-inducing protein, transcript variant Tc-mip			NM_030629.1	-2.54	5.0	7.3E-04
EFHD2	EF-hand domain family, member D2	CD14+		NM_024329.4	-2.54	7.8	5.7E-04
ADA	adenosine deaminase			NM_000022.2	-2.53	2.9	2.0E-04
RRAGD	Ras-related GTP binding D			NM_021244.2	-2.53	18.1	8.2E-03
MYO5A	myosin VA			NM_000259.1	-2.53	10.4	1.1E-03
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa, transcript variant 1	CD4+ T cells		NM_016817.2	-2.53	9.5	1.3E-03
STK10	serine/threonine kinase 10	CD56+ NK		NM_005990.2	-2.52	18.8	2.8E-03
LILRA2	leukocyte immunoglobulin-like receptor, subfamily A, member 2	BDCA4+ DC		NM_006866.1	-2.52	5.6	1.3E-03
CARD15	caspase recruitment domain family, member 15			NM_022162.1	-2.52	4.9	1.6E-04
IQGAP3	IQ motif containing GTPase activating protein 3			NM_178229.3	-2.52	3.3	2.1E-03
SIGLEC9	sialic acid binding Ig-like lectin 9			NM_014441.1	-2.51	7.3	4.8E-04

HPCAL1	hippocalcin-like 1, transcript variant 1			NM_002149.2	-2.51	10.8	2.7E-04
SH3TC1	SH3 domain and tetratricopeptide repeats 1			NM_018986.2	-2.51	1.7	1.5E-04
NCF4	neutrophil cytosolic factor 4, 40kDa, transcript variant 2	BDCA4+ DC		NM_013416.2	-2.50	2.3	3.7E-04
TPP1	tripeptidyl peptidase I	CD14+		NM_000391.2	-2.50	2.5	1.4E-04
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa, transcript variant 2,2',5'-oligoadenylate synthetase 1, 40/46kDa, transcript variant 3	BDCA4+ DC		NM_001032409.1,NM_002534.2	-2.49	13.9	6.9E-03
ZNF385	zinc finger protein 385			NM_015481.1	-2.48	14.8	5.3E-04
MYO1G	myosin IG			NM_033054.1	-2.48	7.7	2.0E-03
IL4I1	interleukin 4 induced 1, transcript variant 1			NM_152899.1	-2.48	23.4	1.1E-02
CACNA2D4	calcium channel, voltage-dependent, alpha 2/delta subunit 4, transcript variant 1			NM_172364.3	-2.47	21.0	1.5E-03
RASSF4	Ras association domain family 4, transcript variant 2	CD56+ NK		NM_178145.1	-2.47	15.6	3.0E-03
DOK2	docking protein 2, 56kDa, transcript variant 2	CD14+		NM_201349.1	-2.46	11.6	3.5E-03
LIMK1	LIM domain kinase 1, transcript variant dLIMK			NM_016735.1	-2.46	11.6	3.3E-04
ATP6V1B2	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 2			NM_001693.2	-2.44	12.8	3.4E-04
BID	BH3 interacting domain death agonist, transcript variant 2	CD14+		NM_001196.2	-2.44	1.8	1.0E-04
SNCA	synuclein, alpha, transcript variant NACP112			NM_007308.1	-2.43	13.9	4.0E-03
TACC3	transforming, acidic coiled-coil containing protein 3			NM_006342.1	-2.43	5.2	1.8E-04
CSK	c-src tyrosine kinase	CD56+ NK		NM_004383.1	-2.42	2.2	2.1E-04
FCGRT	Fc fragment of IgG, receptor, transporter, alpha			NM_004107.3	-2.42	16.0	2.0E-03
CDC20	CDC20 cell division cycle 20 homolog			NM_001255.1	-2.42	11.5	6.8E-04
C12orf59	chromosome 12 open reading frame 59			NM_153022.1	-2.41	2.7	1.3E-04
FSCN1	fascin homolog 1, actin-bundling protein			NM_003088.2	-2.41	9.0	1.6E-04
TRIB1	tribbles homolog 1			NM_025195.2	-2.41	6.1	1.4E-03
KIAA1598	KIAA1598			NM_018330.3	-2.40	5.9	4.5E-04
MFNG	manic fringe homolog			NM_002405.2	-2.39	1.2	9.1E-05
AADACL1	arylacetamide deacetylase-like 1			NM_020792.3	-2.39	19.7	1.4E-03
CMTM3	CKLF-like MARVEL transmembrane domain containing 3, transcript variant 4			NM_181555.1	-2.39	3.1	5.1E-04
OSTF1	osteoclast stimulating factor 1			NM_012383.3	-2.39	5.2	1.8E-04
LAIR2	leukocyte-associated Ig-like receptor 2, transcript variant 1	CD14+		NM_002288.3	-2.38	6.4	4.5E-04
PIM1	pim-1 oncogene			NM_002648.2	-2.38	16.2	5.0E-03
APOB48R	apolipoprotein B48 receptor			NM_018690.1	-2.37	13.7	1.1E-03
ATF5	activating transcription factor 5			NM_012068.2	-2.35	12.3	7.2E-03
DEF6	differentially expressed in FDCP 6 homolog			NM_022047.2	-2.35	9.4	2.0E-03
PARVG	parvin, gamma			NM_022141.4	-2.34	5.8	4.3E-04
ABCC3	ATP-binding cassette, sub-family C, member 3, transcript variant MRP3A			NM_020037.1	-2.34	7.0	2.5E-03
RALA	v-ral simian leukemia viral oncogene homolog A			NM_005402.2	-2.34	13.5	2.8E-03
SLC16A3	solute carrier family 16, member 3	CD14+		NM_004207.1	-2.34	4.6	5.0E-03
FGD3	FYVE, RhoGEF and PH domain containing 3			NM_033086.1	-2.33	6.4	1.2E-03
DUSP10	dual specificity phosphatase 10, transcript variant 2			NM_144728.1	-2.32	4.2	5.7E-05
SCD	stearoyl-CoA desaturase		lipid metabolism	NM_005063.4	-2.32	15.4	5.1E-03
TUBB3	tubulin, beta 3			NM_006086.2	-2.31	5.2	3.0E-02
SDSL	serine dehydratase-like			NM_138432.2	-2.31	23.7	1.5E-03
CCR1	chemokine receptor 1			NM_001295.2	-2.30	13.5	1.6E-03
MT1G	metallothionein 1G			NM_005950.1	-2.30	6.7	7.2E-03
CSF2RA	colony stimulating factor 2 receptor, alpha, low-affinity, transcript variant 6	CD14+		NM_172249.1	-2.29	2.9	7.8E-05
OSM	oncostatin M			NM_020530.3	-2.29	19.0	1.6E-02
HAK	heart alpha-kinase			NM_052947.2	-2.29	14.4	2.2E-03
TLR8	toll-like receptor 8, transcript variant 1,toll-like receptor 8, transcript variant 2			NM_016610.2,NM_138636.2	-2.29	6.6	5.9E-04
CHST13	carbohydrate sulfotransferase 13			NM_152889.1	-2.28	12.6	1.4E-01
PNKD	paroxysmal nonkinesiogenic dyskinesia, transcript variant 2			NM_022572.2	-2.28	12.1	2.9E-04
SLC39A11	solute carrier family 39, member 11			NM_139177.2	-2.28	12.2	6.5E-04
FN5	FN5 protein			NM_020179.1	-2.27	6.6	3.8E-04
GPR92	G protein-coupled receptor 92			NM_020400.4	-2.27	17.1	3.6E-03
NP	nucleoside phosphorylase			NM_000270.1	-2.27	12.6	2.2E-03
PSCD1	pleckstrin homology, Sec7 and coiled-coil domains 1(cytohesin 1), transcript variant 2			NM_017456.1	-2.27	8.3	1.1E-03
IL6	interleukin 6			NM_000600.1	-2.27	12.4	5.9E-01
TM7SF4	transmembrane 7 superfamily member 4	CD14+		NM_030788.2	-2.26	11.6	2.7E-03
BLNK	B-cell linker	BDCA4+ DC		NM_013314.2	-2.26	10.0	2.2E-04
TLR7	toll-like receptor 7	BDCA4+ DC		NM_016562.3	-2.26	10.2	2.0E-03
GIMAP4	GTPase, IMAP family member 4			NM_018326.2	-2.26	2.2	3.9E-04
MGAT4A	mannosyl-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme A			NM_012214.1	-2.25	1.5	3.3E-05
DFNA5	deafness, autosomal dominant 5			NM_004403.1	-2.25	14.8	2.1E-03
LRRC15	leucine rich repeat containing 15			NM_130830.2	-2.25	3.5	3.2E-04
MGC42367	similar to 2010300C02Rik protein			NM_207362.1	-2.25	6.0	1.3E-04
PRDM1	PR domain containing 1, with ZNF domain, transcript variant 1			NM_001198.2	-2.25	7.1	1.8E-03
ENC1	ectodermal-neural cortex			NM_003633.1	-2.24	12.9	1.1E-04
SLC22A18AS	solute carrier family 22, member 18 antisense			NM_007105.1	-2.23	15.4	5.3E-03
ASCL2	achaete-scute complex-like 2			NM_005170.2	-2.22	18.0	1.0E-03
IRAK1	interleukin-1 receptor-associated kinase 1, transcript variant 2			NM_001025242.1	-2.21	8.5	3.2E-04
IRF7	interferon regulatory factor 7, transcript variant c	BDCA4+ DC		NM_004030.1	-2.20	16.5	3.1E-03
CCL23	chemokine ligand 23, transcript variant CKbeta8-1			NM_005064.3	-2.20	7.1	4.2E-03
PRKCB1	protein kinase C, beta 1, transcript variant 2	BDCA4+ DC		NM_002738.5	-2.18	17.1	3.8E-03
CD28	CD28 antigen			NM_006139.1	-2.18	19.1	7.5E-03
SPOCD1	SPOC domain containing 1			NM_144569.3	-2.18	7.8	1.9E-03
TMEM2	transmembrane protein 2			NM_013390.1	-2.18	20.1	1.6E-03
SLC7A5	solute carrier family 7, member 5			NM_003486.5	-2.17	8.5	7.0E-03
CKLF	chemokine-like factor, transcript variant 3			NM_016326.2	-2.17	12.1	5.6E-04
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein			NM_001343.1	-2.17	13.8	8.7E-03
ADAMDEC1	ADAM-like, decysin 1	CD14+		NM_014479.2	-2.17	20.4	6.9E-03
RCC2	regulator of chromosome condensation 2			NM_018715.1	-2.16	8.7	1.2E-03
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa, transcript variant 1	CD56+ NK		NM_001017915.1	-2.16	2.4	2.8E-04
RASSF2	Ras association domain family 2, transcript variant 2			NM_170773.1	-2.15	12.6	8.8E-04
SIPA1	signal-induced proliferation-associated gene 1, transcript variant 1	CD56+ NK		NM_153253.2	-2.15	16.1	9.1E-04
TOP2A	topoisomerase II alpha 170kDa			NM_001067.2	-2.15	24.2	6.3E-03
PLCG2	phospholipase C, gamma 2	CD56+ NK		NM_002661.1	-2.15	1.7	2.9E-04
CORO7	coronin 7			NM_024535.1	-2.14	15.1	2.6E-03
COQ2	coenzyme Q2 homolog, prenyltransferase			NM_015697.5	-2.14	5.8	5.4E-04
NALP12	NACHT, leucine rich repeat and PYD containing 12, transcript variant 1			NM_033297.1	-2.12	1.7	1.8E-04
FLJ20718	hypothetical protein FLJ20718, transcript variant 1			NM_017939.1	-2.11	19.7	1.8E-03
OPN3	opsin 3, transcript variant 3	BDCA4+ DC		NM_001030012.1	-2.11	14.6	7.7E-04
MYD88	myeloid differentiation primary response gene			NM_002468.2	-2.11	12.8	6.3E-04
VENTX	VENT homeobox homolog			NM_014468.2	-2.09	6.8	2.9E-04
NEK6	NIMA-related kinase 6			NM_014397.3	-2.08	17.2	4.0E-03
LTB4R	leukotriene B4 receptor			NM_181657.1	-2.07	13.6	3.2E-03
RUNX3	runt-related transcription factor 3, transcript variant 2			NM_004350.1	-2.07	21.5	5.1E-02
C1orf85	chromosome 1 open reading frame 85			NM_144580.1	-2.07	12.3	2.2E-03
KIAA0746	KIAA0746 protein	BDCA4+ DC		NM_015187.1	-2.07	5.2	4.0E-04
SLC43A3	solute carrier family 43, member 3			NM_199329.1	-2.06	13.2	1.1E-03
TCN2	transcobalamin II; macrocytic anemia			NM_000355.2	-2.05	12.6	2.4E-03
C20orf31	chromosome 20 open reading frame 31			NM_018217.1	-2.05	11.1	9.3E-04

