Leukocyte activation and inflammation in cardiovascular disease

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Introduction and aims of the thesis

Mechanisms and correlates of inflammation in atherosclerosis

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INTRODUCTION

Atherosclerosis is the name for a complex of pathological changes affecting the large and middle large arteries of the human body. It ultimately results in occlusive disease of the coronary arteries, carotid or cerebral arteries and peripheral arteries, manifesting itself as (unstable) angina pectoris, acute myocardial infarction, ischaemic stroke or intermittent claudication. This chapter aims to provide a short review of the historical views, epidemiological features and the current pathophysiological concepts of atherosclerotic vascular disease. Furthermore, it will focus on correlates of the inflammatory response in a clinical perspective.

1. HISTORICAL OVERVIEW

Atherosclerosis and its clinical correlates are of all times. Atherosclerotic lesions have already been found in Egyptian mummies dating from 1580 B.C. [1] The term atherosclerosis was brought up by Marchand in 1904 [2]. He described the ‘gruel-like’ (athere) soft contents of the plaque core (atheroma) that is covered by a hardened (scleros) fibrous cap. Before that time the disease was known as arteriosclerosis [3]. The first description of the disease is ascribed to Parry who, in 1799, recounts an anecdote in which, during the course of an autopsy, he discovered something hard and gritty in the coronary arteries and "well remembered looking up to the ceiling, which was old and crumbling, conceiving that some plaster (sic) had fallen down." From his discovery of hardened, “ossified” vessels he proposed that a principle cause of the syncope anginosa is to be looked for in disordered coronary arteries [4].

A few decades later, it was the surgeon Joseph Hodgson who published a monograph on vascular disease, claiming that inflammation was the underlying cause of atherosclerosis in spite of being a natural degenerative manifestation of the aging process. He also identified that the disease process occurred in the intima, between the lumen and the media of the diseased vessels [5]. After the initial studies based solely on descriptive studies, three theories have been dominating the discussion on the pathophysiology of atherosclerosis: the thrombogenic theory propagated by Rokitansky, the inflammatory theory of which Virchow was the advocate, and the lipid/insudation theory. The last theory has been adapted by Ross, who unified the concepts of atherogenesis in the current response-to-injury theory [6;7].

Thrombosis has been suggested as a cause of occlusive vascular disease by Rokitansky in 1841. He proposed that the deposits observed in the inner layer of the arterial wall were primarily derived from fibrin and other blood elements. The atheroma was the result of a pre-existing ‘crasis’ of blood components [8]. Rokitansky was opposed by Virchow who gave an extensive description of the various stages of atherosclerotic disease that he named "endarteritis deformans". By this, he meant that the atheroma was a product of an inflammatory process within the intima, and that the fibrous thickening had evolved as a consequence of a reactive fibrosis induced by proliferating connective tissue cells. He maintained that mechanical forces initiated the irritative stimulus and that the endarteritis was part of a repair mechanism. To date, Virchow’s concept of local intima injury as the initiating irritative stimulus is still accepted and it has been extended to include other factors besides mechanical factors [9]. After Virchow’s discoveries the focus gradually moved to the concept of a slowly progressive disease starting at a young age in which lipids accumulated in the vascular wall until a significant stenosis occurred later in life. Cholesterol, a main composite of the atherosclerotic plaque, emerged as a
major risk factor for atherosclerotic cardiovascular disease emerged [10]. One of the most important contributors to this discussion was the Russian scientist, Ignatovski, who noticed that rabbits that were fed a diet of milk and egg yolk, developed severe atherosclerosis [10]. These findings formed the basis for large epidemiological trials that confirmed the importance of serum cholesterol levels as a major cardiovascular risk factor. Experimental atherosclerosis was further investigated by Anischkov. His view was that atherosclerosis was merely an infiltrative rather than a degenerative disease, this view was further explored in the insudation theory. He was the first to acknowledge that the cause was most probably multifactorial and the consequence of a metabolic disturbance at the level of the vessel wall [11]. The insudation theory claimed that noxious elements in the blood damaged the endothelium [12] and it formed the basis for the response-to-injury theory of Ross. First the focus was on mechanical stress that would injure the endothelium. Later, inflammation came into focus again, and as molecular techniques provided new opportunities to gain insight in cellular and molecular mechanisms, it became clear that atherosclerosis was an inflammatory disease [13-15].

2. EPIDEMIOLOGICAL FEATURES OF ATHEROSCLEROSIS AND VASCULAR DISEASE

Acute coronary syndromes, stroke and peripheral artery disease are all clinical manifestations of a common pathophysiology: atherosclerosis of the large arteries. Taken together these diseases are still the leading cause of mortality and morbidity in the Western society and emerging as a major cause of death in other parts of the world [16;17]. Although declining, still 36% (49,952 persons) of all deaths in the Netherlands in 2000 was caused by cardiovascular disease. The major cause of death in this group was ischaemic heart disease 34.9% (17,443 persons) followed by cerebrovascular disease that accounted for 19.7% of cardiovascular mortality. Cardiovascular morbidity led to 262,121 hospitalizations in 2001. The majority occurred because of ischaemic heart disease (37.1% of all men and 24.8% of all women) [18] with 25,864 patients suffering a heart attack, 33,416 patients suffering from unstable angina. Forty-four percent of the hospitalized patients for cardiovascular disease was younger than 65 years [18]. Thus, great efforts are still needed to reduce the morbidity and mortality of cardiovascular disease due to widespread atherosclerosis.