ACP2	acid phosphatase 2, lysosomal	CD14+		NM_001610.1	-2.05	14.6	2.4E-03
ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F			NM_004231.2	-2.05	22.6	2.7E-03
IFNGR1	interferon gamma receptor 1			NM_000416.1	-2.05	17.1	2.7E-03
ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit c"			NM_004047.2	-2.04	12.7	1.4E-03
NUDT14	nudix-type motif 14			NM_177533.2	-2.04	20.8	1.9E-03
TLR1	toll-like receptor 1			NM_003263.3	-2.02	9.3	2.9E-04
AP1B1	adaptor-related protein complex 1, beta 1 subunit, transcript variant 1			NM_001127.2	-2.02	14.7	4.4E-03
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4			NM_005099.3	-2.01	18.4	6.4E-01
LRRN1	leucine rich repeat neuronal 1			NM_020873.3	2.00	1.3	6.1E-05
SMTN	smoothelin, transcript variant 3			NM_006932.3	2.00	12.7	3.3E-03
Ells1	hypothetical protein Ells1			NM_152793.1	2.01	24.5	4.1E-03
C14orf28	chromosome 14 open reading frame 28			NM_001017923.1	2.02	10.7	8.6E-04
FLJ37440	hypothetical protein FLJ37440			NM_153214.1	2.02	2.9	6.1E-05
C10orf65	chromosome 10 open reading frame 65			NM_138413.2	2.02	8.1	5.4E-04
ZNF503	zinc finger protein 503			NM_032772.3	2.02	22.3	6.6E-03
TMEM16A	transmembrane protein 16A			NM_018043.4	2.02	9.8	2.3E-03
SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3, transcript variant 2			NM_003078.3	2.02	23.3	2.7E-03
C1orf24	chromosome 1 open reading frame 24, transcript variant 2			NM_052966.1	2.02	19.0	2.7E-02
SLC16A9	solute carrier family 16, member 9			NM_194298.1	2.03	4.7	1.7E-03
RBPMS	RNA binding protein with multiple splicing, transcript variant 4			NM_006867.2	2.03	23.9	3.8E-02
LOC387758	similar to RIKEN cDNA 1110018M03			NM_203371.1	2.03	16.4	5.8E-03
FBXO17	F-box protein 17, transcript variant 2			NM_024907.5	2.03	4.2	3.6E-04
MARK1	MAP/microtubule affinity-regulating kinase 1			NM_018650.2	2.04	4.2	1.8E-04
NCALD	neurocalcin delta	CD56+ NK		NM_032041.1	2.05	22.4	2.1E-02
RAB23	RAB23, member RAS oncogene family, transcript variant 1			NM_016277.3	2.05	17.8	2.1E-03
MAP1B	microtubule-associated protein 1B, transcript variant 2	VSMC		NM_032010.1	2.05	23.4	4.4E-03
CRI1	CREBBP/EP300 inhibitor 1			NM_014335.2	2.06	24.3	6.4E-03
TGFB1I1	transforming growth factor beta 1 induced transcript 1			NM_015927.3	2.06	23.2	8.7E-03
SORBS1	sorbin and SH3 domain containing 1, transcript variant 2			NM_015385.1	2.06	9.3	1.2E-02
LHFP	lipoma HMGIC fusion partner			NM_005780.1	2.07	17.1	8.6E-03
PLN	phospholamban			NM_002667.2	2.07	22.2	1.2E-02
PLSCR4	phospholipid scramblase 4			NM_020353.1	2.07	16.4	5.6E-03
PYGB	phosphorylase, glycogen; brain			NM_002862.3	2.08	23.7	9.9E-03
TRIM36	tripartite motif-containing 36, transcript variant 2			NM_001017397.1	2.08	14.8	8.5E-03
CAMK2G	calcium/calmodulin-dependent protein kinase II gamma, transcript variant 2			NM_172169.1	2.10	11.4	4.6E-03
TANC1	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1			NM_033394.1	2.10	11.0	3.0E-03
CSRP2	cysteine and glycine-rich protein 2			NM_001321.1	2.10	18.5	7.3E-03
PDLIM3	PDZ and LIM domain 3			NM_014476.1	2.10	14.5	2.3E-02
OGN	osteoglycin, transcript variant 2			NM_024416.2	2.12	8.8	5.9E-02
CNN1	calponin 1, basic, smooth muscle	VSMC		NM_001299.4	2.12	14.4	1.7E-02
GULP1	GULP, engulfment adaptor PTB domain containing 1			NM_016315.2	2.12	8.9	5.6E-04
LRCH2	leucine-rich repeats and calponin homology domain containing 2			NM_020871.2	2.13	23.8	1.0E-02
PDZRN3	PDZ domain containing RING finger 3			NM_015009.1	2.14	2.0	1.3E-04
MEIS2	Meis1, myeloid ecotropic viral integration site 1 homolog 2, transcript variant e			NM_020149.2	2.14	12.7	5.0E-03
MXRA7	matrix-remodelling associated 7, transcript variant 1			NM_001008528.1	2.15	20.7	2.8E-03
RGS5	regulator of G-protein signalling 5			NM_003617.2	2.15	6.5	4.8E-02
NXN	nucleoredoxin			NM_022463.3	2.15	10.7	1.1E-03
IL17D	interleukin 17D			NM_138284.1	2.15	1.7	4.2E-05
PKD2	polycystic kidney disease 2			NM_000297.2	2.16	14.5	6.0E-03
PPP1R14A	protein phosphatase 1, regulatory subunit 14A			NM_033256.1	2.17	23.6	3.3E-02
SLC22A3	solute carrier family 22, member 3			NM_021977.2	2.18	7.8	1.9E-03
HSPB8	heat shock 22kDa protein 8			NM_014365.2	2.19	13.6	4.5E-03
ZNF651	zinc finger protein 651			NM_145166.2	2.20	13.5	6.2E-04
KLHDC5	kelch domain containing 5			NM_020782.1	2.21	16.5	1.8E-03
TSPAN2	tetraspanin 2			NM_005725.3	2.21	12.7	5.0E-03
SLMAP	sarcolemma associated protein			NM_007159.2	2.21	15.2	3.9E-03
PLCE1	phospholipase C, epsilon 1			NM_016341.2	2.22	15.1	5.3E-03
LAMC3	laminin, gamma 3		ECM_regulation	NM_006059.2	2.23	1.6	1.1E-04
EPHA3	EPH receptor A3, transcript variant 1			NM_005233.3	2.24	3.3	1.5E-04
SSPN	sarcospan			NM_005086.3	2.24	18.0	1.5E-02
TMEM47	transmembrane protein 47			NM_031442.2	2.25	12.5	1.7E-02
FYCO1	FYVE and coiled-coil domain containing 1			NM_024513.1	2.25	11.2	1.4E-03
CAV2	caveolin 2, transcript variant 1			NM_001233.3	2.26	22.8	3.0E-02
FMOD	fibromodulin			NM_002023.3	2.26	12.2	6.0E-03
LMOD1	leiomodin 1			NM_012134.1	2.26	12.3	2.0E-02
NFIA	nuclear factor I/A			NM_005595.1	2.27	7.1	1.2E-03
VIPR2	vasoactive intestinal peptide receptor 2			NM_003382.3	2.27	6.4	5.2E-03
HDC	histidine decarboxylase			NM_002112.1	2.28	19.5	2.8E-03
PSIP1	PC4 and SFRS1 interacting protein 1, transcript variant 2			NM_033222.2	2.28	6.4	6.4E-04
DNER	delta-notch-like EGF repeat-containing transmembrane			NM_139072.2	2.31	6.5	2.8E-05
MSRB3	methionine sulfoxide reductase B3, transcript variant 1			NM_198080.2	2.31	18.2	1.1E-02
HEY2	hairy/enhancer-of-split related with YRPW motif 2			NM_012259.1	2.31	2.7	3.0E-03
SGCE	sarcoglycan, epsilon	VSMC		NM_003919.1	2.32	19.4	1.4E-02
AGTR1	angiotensin II receptor, type 1, transcript variant 5			NM_032049.1	2.33	3.0	7.6E-05
AMPH	amphiphysin, transcript variant 1			NM_001635.2	2.33	5.2	4.2E-03
PDE8B	phosphodiesterase 8B, transcript variant 3			NM_001029851.1	2.38	2.9	1.4E-03
ZCCHC5	zinc finger, CCHC domain containing 5			NM_152694.1	2.38	4.2	1.7E-04
BVES	blood vessel epicardial substance, transcript variant A			NM_007073.3	2.39	9.0	6.6E-04
SOST	sclerosteosis			NM_025237.2	2.39	8.8	1.0E-02
PPP2R2B	protein phosphatase 2, regulatory subunit B, beta isoform, transcript variant 4	CD56+ NK		NM_181676.1	2.39	20.7	5.9E-03
FILIP1	filamin A interacting protein 1			NM_015687.2	2.40	5.8	6.9E-04
MN1	meningioma 1			NM_002430.2	2.41	6.8	7.0E-04
CNTN3	contactin 3			NM_020872.1	2.41	10.8	3.9E-03
NAP1L3	nucleosome assembly protein 1-like 3			NM_004538.3	2.42	10.0	2.9E-03
HSPA2	heat shock 70kDa protein 2			NM_021979.2	2.42	21.2	8.4E-03
SORBS2	sorbin and SH3 domain containing 2, transcript variant 1			NM_003603.4	2.42	20.1	4.3E-02
SYNC1	syncoilin, intermediate filament 1			NM_030786.1	2.43	13.9	1.6E-03
DKFZP686A01247	hypothetical protein			NM_014988.1	2.45	12.2	2.6E-03
C1QTNF7	C1q and tumor necrosis factor related protein 7			NM_031911.3	2.46	5.8	1.5E-03
SFRP1	secreted frizzled-related protein 1	VSMC		NM_003012.3	2.47	1.3	1.1E-04
C9orf65	chromosome 9 open reading frame 65			NM_138818.2	2.48	9.6	1.3E-02
MRGPRF	MAS-related GPR, member F			NM_145015.2	2.50	13.2	4.2E-03
CAP2	CAP, adenylate cyclase-associated protein, 2			NM_006366.2	2.51	14.0	6.9E-03
RERG	RAS-like, estrogen-regulated, growth inhibitor			NM_032918.1	2.52	7.2	3.9E-02
HLF	hepatic leukemia factor			NM_002126.3	2.52	1.8	1.5E-05
LOC285382	hypothetical gene supported by AK091454			NM_001025266.1	2.53	0.8	3.0E-05
CAV1	caveolin 1, caveolae protein, 22kDa			NM_001753.3	2.54	6.8	1.2E-03
GPC3	glypican 3			NM_004484.2	2.54	3.3	2.2E-04
GPM6B	glycoprotein M6B, transcript variant 1,glycoprotein M6B, transcript variant 4	BDCA4+ DC		NM_001001994.1,NM_001001995.1	2.54	1.1	5.0E-07
OLFML3	olfactomedin-like 3			NM_020190.2	2.59	12.7	1.5E-03
PGM5	phosphoglucomutase 5			NM_021965.3	2.60	8.4	6.9E-04
C6orf117	chromosome 6 open reading frame 117			NM_138409.1	2.60	7.1	3.4E-02
TES	testis derived transcript, transcript variant 2			NM_152829.1	2.60	6.6	6.8E-04