3. RISK FACTORS AND MARKERS

Although there is a vast amount of evidence that links ‘traditional’ risk factors to atherosclerotic vascular disease, only half of the cases of clinical atherosclerotic disease can be explained by such risk factors [19]. A huge number of risk factors has been identified [20] and new ones still arise [21]. Although most risk factors have a genetic background, risk-reduction by either lifestyle modification or pharmacotherapy can be achieved for many risk factors (table 1). However, the incidence and prevalence of important contributors to cardiovascular disease such as obesity and diabetes [22] are still rising. Regardless of the fact that important improvements have been made in the treatment of dyslipidemia, cholesterol levels in patients with coronary heart disease are still far from ideal [23]. Notably, a number of important risk factors is thought to contribute to atherogenesis by inducing an inflammatory response [24].

4. INFLAMMATION AND THE IMMUNE SYSTEM

Inflammation is the answer to noxious stimuli that threaten the organism. In humans there are two distinctive defence mechanisms that form the base of any
### Table 1. Genetic and environmental risk factors for atherosclerosis.

<table>
<thead>
<tr>
<th>Factors with a strong genetic background</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated LDL and VLDL cholesterol</td>
<td>Abundant evidence from epidemiological studies. Several genotypes identified. Modifiable risk factor with strong beneficial results.</td>
</tr>
<tr>
<td>Low HDL-cholesterol</td>
<td>Several genotypes identified, also non-genetic causes. Partly modifiable risk factor with results perhaps equal to LDL-lowering.</td>
</tr>
<tr>
<td>Elevated levels of Lp(a)</td>
<td>Single gene responsible. Unclear if modifiable, unclear if beneficial</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Several genes identified. Modifiable risk factor, beneficial effects.</td>
</tr>
<tr>
<td>Hyperhomocysteinemia</td>
<td>Multiple genes identified. Modifiable risk factor, effects not yet determined.</td>
</tr>
<tr>
<td>Family history for vascular disease</td>
<td>Multiple genetic causes identified. Partly modifiable, beneficial effects.</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Epidemiological data. Probably modifiable, effects are yet under investigation.</td>
</tr>
<tr>
<td>Depression and other behavioural traits</td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>Several genes identified. Possibly modifiable risk factor, effects currently under investigation.</td>
</tr>
<tr>
<td>Systemic inflammation</td>
<td>Partly modifiable risk factor, effects currently under investigation.</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>Several causes identified.</td>
</tr>
<tr>
<td>Auto-immunity</td>
<td></td>
</tr>
<tr>
<td>Maternal hypercholesterolaemia</td>
<td></td>
</tr>
</tbody>
</table>

### Environmental factors

| High-fat diet                             | Causal relationship not yet established. Possibly modifiable risk factors, currently under investigation. |
| Smoking                                  |                                                  |
| Low anti-oxidant status                  |                                                  |
| Lack of exercise                         |                                                  |
| Infectious agents                        |                                                  |

Inflammatory response: innate and adaptive immunity. Both systems have their own mechanisms of action but act in close co-operation (figure 1). The innate immune system provides the fastest but least specific response to toxic or inflammatory stimuli. It forms the first line of defence against micro-organisms and it is based on the recognition of pathogen-associated molecular patterns (PAMP). Its cellular components comprise monocytes /macrophages, ‘natural killer’ cells neutrophils, and mast cells. Once activated through contact with a PAMP or upon IFNγ stimulation by T helper type 1 lymphocytes, the cells of the innate immunity start producing substances (e.g. cytokines, growth factors, chemokines, and lysosomal enzymes such as myeloperoxidase) that mount a local inflammatory response and/or start with phagocytosis and lysosomal degradation of micro-organisms and/or presented particles. Moreover, through the expression of major histocompatibility complex (MHC) class II receptors on their surface, they act as antigen presenting cells to T lymphocytes.

The adaptive immune system elicits a slower but more specific immune response. Its cellular components are the B and T lymphocytes. Due to a process called somatic rearrangement these cells are able to recognise a large number of antigens by generating T cell receptors and immunoglobulins. Once T cells recognise foreign antigens presented to them, they initiate adaptive immune responses directed precisely against these antigens. These responses include a direct attack against the antigen by cytotoxic T cells, stimulation of B lymphocytes to become plasma cells and subsequently to produce antibodies against the antigens and stimulation of T helper 1 cells resulting in the induction of inflammation through activation of the innate immune system at the site where the antigen is present. An inappropriate adaptive immune response can cause auto-immune diseases [25-28].
5. THE PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis develops silently and is thought to begin already in fetal life [29]. It was Ross [6,7] who brought up the response-to-injury theory as an explanatory mechanism for the pathogenesis of atherosclerosis, a theory that is still in force these days. While it was first suggested that the injury had a mechanical character, he later proposed that it was an inflammatory response evoked by a huge number of stressors (atherogenic risk factors) that led to the atherosclerotic changes in the vessel wall [15]. Nowadays, the concept is that chronic inflammation results from interaction between genetic and environmental risk factors, especially modified lipoproteins, monocytes/macrophages, T lymphocytes and the normal cellular elements of the vessel wall [30]. The following paragraphs will provide a brief overview of the different stages in atherogenesis.