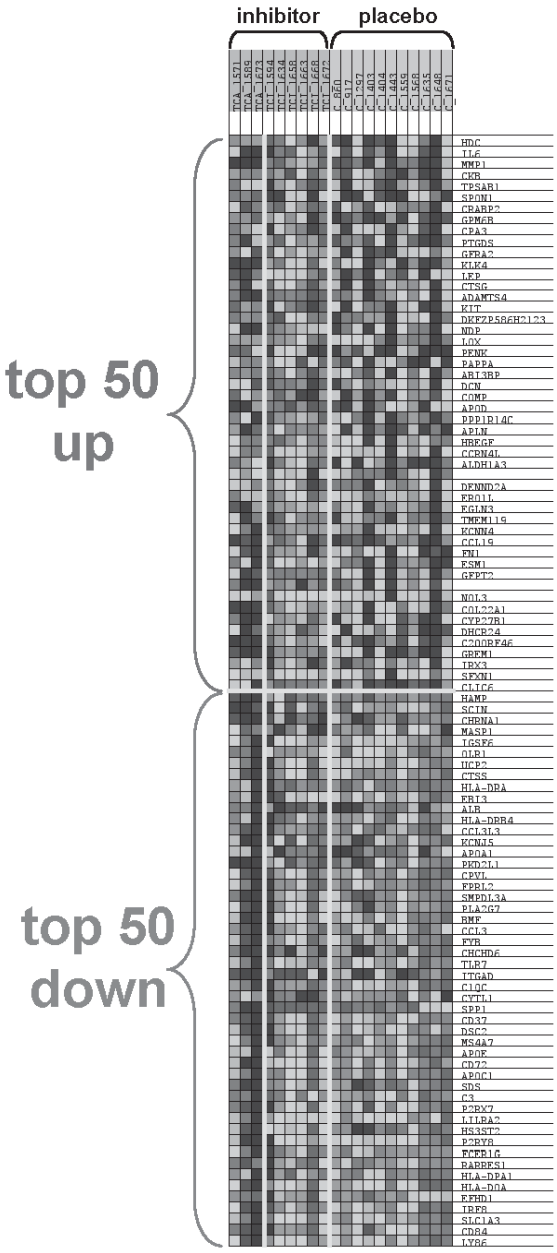
RASL12	RAS-like, family 12			NM_016563.2	2.60	12.6	9.4E-03
SOX15	SRY-box 15			NM_006942.1	2.61	1.8	1.7E-04
FXYD1	FXYD domain containing ion transport regulator 1, transcript variant a			NM_005031.3	2.66	4.2	1.0E-03
DOC1	downregulated in ovarian cancer 1, transcript variant 1,downregulated in ovarian cancer 1, transcript variant 2			NM_014890.1,NM_182909.1	2.66	6.2	3.8E-04
NLGN1	neuroligin 1			NM_014932.2	2.67	24.5	4.8E-03
ANKRD15	ankyrin repeat domain 15, transcript variant 2			NM_153186.3	2.68	12.1	8.1E-03
REEP1	receptor accessory protein 1			NM_022912.1	2.68	7.1	1.7E-03
TMOD1	tropomodulin 1			NM_003275.1	2.69	2.3	1.6E-04
ECM2	extracellular matrix protein 2, female organ and adipocyte specific			NM_001393.2	2.70	12.1	6.5E-03
LAYN	layilin			NM_178834.2	2.71	8.3	4.8E-03
RAVER2	hypothetical protein FLJ10770			NM_018211.2	2.72	15.2	9.5E-03
DKFZP586H2123	regeneration associated muscle protease, transcript variant 2	VSMC		NM_001001991.1	2.74	7.0	7.1E-05
PCDH7	BH-protocadherin, transcript variant c			NM_032457.1	2.74	18.6	1.2E-02
CKB	creatine kinase, brain			NM_001823.3	2.78	21.9	7.0E-03
PLD5	phospholipase D family, member 5			NM_152666.1	2.79	0.6	1.9E-05
LGR6	leucine-rich repeat-containing G protein-coupled receptor 6, transcript variant 1			NM_001017403.1	2.81	15.6	1.3E-02
LDB3	LIM domain binding 3			NM_007078.1	2.85	11.5	2.9E-03
ALDH1L1	aldehyde dehydrogenase 1 family, member L1			NM_012190.2	2.86	1.7	3.4E-05
NEXN	nexilin			NM_144573.1	2.89	5.8	8.1E-04
CRISPLD1	cysteine-rich secretory protein LCCL domain containing 1			NM_031461.3	2.90	4.9	1.1E-03
FOXC1	forkhead box C1			NM_001453.1	2.93	12.1	1.5E-02
C14orf132	chromosome 14 open reading frame 132			NM_020215.2	2.96	5.8	4.4E-04
SPARCL1	SPARC-like 1			NM_004684.2	2.97	5.8	1.2E-03
DMN	desmuslin, transcript variant B			NM_015286.4	2.98	12.9	2.1E-03
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9			NM_182920.1	2.99	13.2	1.3E-03
TMEM97	transmembrane protein 97			NM_014573.1	3.04	19.2	8.1E-04
ACADL	acyl-Coenzyme A dehydrogenase, long chain, nuclear gene encoding mitochondrial protein			NM_001608.2	3.05	6.3	6.7E-04
ENAH	enabled homolog, transcript variant 2	VSMC		NM_018212.4	3.10	4.2	4.1E-04
NPNT	nephronectin		ECM_regulation	NM_001033047.1	3.15	12.6	9.1E-03
PHGDH	phosphoglycerate dehydrogenase			NM_006623.2	3.22	4.6	2.6E-04
KIAA0367	KIAA0367			NM_015225.1	3.25	12.1	7.6E-03
CCDC3	coiled-coil domain containing 3			NM_031455.2	3.26	16.2	1.9E-02
KCNK17	potassium channel, subfamily K, member 17			NM_031460.2	3.29	2.3	2.4E-04
KIAA1505	KIAA1505 protein			NM_020879.1	3.39	1.8	3.8E-05
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1			NM_002130.4	3.58	5.2	2.4E-05
PTPLA	protein tyrosine phosphatase-like, member a			NM_014241.2	3.64	21.7	8.2E-03
RAMP1	receptor activity modifying protein 1			NM_005855.2	3.66	23.7	1.2E-02
LDOC1	leucine zipper, down-regulated in cancer 1			NM_012317.2	4.08	3.1	1.7E-03
SPON1	spondin 1, extracellular matrix protein		ECM_regulation	NM_006108.1	4.15	0.2	1.0E-06
ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide			NM_000702.2	4.42	1.5	6.9E-05
CASQ2	calsequestrin 2			NM_001232.1	5.32	1.1	4.1E-05
LEFTY2	left-right determination factor 2			NM_003240.2	5.69	3.3	2.6E-04
SCRG1	scrapie responsive protein 1			NM_007281.1	6.04	5.8	2.0E-03

* p-values obtained by a Bayesian oneway Anova including Tukey's postHoc test. N=541 genes had differential gene expression levels between the three patients with a Good MMF response and the eleven Placebo treated patients (N=403 down in MMF, and N=138 up in MMF).

Supplement 1B. N=25 genes with differential mRNA expression between poor responders to MMF treatment and Placebo

Symbol	Description	celltype signature	Function	Accession	fold difference	FDR (%)	p-value*
MMP7	matrix metallopeptidase 7		MMPs	NM_002423.3	4.46	0.02	2.1E-02
IL6	interleukin 6	VSMC		NM_000600.1	3.66	0.12	3.5E-03
PTGDS	prostaglandin D2 synthase 21kDa			NM_000954.5	3.33	0.13	8.1E-03
IL1RN	interleukin 1 receptor antagonist, transcript variant 1			NM_173842.1	2.73	0.02	5.0E-03
CRABP2	cellular retinoic acid binding protein 2			NM_001878.2	2.68	0.13	3.3E-03
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4			NM_005099.3	2.65	0.18	6.1E-03
C15orf48	chromosome 15 open reading frame 48, transcript variant 2			NM_032413.2	2.65	0.02	2.3E-02
MMP9	matrix metallopeptidase 9		MMPs	NM_004994.2	2.59	0.00	1.9E-02
TUBB3	tubulin, beta 3			NM_006086.2	2.57	0.05	5.5E-03
IL1B	interleukin 1, beta	VSMC		NM_000576.2	2.54	0.13	3.4E-02
GFPT2	glutamine-fructose-6-phosphate transaminase 2	VSMC		NM_005110.1	2.52	0.23	1.3E-02
GREM1	gremlin 1, cysteine knot superfamily, homolog	VSMC		NM_013372.5	2.49	0.15	2.0E-02
MT1G	metallothionein 1G			NM_005950.1	2.48	0.07	3.3E-02
MMP1	matrix metallopeptidase 1	VSMC	MMPs	NM_002421.2	2.39	0.02	5.9E-04
GAL	galanin			NM_015973.2	2.25	0.15	3.0E-02
MCEMP1	mast cell-expressed membrane protein 1			NM_174918.1	2.17	0.15	1.2E-02
DARC	Duffy blood group, chemokine receptor			NM_002036.2	2.15	0.15	2.9E-02
ITGA10	integrin, alpha 10		ECM_regulation	NM_003637.3	-2.04	0.19	1.6E-02
HEY2	hairy/enhancer-of-split related with YRPW motif 2			NM_012259.1	-2.08	0.03	8.8E-03
COL4A5	collagen, type IV, alpha 5, transcript variant 3			NM_033381.1	-2.12	0.17	3.7E-02
FRZB	frizzled-related protein			NM_001463.2	-2.27	0.22	3.9E-02
PPP1R3C	protein phosphatase 1, regulatory subunit 3C			NM_005398.3	-2.39	0.14	2.4E-02
RGS5	regulator of G-protein signalling 5			NM_003617.2	-2.39	0.06	6.0E-03
ADH1A	alcohol dehydrogenase 1A, alpha polypeptide		lipid metabolism	NM_000667.2	-2.49	0.11	8.4E-03
CYTL1	cytokine-like 1			NM_018659.2	-3.54	0.22	1.4E-02