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**Figure 1.** Interplay between adaptive and innate immunity during atherogenesis. The principal effector cell of innate immunity, the macrophage, elaborates cytokines that critically regulate many functions of atheroma-associated cells involved with disease initiation, progression, and complication, as well as thrombosis. IFNγ, a product of the activated T-cell, activates a number of these functions of the macrophage. In turn, the activated macrophage expresses high levels of MHC class II antigens, needed for antigen-dependent activation of T-cells. Adapted from ref. 26.
The normal artery

The normal arterial wall consists of three layers. The inner layer or intima consists of the endothelium and the basal lamina that borders the media, the middle layer that is build up by smooth muscle cells together with connective tissue, mainly collagen and elastin. The outer layer is called the adventitia, this layer is merely composed of concentrically layered elastin fibres. Furthermore, it contains small blood vessels, the vasa vasorum, lymph vessels and nerves [3].

Endothelial dysfunction

In the normal vasculature, the endothelium forms the first barrier between the blood and the surrounding tissues. Under physiological circumstances the endothelium responds to physical and chemical stimuli by expressing a variety of chemical substances to prevent smooth muscle cell contraction and proliferation, platelet aggregation, thrombosis and leukocyte cell adhesion. All stages in atherogenesis are characterized by functional and morphological changes to the endothelium known as endothelial dysfunction (figure 2). Endothelial dysfunction is most likely to represent the earliest changes in atherogenesis. Endothelial dysfunction is a collective term that incorporates a number of changes that the endothelium undergoes during atherogenesis: loss of anticoagulant properties, an increased expression of cellular adhesion molecules and an increase in vascular tone due to loss of bioavailability of the vasodilatory and possibly anti-inflammatory endothelial nitric oxide (NO) [31;32]. Atherosclerotic lesions are preferentially located at sites of arterial branching or curvature, where there is an altered blood flow exposing the endothelium to an enhanced shear stress gradient. The endothelium at these places is more permeable and thus provides a location for macromolecules, such as low-density lipoprotein (LDL)-cholesterol, to deposit [33]. Multiple genes have been identified that are susceptible to changes in blood flow, such as platelet derived growth factor, transforming growth factor-β, tissue plasminogen activator, the adhesion molecules ICAM-1 and VCAM-1 and, recently identified, lung Kruppel-like factor [33-35]. It is believed that the upregulation of vascular adhesion molecule-1 by the endothelium is of crucial importance in the occurrence of atherosclerotic plaque formation. Most known risk factors either genetic or environmental are known to induce endothelial dysfunction. Especially oxidatively modified forms of LDL-cholesterol (oxLDL) are thought to be important inducers of endothelial dysfunction, but other risk factors most of them mentioned in table 1, alone or in concordance, also contribute to this process [36-39]. OxLDL induces phenotypical changes to the endothelial cells as they start expressing adhesion molecules, that facilitate adhesion of monocytes and T-lymphocytes to the endothelium [40], and produce chemokines that attract inflammatory cells from the circulation [41]. The atherogenic potential of oxLDL is summarised in table 2. The role of adhesion molecules will be discussed in more detail below.

In conclusion, due to environmental and genetic risk factors, the endothelium undergoes functional and structural changes at a very early age. Endothelial dysfunction induces monocyte adherence to the vessel wall.

Fatty streak formation

The first ‘visible’ manifestation of atherosclerosis is called the ‘fatty streak’. These are local elevations of the intima that consist of accumulations of so-called ‘foam cells’, transformed macrophages, loaded with oxLDL [3;15]. Since fatty streaks are located at sites where atherosclerosis is unlikely to develop, it is still disputed if these lesions are the precursors of the later complicated atherosclerotic plaques [3].
Chapter 1

Figure 2. Initiating events in the development of a fatty streak lesion. Monocytes attach to endothelial cells that have been induced to express adhesion molecules by several inflammatory stimuli, e.g. cardiovascular risk factors. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. Adapted from ref. 39.

Figure 3. Lesion progression. Interactions between macrophages and foam cells. Th1 and Th2 cells establish a chronic inflammatory process. Cytokines secreted by lymphocytes and macrophages exert both pro- and antiatherogenic effects of the cellular elements of the vessel wall. Smooth muscle cells migrate from the medial portion of the arterial wall, proliferate and secrete extracellular matrix proteins that form a fibrous plaque.

Figure 4. Plaque rupture and thrombosis. Necrosis of macrophage and smooth muscle cell-derived foam cells leads to the formation of a necrotic core and accumulation of extracellular cholesterol. Macrophage secretion of matrix metalloproteinases and neovascularisation contribute to weakening of the fibrous plaque. Plaque rupture exposes blood components to tissue factor, initiating coagulation, the recruitment of platelets, and the formation of a thrombus.
Monocytes are attracted from the circulation and adhere to the activated endothelial cells. Then, they transmigrate to the sub-endothelial layer where they transform into a specific subset of macrophages that express scavenger receptors. These receptors facilitate the uptake of oxLDL (figure 3). Accumulated foam cells set the pace for progression of atheroma since they are capable of producing a large number of substances such as cytokines, growth factors, chemokines and proteolytic enzymes [42].

**Advanced plaque**

Next, the smooth muscle cells (SMC) start growing and proliferating due to growth factor release from activated monocytes. Moreover, they migrate from the media to the subendothelial space. Here, they start producing extracellular matrix proteins that lead to the formation of the fibrous cap. The atheromatous lesion itself grows as well, due to monocyte/macrophage proliferation, inhibition of smooth muscle cell and leukocyte apoptosis and recruitment of T lymphocytes and monocytes from the circulation. Significant cross talk appears to occur among the cellular elements of the developing lesions, i.e. all participating cell types produce signalling molecules that influence the activity of the others. Lesional T cells are activated expressing both Th1 and Th2 cytokines. Macrophages, endothelial cells and SMCs are activated as well, since MHC Class II receptors are expressed and inflammatory products such as tumor necrosis factor-α (TNFα), monocyte chemotactic protein-1 (MCP-1) and interleukin-6 are produced [43;44]. The endothelial cells overlying the lesion gradually decline and a fibrous cap is formed, protecting the blood from the highly thrombogenic lipid core of the plaque. Initially, the vessel lumen is not affected by the proliferating cells due to a process called ‘remodelling’. By expanding outwards in ‘compensatory enlargement’ the artery accommodates the growing lesion. Eventually, the vessel lumen does diminish and a critical stage in atherogenesis has started.

**The vulnerable or unstable plaque**

The clinical consequences of atherosclerosis are the result of luminal obstruction. The cause for acute luminal obstruction in most cases, is the formation of a thrombus. Thus, the final push is being given by the coagulation system. However,

| Table 2. Possible mechanisms by which oxLDL can exert atherogenic effects. |
|--------------------------|--------------------------|
| **Effect**               | **Mechanism**            |
| Increased monocyte adhesion | Increased expression of adhesion molecules on endothelial cells |
| Increased monocyte and T-cell chemotaxis | Direct effects on monocytes and T cells and indirect effects due to stimulation of chemokine production (e.g. MCP-1) |
| Increased scavenger receptor A expression | Activation of AP-1 and its transcription factors |
| Increased CD36 expression | Activation of PPARγ |
| Increased foam cell formation | Enhanced uptake of oxLDL mediated by scavenger receptor |
| Induction of proinflammatory genes | Activation of NFκB |
| Induction of cellular and humoral immune responses | Neoepitope formation |
| Increased apoptosis and necrosis | Induction of tissue factor, increased platelet aggregation |
| Activation of programmed cell death, formation of cholesterol crystals, Loss of membrane integrity | |
| Enhanced procoagulant activity | |
it is the underlying atherosclerotic plaque and its ongoing inflammatory response that enhances thrombosis [45]. Most cases of acute myocardial infarction or sudden cardiac death are the result of the rupture of a so-called ‘vulnerable plaque’, whereas a minority of the cases is due to superficial thrombosis on a disrupted endothelium [46]. The atheromatous, highly thrombogenic, contents of the plaque core are protected from the circulation by the fibrous cap. Rupture of this cap causes exposure of the thrombogenic contents to the blood, subsequently leading to thrombosis (figure 4). The fibrous cap constitutes of interstitially formed collagen fibres. These fibres are mainly produced by smooth muscle cells under control of TGF-β. In a mouse model lack of TGF-β was associated with an unstable plaque phenotype [47].

There are several mechanisms that threaten the cap’s integrity. The production of interferon-γ (IFN-γ) by T cells that are present in the plaque, limits the production of collagen types I and III by smooth muscle cells, thus leading to thinner fibrous caps [48;49]. If produced in excess, the production of collagen degrading enzymes, such as matrix metalloproteinases (MMP), also takes place in a plaque, leading to a weaker fibrous cap. These MMPs are produced by macrophages and SMCs, and their production is enhanced by several pro-inflammatory mediators [50;51]. Thus, both macrophages and SMCs, probably directed by T lymphocytes, contribute to the transformation to an unstable, rupture prone plaque phenotype. It is currently thought that if possible switching the plaque’s balance from the unstable inflammatory to the hemodynamically stable fibrotic phenotype may prove beneficial in preventing the clinical complications of atherosclerosis [52;53].

Another possible contributor to plaque instability is the mast cell. Mast cells contain a large number of granules containing many mediators that may be involved in plaque rupture. Among these mediators are the proteolytical enzymes chymase and tryptase [54]. These enzymes are capable of activating MMPs [55]. Multiple studies support the view that mast cell activation could be linked with plaque rupture, since they documented accumulation of mast cells and increased expression of MMPs in the shoulder region of the plaque [56]. In pathological studies it proved that the amount of mast cells seen in the plaque was far less than that of monocytes/macrophages or T lymphocytes [57]. If mast cells indeed contribute to plaque instability, has not yet been established.

In conclusion, atherosclerotic plaque formation is a long-term process, in which a central role is attributed to infiltration, activation and proliferation of monocytes/macrophages. However, other cells are potentially active contributors as well, e.g. endothelial cells, vascular smooth muscle cells, T lymphocytes, mast cells and blood platelets. Remarkably, each of these cells is able to adapt an inflammatory phenotype. In other words, these cells are capable of producing pro-inflammatory (and anti-inflammatory) atherogenic and thrombogenic substances, that may contribute to the formation of a vulnerable, rupture-prone, atherosclerotic plaque. The complex interplay between these different cell types most likely involves a substantial number of pathways that if their pathogenetic role is established may eventually lead to potential sites for intervention.

6 ACCELERATED OR PREMATURE ATHEROSCLEROSIS; INFECTION, AUTO-IMMUNITY OR BOTH?

Whereas most cardiovascular atherosclerotic diseases become clinically manifest in the sixth and seventh decade [58], a number of patients presents with manifest atherosclerotic disease before the age of 55. This is defined as premature atherosclerosis. Why these patients suffer from premature atherosclerosis is not
known, but it is evident that most patients are more exposed to multiple established risk factors as compared to their controls [59;60].