* p-values obtained by a Bayesian oneway Anova including Tukey's postHoc test. N=25 genes fhad differential gene expression levels between the six patients with a poor MMF response and the eleven placebo treated patients (N=17 up in MMF, and N=8 down in MMF)



Supplemental Figure 1.

T cell inhibitor versus Placebo: Gene Set Enrichment Analysis (GSEA) large variation, and two treatment sub-groups.

Summary and Future Perspectives

Inflammation plays a major role during all phases of atherogenesis from plaque initiation up to plaque rupture (outlined in **chapter 1**). In this thesis the role of inflammation in the pathophysiology of atherosclerosis is examined from different angles. In part I the effect of various pro-inflammatory mediators is examined in the context of atherogenesis. In part II the relation of atherosclerosis with chronic inflammatory disorders is explored. Part III focuses on the anti-atherosclerotic effects of various immunomodulatory interventions.

Part I: Inflammation and Atherosclerosis

Summary

In **chapter 2** we evaluated whether mannose-binding lectin (MBL) plays a role in the development of atherosclerosis. MBL is a part of the complement cascade and plays an important role in the first line of defense of the innate immune system against pathogenic micro-organisms. We addressed this hypothesis in a large prospective case-control study nested in the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) population study. We showed that serum concentrations of MBL are associated with an increased risk of future coronary artery disease (CAD) in apparently healthy men. This relationship was independent of other cardiovascular risk factors. In women no such relation was observed.

The same EPIC-Norfolk study was used in **chapter 3** to explore the relation between chemokine (C-C motif) ligand 2 (CCL2) polymorphisms, monocyte chemoattractant protein-1 (MCP-1) serum levels and cardiovascular risk. Although MCP-1 has been implicated in promoting the atherosclerotic process in various animal studies, human genetic evidence is contradictory. We found that individuals possessing the MCP-1-2136T alleles or the MCP-1+764G allele had higher MCP-1 levels. In addition, we showed that homozygotes of the MCP-1-3726C allele have an increased risk of CAD and that the presence of the MCP-1-2835A allele and that in males the MCP-1-2578G

allele is associated with an increased risk of CAD. Finally, we observed that CCL2 genotype is associated with both MCP-1 serum concentrations and the risk of future CAD. We therefore provide firm evidence that MCP-1 is involved in the pathogenesis of atherosclerosis and/or the progression into CAD.

In contrast to the prolonged exposure of the apparently healthy subjects of EPIC-Norfolk to (elevated) levels of MBL and MCP-1 the remainder of part I focuses on the atherosclerotic effects of acute inflammatory stimuli induced by either C-reactive protein (CRP) or endotoxin. CRP has recently been shown to exert various atherothrombotic effects and has emerged as a strong and independent predictor not only for cardiovascular risk but also the development of type 2 diabetes. In **chapter 4** we report the results of a proof-of-principle study designed to elucidate whether CRP affects glucose homeostasis. Seven healthy male volunteers were randomly assigned to receive a single bolus infusion of highly purified recombinant human rhCRP or CRP-free diluent in a crossover design. We were able to show that CRP induces an inflammatory response followed by increased norepinephrine and cortisol levels, which results in increased gluconeogenesis.

The abovementioned atherothrombotic actions mediated by CRP were recently shown to be counterregulated by high-density lipoprotein cholesterol (HDL) in an in vitro study. In **chapter 5** we studied whether HDL has the ability to neutralize atherothrombotic effects of CRP in humans. Fifteen healthy male volunteers received an infusion of recombinant human rhCRP. In 8 of these volunteers, an infusion of human apoAI reconstituted with phosphatidylcholine (apoAI-PC) preceded rhCRP infusion. Infusion of apoAI-PC prior to rhCRP apoAI-PC blunted the release of the pro-inflammatory cytokines IL-6 and IL-8 and diminished thrombin generation and fibrinolysis activation as illustrated by decreased levels of F1+2 and D-dimer, respectively.

In order to gain more insight in the atheroprotective effects of HDL by evaluating its ability to neutralize pro-inflammatory stimuli, we extended these findings in another model in **chapter 6**. Thirteen healthy men with genetically defined isolated

low HDL and 14 age-, and body weight-matched men with normal/high HDL levels were challenged by infusion of endotoxin. Both the inflammatory response as well as thrombin generation was significantly increased in the low HDL-group upon endotoxin challenge.

Future perspectives

Although it has now been firmly established that inflammation plays a major role during all phases of atherothrombosis, there is a lot that remains to be elucidated with regard to the (variable) roles of the numerous implicated biomarkers, chemokines, cytokines and immune cells during the different stages of the atherosclerotic process. In particular, clarification of the underlying mechanisms responsible for continuous immune activation and attenuated plaque stability are a focus of attention. A wide variety of approaches and study models can be employed to achieve these goals. A promising technique in this regard is in vivo cellular and molecular imaging, which enables the visualization of cellular recruitment in atherogenesis and subsequent atheroma inflammation. It remains to be established whether a single dominant mediator can be identified of which inhibition leads to profound beneficial effects in humans, as is the case with tumor necrosis factor-a in rheumatoid arthritis.

Part II: The Echo of Inflammation

Summary

In **chapter 7** we review a validated surrogate marker for atherosclerotic vascular disease, the ultrasonographic measurement of the intima-media thickness (IMT) of the carotid arterial walls. It enables reproducible and noninvasive assessment of the carotid artery wall and can be used to identify populations at risk as well as to assess drug efficacy.

To evaluate whether Crohn's disease (CD), characterized by inflammatory exacerbations, is associated with an increased progression of the atherosclerotic process we used IMT in an exploratory study (**chapter 8**). Carotid IMT in patients

with CD was significantly increased compared to healthy volunteers. In addition, CD patients during an inflammatory exacerbation were characterized by profoundly decreased levels of HDL combined with biochemical changes of the HDL particle such as an increased content of serum amyloid A (SAA) and a reduced ability to attenuate oxidation compared to controls.

We are also performing a study in which IMT measurements are performed in patients who have been diagnosed with rheumatoid arthritis (RA) within the last six months as well as subjects with elevated serum levels of RA specific autoantibodies. We now performed an interim analysis of this study (**chapter 9**), which revealed that individuals with RA specific autoantibodies are characterized by increased mean IMT indicative of accelerated atherosclerosis. Moreover, IMT was associated with age, ESR and ACPA levels in univariate analysis. More patients recently diagnosed with RA will need to be included.

Finally, we provide an overview of the association of several chronic inflammatory disorders and acceleration of the atherosclerotic process (**chapter 10**). Direct and indirect consequences of a systemic inflammatory state that mediate atherothrombotic disease are discussed as well as pathophysiological mechanisms specific to SLE (systemic lupus erythematosus) or RA that can also contribute to this. Especially considering the fact that pathophysiological processes involved in these disorders can occur at a subclinical level years before a diagnosis is made, it was concluded early cardiovascular prevention seems essential in these patients.