Autoimmunity has been suggested as a causative factor in atherosclerosis, especially in accelerated forms of atherosclerosis, and several auto-antibodies are associated with an enhanced form of atherosclerosis, such as anti-cardiolipin antibodies (ACLA), antibodies against oxidized LDL (anti oxLDL), and antibodies against heat shock protein 65 (anti-HSP65) [61-64]. On the other hand it is known that patients suffering from autoimmune diseases such as systemic lupus erythematosus (SLE) and antiphospholipid syndrome are known to have more aggressive forms of atherosclerosis [65]. In addition, there is evidence that autoimmune processes are involved in the development of atherosclerosis as well [66;67].

Elevated levels of Lp(a) are an independent risk factor for cardiovascular disease, especially in patients with premature atherosclerosis [68]. Although not immunogenic itself, the presence of Lp(a) is known to facilitate the atherosclerotic process possibly by providing a prothrombotic environment since its molecular structure strongly resembles plasminogen. Moreover, it induces expression of cell adhesion molecules on coronary endothelial cells in vivo [69] and, in SLE patients, elevated levels of Lp(a) were associated with increased levels of oxLDL-containing immune complexes, that are thought to be easier phagocyted by macrophages [70]. Higher levels of Lp(a) have been reported in patients with SLE and anti-phospholipid syndrome as well [71-73].

A number of infectious agents, the most important being Chlamydia Pneumoniae, Helicobacter Pylori, and cytomegalovirus, have been linked with atherosclerosis. Numerous studies have linked antibody titers to these pathogens with the incidence of vascular disease and others were able to isolate microorganisms from atheromatous plaques [74;75]. However, results from a recent clinical trial where antibiotics were given to prevent atherosclerotic vascular diseases, were rather disappointing [76]. Although the circumstantial evidence is abundant, until today no causal relationship between infection and atherosclerotic vascular disease has been established.

An interesting hypothesis that is currently under investigation links autoimmunity with infection. It is suggested that an autoimmune response is initiated through molecular mimicry, whereby bacterial antigens are recognised that share homologous sequences with the host antigens. For example, all bacteria encode for heat shock proteins, a group of stress proteins that is evolutionary well preserved and shares a high homology with mammalian heat shock proteins. Antibodies against HSP are frequently found in patients with vascular disease and they may probably facilitate atherogenesis [77]. Another mechanism linking atherosclerosis with infectious disease is that of the increased infectious burden, i.e. not just one pathogen but repetitive infections with a larger number of microbes, is responsible for inducing atherogenesis. It has even been suggested that the exceeding of a certain infectious burden, as reflected by elevated antibody titres against multiple pathogens, accounts for the elevated levels of acute phase reactants that are often found in patients at risk for vascular disease [74;78].

7. INFLAMMATORY MARKERS IN CARDIOVASCULAR DISEASE

The concept of inflammation as a major determinant of atherosclerosis has initiated a gold rush in seroepidemiologic studies. Since only 50% of patients suffering from vascular disease can be explained by means of traditional risk factors [79], the inflammatory theory has brought up new
directions in which to search for (risk) markers that can help the physician identify those patients at risk for (recurrent) cardiovascular disease. In the last fifteen years numerous studies have been published, with the purpose of evaluating the prospective value of several biomarkers in cardiovascular disease. The next paragraphs provide a brief review on the most prominent biomarkers in atherosclerotic vascular disease.

**C-Reactive Protein**

Of all biomarkers, the acute phase reactant C-reactive protein (CRP) has gained most attention and is most likely to proceed from a research tool to a clinical tool. CRP is produced by the liver upon stimulation by the pro-inflammatory cytokine interleukin-6 [80]. CRP levels tend to rise quite easily in response to a large spectrum of conditions such as trauma, infectious disease, malignancy or auto-immune disease. It has been in use for clinical practise since its discovery in 1930 [81] and levels below 10 mg/L were always considered normal [82].

With the development of highly sensitive CRP-assays, it became clear that CRP-levels were elevated in patients with unstable angina [83], with approximately 50% of the patients having levels > 3.0 mg/L at discharge [84]. Furthermore, it proved that CRP-levels, albeit still within the normal range, could serve as a prognostic marker for disease outcome in these patients. [84;85].

Also, patients at risk for coronary heart disease, stroke and peripheral vascular disease have elevated levels of CRP [86-90]. However, there is still a debate going on about the accuracy and reproducibility of CRP measurements in patients whose levels are in the subnormal ranges [91], some claiming that it would take as much as 18 repetitive measurements in an individual patient to establish a reliable value [92]. It is still unclear whether elevated CRP levels reflect an inflamed arterial system, or that the inflammatory stimulus resides in adipose tissue [93] or that CRP itself has pathogenic properties. General coronary inflammation was suggested by the observation that in acute coronary syndromes neutrophil activation occurred in the whole coronary system and not only at the site of the culprit plaque [94]. On the other hand, there are reports claiming a direct inflammatory action of CRP on monocytes and endothelial cells [38;95;96]. Also CRP is present in atherosclerotic lesions and co-localises with activated complement factors suggesting an active role for CRP in the inflammatory response in plaque [97].

Interestingly, CRP is claimed to be an independent risk marker in previously healthy women that is stronger in predicting cardiovascular events than elevated levels of LDL-cholesterol [98]. In a post-hoc analysis of the CARE study, it was suggested that statin therapy favoured especially those patients in whom CRP was elevated at baseline (3 months after a myocardial infarction) [99].

Thus far, we know that CRP is a strong and independent risk marker for cardiovascular events. It certainly has the potential of becoming a tool in management of patients at risk for cardiovascular disease, especially those patients with acute coronary syndromes. Although several drugs, such as statins [100], aspirin [101], fibrates [102] and thiazolidinediones [103] are capable of lowering CRP levels, it is still unknown if this is beneficial. Studies are needed that assess CRP-guided treatments [104].

**Cytokines**

Inflammation requires interplay between several cell types. In the case of atherosclerosis, inflammation is due to a coalition of monocytes/macrophages, T lymphocytes, endothelial cells, vascular smooth muscle cells and blood platelets. The interactions of these cell types are orchestrated by a group of signaling proteins better known as cytokines. Currently there are three ‘families’ of
cytokines, the interleukins, the tumor necrosis factors and the interferons. Cytokines were initially thought to be produced solely by hematopoietic cells but also endothelial cells, smooth muscle cells and adipocytes respond to and produce particular subsets of cytokines [93;105]. Some cytokines such as TNFα and IL-1 act strictly pro-inflammatory whereas IL-4 and IL-10 are mainly anti-inflammatory cytokines. However, most other cytokines exert both pro- and anti-atherogenic actions, depending on the environmental circumstances (table 3). The balance between pro- and anti-inflammatory cytokines is thought to depend on certain T lymphocyte subsets. The T-helper type 1 (Th1) subset favors secretion of pro-inflammatory cytokines such as IFNγ and TNFα, whereas the T helper type 2 subset is associated with the production of interleukins-4 and -10 [106].

Most cytokines have been studied in pre-clinical, experimental settings, but a number of them have also been studied using human material. In human atheromatous plaques, TNFα [107] as well as the pro-inflammatory cytokines IL-2 and IFNγ were abundantly present [108]. This has strengthened the concept of atherosclerosis as a Th1-driven disease. Also [109], IL-12 and IL-10 have been demonstrated in human plaque and it was suggested that IL-10 inhibited the production of IL-12. Others reported that the presence of IL-10 in plaques was associated with reduced activity of iNOS and less apoptosis in macrophages and smooth muscle cells [110]. The latter observation is quite interesting since a number of studies have suggested a beneficial role for IL-10 in reducing atherogenesis [120]. It later became clear that this adherence was due to the expression of cell adhesion molecules by both the activated endothelium and leukocytes [121;122]. Currently four families of adhesion molecules are recognized [123]. Selectins (E-selectin and P-selectin) are responsible for the rolling of leukocytes to the endothelium. Their expression on endothelial cells is enhanced by several stimuli such as cytokines and bacterial endotoxins [40]. Firm adhesion of leukocytes to the endothelial cells is facilitated by members of the immunoglobulin superfamily and their

with CRP, various cytokine levels have been extensively investigated as prognostic markers in patients with cardiovascular disease. Levels of TNFα, IL-1, IL-6 and IL-18 were predictive for adverse events in patients with different stages of coronary artery disease [116-118]. Also, IL-6 proved predictive for adverse cardiovascular events in apparently healthy physicians [119].

In conclusion, cytokines are important in orchestrating the inflammatory response in atherosclerosis. Both pro- and anti-inflammatory cytokines are active in different stages of the disease. There is growing evidence that anti-inflammatory cytokine production is compromised in acute coronary syndromes. On the other hand there is evidence that supports a beneficial role for IL-10 in atherosclerosis, providing a basis for future therapies. Also, cytokine levels provide prognostic information in patients at risk for cardiovascular disease albeit not as convincing as is the case with C-reactive protein.