Future perspectives

Direct and indirect consequences of a systemic inflammatory state mediate atherothrombotic disease. Indeed several chronic inflammatory disorders, such SLE and RA are associated with an increased incidence of cardiovascular disease. As such, it is likely that in the future an association with atherosclerotic vascular disease will be confirmed in other inflammatory disorders as well. Although the association between RA as well as SLE with atherosclerotic vascular disease was identified decades ago, surprisingly enough no progress whatsoever has been made with regard to (primary)

prevention. In comparison, a multicenter randomized placebo-controlled trial (RCT) evaluating statin therapy in patients with diabetes has been performed years ago demonstrating robust clinical benefit. Clearly, similar studies are crucial in SLE and RA. Unfortunately, such a trial in SLE has already failed due to miscellaneous problems (Arthritis Rheum. 2005 Oct 15;53(5):718-23). Recently, the effect of fluvastatin on cardiac events was evaluated in renal transplant recipients with SLE in the ALERT trial (Assessment of LEscol in Renal Transplantation). Compared with placebo-treated patients, patients randomized to receive fluvastatin exhibited a 73.4% reduction in the risk of major cardiac events (Arthritis Rheum. 2009 Apr;60(4):1060-4). A RCT comparing statin therapy to placebo in SLE will nevertheless need to be performed and the same holds true for RA. Such a trial has already been performed in RA, unfortunately the authors neglected to include surrogate markers of atherosclerotic vascular disease (Lancet. 2004 Jun 19;363(9426):2015-21). Currently the TRACE RA study (Trial of Atorvastatin for the primary prevention of Cardiovascular Events and overall improvement of RA outcome) has included close to thousand patients. This is a prospective, 5-year, multi-centre, randomized, double blind, placebo-controlled trial that will assess the hypothesis that atorvastatin is more effective than placebo in the primary prevention of cardiovascular events in patients with RA. Hopefully, the results of the ALERT trial and TRACE RA study will finally usher in a new age for the prevention and treatment of CVD in patients with inflammatory conditions such as SLE and RA.

Part III: Immunomodulation and atherogenesis

Summary

There is an abundance of evidence supporting an atheroprotective role of HDL. Indeed, HDL appears to exert various anti-atherothrombotic mechanisms, one of which is its anti-inflammatory potential as was also demonstrated in chapters 5 and 6. As such, raising HDL levels is an attractive anti-atherosclerotic strategy. Over the past few years, attempts to raise HDL levels have been particularly successful with

small molecule inhibitors of cholesteryl ester transfer protein (CETP). **Chapter 11** describes the results of a RCT with the CETP inhibitor torcetrapib (RADIANCE 1 Rating Atherosclerotic Disease change by Imaging with a new CETP Inhibitor). In patients with heterozygous familial hypercholesterolemia (FH), the use of torcetrapib with atorvastatin did not result in further reduction of progression of atherosclerosis as assessed by a combined measure of carotid arterial wall thickness and, when restricted to the common carotid segment, caused progression of disease. These effects occurred despite an unparalleled increase of HDL and a substantial additional decrease of low-density lipoprotein cholesterol (LDL) and triglyceride levels.

In order to gain more insight into the mechanism(s) underlying the adverse outcome of these studies with torcetrapib, we merged the databases of the RADIANCE 1 and 2 studies and performed exploratory analyses into the parameters that were associated with cIMT progression and on-trial blood pressure changes (**chapter 12**). In these analyses we focused primarily on parameters that are related to CETP inhibition as a mechanism (i.e. on-trial changes in lipoproteins) as well as on parameters that are presumably connected to off-target toxicity (i.e. on-trial blood pressure and electrolyte changes). These analyses provide evidence in support of off-target toxicity, relating to mineralocorticoid excess, which might have contributed to the adverse outcome of torcetrapib use.

Because chronic systemic inflammation is a hallmark in both RA and atherosclerosis where macrophage migration inhibitory factor (MIF) and TNFa both play a crucial role we hypothesized that TNFa blockade may attenuate the increased cardiovascular risk associated with RA by decreasing not only local joint inflammation but also systemic inflammation (**chapter 13**). Sixteen weeks after initiation of TNFa blockade, MIF levels were significantly decreased which was sustained up to week 52. HDL cholesterol levels increased at week 16, but returned to baseline at week 52. ApoAI levels increased at week 16 and remained elevated up to week 52 which resulted in an improved ApoB/ApoAI ratio. The DAS28 score as well as CRP levels and ESR all decreased significantly after 16 and 52 weeks of adalimumab therapy.

In **chapter 14** we provide an overview of the anti-atherosclerotic effects of the immunomodulatory drug mycophenolate mofetil (MMF) on major components of the atherosclerotic plaque such as T-lymphocytes, monocytes/macrophages and the endothelium. MMF can inhibit leukocyte recruitment to the subendothelium and the subsequent reduced activation of leukocytes is suggested to translate into attenuation of subendothelial cross-talk between T-cells and macrophages. It was hypothesized this cascade of events will interrupt the self-perpetuating pro-inflammatory environment within the arterial wall, the hallmark of atherosclerotic vascular disease.

Finally, in **chapter 15** we evaluated whether short-term treatment with MMF in symptomatic patients scheduled for carotid endarterectomy was associated with altered cellular infiltration and/or changes in plaque inflammatory activity, assessed with mRNA expression profiling. Immunostaining analyses revealed a reduction of activated T-cells in MMF-treated patients compared to controls as well as an increase of regulatory T-cells.

Micro-array analyses confirmed beneficial changes to plaque phenotype, showing a strongly reduced expression of pro-inflammatory genes. Profound reduction in expression of metalloproteinases and osteopontin was present in three out of nine MMF-treated patients compared to nil in the placebo group.

Future perspectives

Immumodulatory interventions to attenuate the atherosclerotic process hold great promise for future human therapies. Encouraging results have been reported with immunosuppression, active immunization, induction of tolerance, intravenous immunoglobulin and manipulation of the cytokine network. Similarly, stimulation of endogenous anti-inflammatory mechanisms such as enhancement of the function of regulatory T-cells or T-cell coinhibition was associated with considerably reduced atherosclerotic burden. These strategies however, have almost exclusively been tested in animal models and the promising hypothesis of immunomodulation will now need to addressed in humans to evaluate its efficacy as well as the risks associated with such interventions.

Samenvatting en Toekomstperspectieven

Ontsteking speelt een belangrijke rol gedurende alle stadia van atherogenese, vanaf het ontstaan tot aan de ruptuur van de plaque (zoals samengevat in **hoofdstuk 1**). In dit proefschrift is de rol van ontsteking in de pathofysiologie van atherosclerose onderzocht vanuit verschillende invalshoeken. In deel I is het effect van verscheidene pro-inflammatoire mediatoren in de context van atherogenese onderzocht. In deel II is de relatie tussen atherosclerose en chronische ontstekingsziekten bestudeerd. Deel III behandelt de anti-atherosclerotische effecten van diverse immunomodulatoire interventies.

Deel I: Ontsteking en atherosclerose

Samenvatting

In **hoofdstuk 2** hebben we geëvalueerd of mannose-binding lectin (MBL) een rol speelt bij de ontwikkeling van atherosclerose. MBL is een onderdeel van de complement cascade en speelt een belangrijke rol in de eerste verdedigingslinie van de aangeboren afweer tegen pathogene micro-organismen. Wij hebben deze hypothese toegepast op een grote prospectieve case-control studie, ingebed in de European prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) populatie studie. Wij toonden aan dat serum concentraties van MBL geassocieerd zijn met een verhoogd risico op toekomstig coronairlijden bij ogenschijnlijk gezonde mannen. Deze associatie was onafhankelijk van andere cardiovasculaire risicofactoren. Bij vrouwen werd deze relatie niet waargenomen.