**Cell adhesion molecules**

In response to injury, the endothelium facilitates coagulation and mediates the binding and transendothelial migration of leukocytes. Monocyte adherence to the endothelium in hypercholesterolemic swines was already noted in the earliest stages of atherogenesis [120]. It later became clear that this adherence was due to the expression of cell adhesion molecules by both the activated endothelium and leukocytes [121;122].
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Anti-atherogenic function</th>
<th>Pro-atherogenic function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interleukins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>↑ thrombomodulin</td>
<td>↑ ICAM-1, VCAM-1</td>
</tr>
<tr>
<td></td>
<td>↑ PAI</td>
<td>↑ Tissue Factor</td>
</tr>
<tr>
<td></td>
<td>↑ Collagen synthesis</td>
<td>↑ IL-1</td>
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<tr>
<td></td>
<td>↓ EC Proliferation</td>
<td>↑ Chemokines (IL-8, MCP-1)</td>
</tr>
<tr>
<td></td>
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<td>↑ IL-6</td>
</tr>
<tr>
<td></td>
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<td>↑ Colony stimulating factors</td>
</tr>
<tr>
<td>IL-4</td>
<td>↓ T11 phenotype</td>
<td>↑ Cell death</td>
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<tr>
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<td>↑ T12 phenotype</td>
<td>↑ MMP release and expression</td>
</tr>
<tr>
<td></td>
<td>↓ Scavenger receptor</td>
<td>↑ 15-LO, promotes LDL oxidation</td>
</tr>
<tr>
<td></td>
<td>↓ MMP</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>↑ T and B cell differentiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ CRP production</td>
<td></td>
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<tr>
<td></td>
<td>↑ fibrinogen production</td>
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<tr>
<td></td>
<td>↑ procoagulant</td>
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<tr>
<td>IL-8</td>
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<tr>
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<td></td>
<td>↓ MMP</td>
<td>↑ Angiogenesis</td>
</tr>
<tr>
<td></td>
<td>↓ IL-1β induced TF</td>
<td>↑ IL-12</td>
</tr>
<tr>
<td>IL-12</td>
<td></td>
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<tr>
<td>IL-15</td>
<td>↑ T11 phenotype</td>
<td>↑ T11 phenotype</td>
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<td>↑ Angiogenesis</td>
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<tr>
<td></td>
<td></td>
<td>↑ IL-12</td>
</tr>
<tr>
<td>IL-18</td>
<td>↑ T12 phenotype</td>
<td>↑ T11 phenotype</td>
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<td>↑ IL-4</td>
<td>↑ IL-1β, IL-6, IL-8, TNFα</td>
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<td>↑ NOS</td>
<td>↑ ICAM-1</td>
</tr>
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<td></td>
<td>↑ IL-13</td>
<td>↑ GM-CSF</td>
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<td>↑ IFNγ</td>
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<td>↑ MMP</td>
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<tr>
<td>TNFα</td>
<td>↑ Thrombomodulin</td>
<td>↑ ICAM-1 and VCAM-1</td>
</tr>
<tr>
<td></td>
<td>↑ PAI-1</td>
<td>↑ Chemokines (IL-8, MCP-1)</td>
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<td></td>
<td>↑ EC proliferation</td>
<td>↑ Scavenger receptor</td>
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<td></td>
<td></td>
<td>↑ MMP</td>
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<tr>
<td></td>
<td></td>
<td>↑ CSF and GF expression</td>
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<tr>
<td></td>
<td></td>
<td>↑ Cell death</td>
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<tr>
<td></td>
<td></td>
<td>↑ Tissue Factor</td>
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<tr>
<td></td>
<td></td>
<td>↑ Tissue Factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ Thrombomodulin</td>
</tr>
<tr>
<td><strong>CD40L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ ICAM-1, VCAM-1, P-selectin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ MMP</td>
<td>↑ angio genesis</td>
</tr>
<tr>
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<td>↑ Chemokines (IL-8, MCP-1)</td>
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<td></td>
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<td>↑ Tissue Factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ Thrombomodulin</td>
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<tr>
<td><strong>Interferons</strong></td>
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</tr>
<tr>
<td>IFNγ</td>
<td>↓ MMP</td>
<td>↑ ICAM-1, VCAM-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ scavenger receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ EC and SMC proliferation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Fas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ Collagen synthesis</td>
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</table>
ligands (integrins) (figure 5). Among the most important adhesion molecules in atherosclerosis are ICAM-1 and VCAM-1. It has been derived from animal studies that VCAM-1 expression is crucial at the earliest stages of atherosclerosis [124]. E-selectin, VCAM-1, P-selectin and ICAM-1 are expressed in advanced atherosclerotic plaques [125]. While the expression of adhesion molecules is difficult to determine on cell surfaces in vivo, soluble forms of adhesion molecules can be detected in the blood. It is thought that these adhesion molecules are ‘shedded’ from the endothelial surface by proteolytical cleavage. Their biological function is unclear. However, several soluble adhesion molecules serve as biomarkers in patients at risk for vascular disease [126].

In both healthy men and women, levels of s-ICAM-1 proved predictive for future myocardial infarction and carotid atherosclerosis [127-129]. Levels of soluble E-selectin, VCAM-1 and ICAM-1 correlated with intima media thickness of the carotid arteries, a supposed marker of preclinical atherosclerosis [130]. However, results with s-VCAM-1 have been contradictory: in an apparently healthy population levels of s-VCAM-1 were not predictive for future cardiovascular events [127] whereas in patients with type II diabetes s-VCAM-1 did predict cardiovascular events [131]. In a large cohort of patients with established cardiovascular disease it proved that s-VCAM-1 was the most powerful marker of subsequent cardiovascular death as compared to hsCRP, lipid levels and other adhesion molecules [132]. These findings do contrast also with the study reported by Malik et al., who found no clear associations between levels of several cell adhesion molecules and adverse cardiovascular events [133]. But, when adjusted for other inflammatory and clinical markers, only a weak association was left. The authors concluded that it is unlikely that measurement of soluble adhesion molecules will add to the predictive value that can be obtained from other current risk markers. Another application of levels of soluble cell adhesion molecules is their use as markers of therapeutic efficacy. Several studies have used soluble cell adhesion molecules as intermediate end points for evaluating the efficacy of an intervention. These studies are summarized in table 4.

### Conclusions

Atherosclerosis and its clinical substrates are the result of a long term, low-grade inflammatory response. The inflammation is triggered by a large number of genetic and environmental risk factors. Interplay of cells of the innate and adaptive immune system, the endothelial cells and smooth muscle cells, finally results in a rupture-prone atherosclerotic plaque. Current understandings have led to the development of prognostic biomarkers for future cardiovascular disease. Especially C-reactive protein proves promising and is likely to add to the information available from traditional measures such as lipid profile. Future developments will probably focus on the genetic mechanisms underlying atherogenesis as well as the development of new methods that provide information useful for the prevention of the major cause of morbidity and mortality in the Western society.

### Aims of the Thesis

Inflammation is a hallmark of atherosclerotic cardiovascular disease. This thesis aims to gain further insights in the clinical application of markers of the inflammatory process with respect to disease severity, therapeutic measures and pathophysiological mechanisms. In the first part the use of several biomarkers of the atherosclerotic process will be evaluated in patients with different stages of disease...
<table>
<thead>
<tr>
<th>First author</th>
<th>Year</th>
<th>Intervention</th>
<th>Number of patients</th>
<th>Clinical presentation</th>
<th>Duration</th>
<th>Design</th>
<th>Markers</th>
<th>Results</th>
</tr>
</thead>
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<tr>
<td>Hackman[134]</td>
<td>1996</td>
<td>statin+/-colestipol</td>
<td>10</td>
<td>hypercholesterolemia</td>
<td>42 weeks</td>
<td>open label</td>
<td>sICAM, sVCAM, sEselectin</td>
<td>sEselectin 77 to 56%</td>
</tr>
<tr>
<td>Abe[135]</td>
<td>1998</td>
<td>n3FA</td>
<td>27</td>
<td>hypertriglyceridemia</td>
<td>28 weeks</td>
<td>open label</td>
<td>sICAM, sVCAM, sEselectin</td>
<td>sICAM -9%, sEselectin -16%</td>
</tr>
<tr>
<td>Johansen[136]</td>
<td>1999</td>
<td>n3FA</td>
<td>54</td>
<td>CAD</td>
<td>4 weeks</td>
<td>open label</td>
<td>sVCAM, sEselectin</td>
<td>sEsel, sVCAM increased,</td>
</tr>
<tr>
<td>Romano[137]</td>
<td>2000</td>
<td>fluvastatin</td>
<td>26</td>
<td>hypercholesterolemia</td>
<td>12 weeks</td>
<td>placebo controlled</td>
<td>p-selectin, sICAM-1</td>
<td>no effects</td>
</tr>
<tr>
<td>Semaan[138]</td>
<td>2000</td>
<td>azithromycin</td>
<td>40</td>
<td>CAD</td>
<td>12 weeks</td>
<td>placebo controlled</td>
<td>sICAM, sVCAM, sEselectin</td>
<td>no effects</td>
</tr>
<tr>
<td>Sardo[139]</td>
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<td>simvastatin</td>
<td>40</td>
<td>hypercholesterolemia</td>
<td>12 weeks</td>
<td>placebo controlled</td>
<td>sICAM, sVCAM, sEselectin</td>
<td>no effects</td>
</tr>
<tr>
<td>Navalkar[140]</td>
<td>2001</td>
<td>irbesartan</td>
<td>33</td>
<td>CAD</td>
<td>24 weeks</td>
<td>open label</td>
<td>VCAM, TNF-alpha</td>
<td>VCAM -36%, TNF-alpha -54%</td>
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<tr>
<td>Prasad[141]</td>
<td>2001</td>
<td>losartan</td>
<td>31</td>
<td>CAD</td>
<td>12 weeks</td>
<td>open label</td>
<td>nog nakijken</td>
<td>no effects</td>
</tr>
<tr>
<td>van Haelst[142]</td>
<td>2001</td>
<td>fluvastatin</td>
<td>10</td>
<td>CAD/PVD</td>
<td>52 weeks</td>
<td>open label</td>
<td>ICAM, sEselectin, neopterin</td>
<td>no effects</td>
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<tr>
<td>van Haelst[143]</td>
<td>2002</td>
<td>quinapril</td>
<td>87</td>
<td>CAD</td>
<td>52 weeks</td>
<td>placebo controlled</td>
<td>ICAM -12%</td>
<td>no effects</td>
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<td>Calabres[143]</td>
<td>2002</td>
<td>fenofibrate</td>
<td>20</td>
<td>low HDL</td>
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<td>sICAM, sVCAM, sEselectin</td>
<td>no effects</td>
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<tr>
<td>Ito[144]</td>
<td>2002</td>
<td>weight reduction</td>
<td>32</td>
<td>obesity</td>
<td>12 weeks</td>
<td>controlled</td>
<td>sICAM, sEselectin</td>
<td>ICAM - sEselectin -23%</td>
</tr>
<tr>
<td>Bemelman[145]</td>
<td>2002</td>
<td>dietary intervention</td>
<td>103</td>
<td>hypercholesterolemia</td>
<td>104 weeks</td>
<td>uncontrolled</td>
<td>sICAM</td>
<td>ICAM - sEselectin -23%</td>
</tr>
<tr>
<td>Hubocka[146]</td>
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<td>quinapril</td>
<td>22</td>
<td>hypertension</td>
<td>12 weeks</td>
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<td>sICAM</td>
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</table>
In chapter 2 the relationship between two hallmarks of early atherosclerosis is investigated. Markers of inflammation as well as endothelial function testing are assessed in patients with familial hypercholesterolemia. Chapters 3 and 4 address the effects of anti-atherosclerotic therapies (HMGCoA-inhibition and ACE-inhibition) on a number of inflammatory markers are investigated. In a large cohort of patients with premature atherosclerosis we sought for a possible contribution of anti-neutrophil cytoplasmatic antibodies (ANCA) as possible contributors to the early occurrence of vascular disease (chapter 5).

The second part will focus on leukocyte function and activity, both as a mechanistic as well as a prognostic measure, in the acute coronary syndromes. Chapter 6 deals with mast cell activation in acute coronary syndromes. A possible role for tryptase, an established biomarker for mast cell activation is investigated. Chapter 7 is a review on neopterin, a marker of monocyte/macrophage activation, in atherosclerosis. The role of monocytes in the pathogenesis of atherosclerosis is dis-cussed. In Chapter 8 the role of neopterin as a prognostic marker is established in patients with acute coronary syndromes.

Finally, in Chapter 9 the functional properties of peripheral monocytes from patients with acute coronary syndromes are investigated. Chapter 10 discusses the thesis and provides future perspectives.

Figure 5. Diagram of the 4 major classes of cell adhesion molecules illustrating their transmembrane structure and their cell counter receptors. Adapted from ref. 122.
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