Dezelfde EPIC-Norfolk studie is gebruikt in **hoofdstuk 3** om de relatie tussen chemokine (C-C motif) ligand 2 (CCL2) polymorphismen, monocyte chemoattractant protein-1 (MCP-1) serum concentratie en cardiovasculair risico te onderzoeken. Hoewel een rol van MCP-1 in het versnellen van het atherosclerotisch proces in diverse dierenstudies wordt gesuggereerd, blijven humane genetische data tegenstrijdig. We vonden dat personen met het MCP-1-2136T allel of het MCP-1+764G allel hogere MCP-1

concentraties hadden. Bovendien toonden we aan dat homozygoten voor het MCP-1-3726C allel een verhoogde kans hebben op coronairlijden en dat de aanwezigheid van het MCP-1-2835A allel en het MCP-1-2578G allel is geassocieerd met een hogere kans op coronairlijden. Tenslotte observeerden we dat het CCL2 genotype is geassocieerd met zowel MCP-1 serumconcentratie als het risico op toekomstig coronairlijden. Derhalve leveren wij solide bewijs voor een rol van MCP-1 in de pathogenese van atherosclerose/progressie tot coronairlijden.

In tegenstelling tot de langdurige blootstelling van de ogenschijnlijk gezonde individuen van EPIC-Norfolk aan (verhoogde) concentraties van MBL of MCP-1, richt de rest van deel I zich op de atherosclerotische effecten van acute inflammatoire stimuli geïnduceerd door C-reactive protein (CRP) of endotoxine. Recent is aangetoond dat CRP verschillende atherotrombotische effecten heeft en heeft het zich ontwikkeld tot een sterke en onafhankelijke voorspeller van zowel cardiovasculair risico als het ontwikkelen van diabetes mellitus type 2. In hoofdstuk 4 rapporteren we de resultaten van een proof-of-principle studie die is ontworpen om te achterhalen of CRP de glucose homeostase beïnvloedt. Zeven gezonde mannelijke vrijwilligers ontvingen willekeurig een infusie van hoogwaardig gezuiverd recombinant humaan CRP of het CRP-vrije oplosmiddel in een cross-over design. We konden aantonen dat CRP een inflammatoire respons induceert gevolgd door een verhoging van norepinefrine en cortisol concentraties, hetgeen resulteerde in toegenomen gluconeogenese.
Van enkele van de reeds vermelde atherotrombotishe effecten van CRP werd recent aangetoond dat ze kunnen worden tegen gegaan door high-density lipoprotein cholesterol (HDL) in een in vitro studie. In hoofdstuk 5 onderzoeken we of HDL de atherotrombotische effecten van CRP kan neutraliseren in mensen. Vijftien gezonde mannelijke vrijwilligers ontvingen een infusie van recombinant humaan CRP. In 8 van deze vrijwilligers ging er een infusie van humaan apoAI gereconstitutioneerd met phosphatidylcholine (apoAI-PC) vooraf aan de CRP infusie. Infusie van apoAI-PC voor CRP infusie voorkwam het vrijkomen van pro-inflammatoire cytokines IL-6 en IL-8 en verminderde thrombine generatie en fibrinolyse activatie zoals geïllustreerd door afgenomen concentraties van respectievelijk F1+2 and D-dimeer.

Teneinde meer inzicht te krijgen in de atheroprotectieve capaciteiten van HDL, door middel van zijn vermogen om pro-inflammatoire stimuli te neutraliseren te evalueren, hebben we deze bevindingen uitgebouwd in hoofdstuk 6. Dertien gezonde mannen met genetisch bepaald geïsoleerd laag HDL en veertien leeftijd- en gewicht gematchte mannen met normaal/hoog HDL ondergingen een infusie met endotoxine. Zowel de inflammatoire respons als thrombine generatie waren significant hoger in de groep met laag HDL.

Toekomstperspectieven

Hoewel nu vaststaat dat ontsteking een belangrijke rol speelt tijdens alle fases van atherotrombose is er nog veel dat uitgezocht dient te worden met betrekking tot de (wisselende) rol van de grote hoeveelheid betrokken biomarkers, chemokines, cytokines en immuuncellen gedurende de verschillende stadia van het atherosclerotisch proces. Opheldering van de onderliggende mechanismen verantwoordelijk voor voortschrijdende immuunactivatie en verminderde stabiliteit van de plaque zijn met name een focus van aandacht. Veel verschillende benaderingen en studiemodellen kunnen worden aangewend om deze doelen te bereiken. Een veelbelovende techniek binnen dit kader is de in vivo cellulaire en moleculaire beeldvorming, hetgeen visualisatie van cellulaire rekrutering in atherogenese en de daaropvolgende atheroom ontsteking mogelijk maakt. Er dient nog te worden vastgesteld of er een enkele dominante mediator kan worden geïdentificeerd waarvan remming leidt tot aanzienlijk gunstige effecten in mensen, zoals het geval is bij tumor necrosis factor-a in reumatoïde artritis.

Deel II: De Echo van Ontsteking

Samenvatting

In **hoofdstuk 7** geven we een overzicht van een gevalideerde surrogaat marker van atherosclerotisch vaatlijden, de ultrasonografische bepaling van de intima-media dikte (IMD) van de wanden van de arteria carotis. Zodoende kan een reproduceerbare en

noninvasieve meting worden verricht aan de vaatwand van de carotiden en kunnen populaties met een verhoogd risico worden geïdentificeerd en kan de efficiënte van farmacologische interventies geëvalueerd worden.

Om te evalueren of de ziekte van Crohn, gekarakteriseerd door inflammatoire exacerbaties, is geassocieerd met een toegenomen progressie van het atherosclerotisch proces hebben we IMD gebruikt in een exploratieve studie (**hoofdstuk 8**). IMD van de carotiden van patiënten met de ziekte van Crohn was significant toegenomen vergeleken met gezonde vrijwilligers. Bovendien waren patiënten met de ziekte van Crohn gedurende een inflammatoire exacerbatie gekarakteriseerd door aanzienlijk verlaagde HDL concentraties gecombineerd met biochemische veranderingen van het HDL partikel zoals een toegenomen inhoud van serum amyloid A (SAA) en een verminderde capaciteit om oxidatie tegen te gaan, wanneer vergeleken met gezonde vrijwilligers.

We zijn ook een studie aan het verrichten waarin IMD metingen worden gedaan in zowel patiënten die zijn gediagnosticeerd met reumatoïde artritis (RA) in de afgelopen 6 maanden als in personen met verhoogde concentraties van RA-specifieke antilichamen. We hebben nu een interim analyse van deze studie uitgevoerd (**hoofdstuk 9**) waaruit blijkt dat personen met verhoogde concentraties van RA-specifieke antilichamen worden gekarakteriseerd door een toegenomen IMD, indicatief voor versnelde atherosclerose. Bovendien was IMD geassocieerd met leeftijd, BSE en ACPA niveau in een invariant analyse. Meer patiënten met recent gediagnosticeerde reumatoïde artritis zullen moeten worden geincludeerd.

Tenslotte geven we een overzicht van de associatie van diverse chronische ontstekingsziekten en versnelling van het atherosclerotisch proces (**hoofdstuk 10**). Directe en indirecte consequenties van een systemische inflammatoire staat die atherotrombose faciliteren worden besproken alsmede pathofysiologische mechanismen specifiek voor SLE (systemic lupus erythematosus) en RA die hieraan bijdragen. Zeker gezien het feit dat de pathofysiologische processen van deze aandoeningen zich jarenlang op een subklinisch niveau kunnen afspelen voordat een diagnose kan worden gemaakt, kan er worden geconcludeerd dat cardiovasculaire preventie in deze patiënten essentieel lijkt.

Toekomstperspectieven

Directe en indirecte consequenties van een systemische inflammatoire staat faciliteren atherotrombose. Verschillende chronische ontstekingsziekten, zoals SLE en RA, zijn inderdaad geassocieerd met een verhoogde incidentie van hart- en vaatziekten. Zodoende is het aannemelijk dat er in de toekomst een associatie met atherosclerotisch vaatlijden in andere chronische ontstekingsziekten zal worden vastgesteld. Hoewel de associatie van zowel RA als SLE met atherosclerotisch vaatlijden enkele decennia geleden al werd geïdentificeerd, is er opmerkelijk genoeg totaal geen vooruitgang geboekt met betrekking tot (primaire) preventie. Ter vergelijking, een multicenter, gerandomiseerde, placebogecontroleerde studie die het effect van statine therapie in diabetes patiënten evalueert werd jaren geleden uitgevoerd en toonde aanzienlijke gunstige klinische effecten. Het is duidelijk dat dergelijke studies in SLE en RA cruciaal zijn. Helaas is een dergelijke studie in SLE al eens mislukt door uiteenlopende oorzaken (Arthritis Rheum. 2005 Oct 15;53(5):718-23). Recent werd het effect van fluvastatine op cardiale events geëvalueerd in SLE-patiënten met een niertransplantatie in de ALERT trial (Assessment of LEscol in Renal Transplantation). Vergeleken met placebobehandelde patiënten, hadden patiënten gerandomiseerd naar fluvastatine een reductie van 73.4% van het risico op grote cardiale events (Arthritis Rheum. 2009 Apr;60(4):1060-4). Een RCT die statine therapie vergelijkt met placebo zal desalniettemin moeten worden verricht in SLE en hetzelfde geldt voor RA. Een dergelijke studie werd al uitgevoerd in RA maar helaas hebben de auteurs nagelaten surrogaatmarkers van atherosclerotisch vaatlijden te meten (Lancet. 2004 Jun 19;363(9426):2015-21). Momenteel heeft de TRACE RA studie (Trial of Atorvastatin for the primary prevention of Cardiovascular Events and overall improvement of RA outcome) bijna duizend patiënten geincludeerd. Dit is een prospectieve, 5-jaar, multicenter, gerandomiseerde, dubbelblinde, placebogecontroleerde studie die de hypothese toetst dat atorvastatine effectiever is dan placebo in de primaire preventie van hart- en vaatziekten in patiënten met RA. Hopelijk leiden de resultaten van de ALERT trial en de TRACE RA studie een nieuw tijdperk in wat betreft de preventie en behandeling van hart- en vaatziekten in patiënten met chronische ontstekingsziekten zoals SLE en RA.

Deel III: Immuunmodulatie en atherogenese

Samenvatting

Er is een overvloed aan bewijs van de atheroprotectieve rol van HDL. HDL heeft verscheiden anti-atherotrombotische effecten, een daarvan is het anti-inflammatoire effect zoals ook aangetoond in hoofdstuk 5 en 6. Het verhogen van HDL niveaus is dus een aantrekkelijke anti-atherosclerotische strategie. Over de afgelopen jaren waren pogingen om HDL niveaus te verhogen het meest succesvol met klein moleculaire remmers van het enzym cholesterol ester transfer protein (CETP). **Hoofdstuk 11** beschrijft de resultaten van een RCT met de CETP-remmer torcetrapib (RADIANCE 1 Rating Atherosclerotic Disease change by Imaging with a new CETP Inhibitor). In patiënten met de heterozygote vorm van familiaire hypercholesterolemie (FH) leidde het gebruik van torcetrapib met atorvastatine niet tot verdere reductie van progressie van atherosclerose zoals geverifieerd door middel van gecombineerde metingen van de IMD van de arteria carotis, en leidde zelfs, wanneer enkel gekeken naar de arteria carotis communis, tot ziekte progressie. Deze effecten traden op ondanks een nog niet eerder vertoonde stijging van HDL en een substantiële additionele daling van low-density lipoprotein cholesterol (LDL) en triglyceride niveaus.

Teneinde meer inzicht te verkrijgen in de mechanisme(n) achter de onverwachte uitkomst van de studies met torcetrapib hebben we de databases van de RADIANCE 1 en 2 studies samengevoegd en hebben we een exploratieve studie uitgevoerd naar de parameters die waren geassocieerd met IMD progressie en veranderingen van bloeddruk (**hoofdstuk 12**). In deze analyses richtten we ons voornamelijk op parameters die zijn gerelateerd aan CETP remming als mechanisme (veranderingen in lipoproteinen) alsmede parameters die mogelijk samenhangen met ongewenste bijwerkingen (veranderingen van bloeddruk en electrolyten). Deze analyses vormen bewijs voor ongewenste bijwerkingen van torcetrapib die samenhangen met een mineralocorticoïd overschot en die mogelijk hebben bijgedragen aan de onverwachte uitkomst van de studies met torcetrapib.

Omdat chronische systemische ontsteking een kenmerk is van zowel RA als atherosclerose, en macrophage migration inhibitory factor (MIF) en TNFa een belangrijke rol spelen in allebei deze aandoeningen, hypothetiseerden wij dat TNF blokkade mogelijk het toegenomen cardiovasculaire risico in RA vermindert niet alleen via afgenomen lokale gewrichtsontsteking maar systemische ontsteking (**hoofdstuk 13**). Zestien weken na de start van TNF blokkade waren MIF niveaus significant verminderd hetgeen aanhield tot 52 weken. HDL was toegenomen op week 16 maar daalde terug tot het baseline niveau op week 52. ApoAI niveaus waren verhoogd op week 16 hetgeen zo bleef tot week 52 en resulteerde in een verbetering van de apoB/apoAI ratio. Zowel de DAS28 score als CRP niveaus en BSE waren allen significant verminderd na 16 en 52 weken adalimumab therapie.

In **hoofdstuk 14** geven we een overzicht van de anti-atherosclerotische effecten van het immuunmodulatoire medicijn mycofenolaat mofetil (MMF) op belangrijke onderdelen van de atherosclerotische plaque zoals T-lymfocyten, monotypen/macrofagen en het endotheel. MMF kan leukocyt rekrutering naar het subendotheel remmen en er wordt gesuggereerd dat de daaropvolgende afname van leukocyt activering zich vertaald in verminderde overspraak tussen T-cellen en macrofagen. Er werd gehypothetiseerd dat deze cascade van gebeurtenissen zal leiden tot een onderbreking van de zichzelf in stand houdende ontstekingshaard in de arteriewand, karakteristiek voor atherosclerotisch vaatlijden.

Tenslotte evalueren we in **hoofdstuk 15** of korte termijn behandeling van symptomatische patiënten die reeds stonden ingepland voor een endarteriëctomie van de arteria carotis met MMF was geassocieerd met een gewijzigde cellulaire infiltratie en/of veranderingen in ontstekingsactiviteit van de plaque, zoals geanalyseerd door middel van mRNA expressie profielen. Immunohistochemische analyses onthulden zowel een afname van geactiveerde T-cellen in MMF-behandelde patiënten vergeleken met controles als een toename van regulatoire T-cellen. Micro-array analyses bevestigden gunstige veranderingen van het plaque fenotype en toonden een sterk afgenomen expressie van pro-inflammatoire genen. Aanzienlijke vermindering in de expressie van metalloproteinases en osteopontin was aanwezig in drie van de negen MMF-behandelde patiënten vergeleken met nul in de placebo groep.

Toekomstperspectieven

Immuunmodulatoire interventies als middel om het atherosclerotisch proces af te remmen vormen een grote belofte voor toekomstige behandelingen in mensen. Aanmoedigende resultaten zijn geboekt met immuunsuppressie, actieve immunizatie, induceren van immuuntoleratie, intraveneuze immunoglobulines en het beïnvloeden van het cytokine netwerk. Stimulatie van endogene anti-inflammatoire mechanismen zoals het versterken van de functie van regulatoire T-cellen of T-cel co-inhibitie was eveneens geassocieerd met een aanzienlijke vermindering van atherosclerose. Deze strategieën zijn echter bijna exclusief getest in diermodellen en de veelbelovende hypothese van immuunmodulatie zal moeten worden getoetst in mensen om de effectiviteit alsmede de geassocieerde risico's te evalueren.

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Curriculum vitae

Sander van Leuven was born on March 1st 1978 in Gouda, the Netherlands. After he graduated from secondary school at the Coenecoop College in Waddinxveen in 1996, he started his medical training at the University of Amsterdam. In 2001, he partcipated as a medical student in a research program at McMaster University in Hamilton, Ontario, Canada. Under supervision of Dr. Janusz Rak he analyzed the expression of vascular endothelial growth factor (VEGF) of various malignant cell lines under pro-inflammatory and pro-coagulant conditions. In 2002, he returned to the Netherlands to start the clinical phase of his medical training. This was completed in 2004 as a student doctor at the Ndala Hospital in the Tabora region, Tanzania.

Sander van Leuven received his medical degree in March 2004 and started his PhD program at the department of vascular medicine at the University of Amsterdam. Most of the research performed during this period is presented in this thesis. In April 2008, he started his residency in Internal Medicine at Tergooiziekenhuizen, Hilversum (under supervision of Dr. S. Lobatto).

Sander van Leuven is a recipient of the 2006 Future Forum Research Grant, 2007 Dutch Atherosclerosis Society Fellowship and 2008 International Atherosclerosis Society Fellowship award.

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