Summary, discussion & future perspectives:

Tackling inflammation in cardiovascular disease
Atherosclerosis is nowadays regarded as an inflammatory disease. The complications as well as the prognosis in atherosclerotic cardiovascular disease are related to inflammation. Moreover, several therapies aiming to prevent cardiovascular disease are thought to exhibit their beneficial effects through modulation of the inflammatory response. The first part of this thesis emphasised the role of the inflammatory response in atherosclerotic cardiovascular disease. The second part focused on the role of leukocyte activation as an important contributor to the pathogenesis and outcome of the acute coronary syndromes.

PART I: THE INFLAMMATORY RESPONSE IN ATHEROSCLEROSIS

The first part of this thesis has focused on the systemic representatives of the ongoing vascular inflammation that characterises atherosclerosis in all its clinical stages. Chapter 1 provided an overview of the current views on the role of inflammation in the pathogenesis of atherosclerosis. From a historical point of view it can be learned that the concept of atherosclerosis as an inflammatory disease is not novel. Already in the nineteenth century, Virchow proposed intimal inflammation to be the underlying cause of ‘endarteritis deformans’, later known as atherosclerosis. Another view on the disease was given by Rokitansky who blamed elements from the blood to be responsible for the vascular occlusion that causes ischemia in tissues. Later, the focus turned to cholesterol as a dietary as well as an inheritable factor that causes lipid accumulation in the vessel wall. Eventually, it turned out that all theories held some truth and Ross integrated them in his response-to-injury theory. It is now conceived that cardiovascular risk factors such as smoking, hypercholesterolemia, diabetes mellitus and hypertension cause endothelial dysfunction and provoke an inflammatory response. The precise mechanism for each risk factor to cause inflammation is not precisely known, but mechanisms involving oxidative stress are considered common denominators. The low-grade inflammation triggers circulating monocytes to attach to the vessel wall. This promotes further inflammation, finally resulting in the complicated atherosclerotic plaque that gives rise to clinical manifestations of cardiovascular disease.

The important role of the immune system in atherogenesis has led to numerous investigations aiming to identify systemic markers of inflammation that would be able to predict the existence and course of cardiovascular disease. The most prominent biomarker in atherogenesis is C-reactive protein (CRP). This acute phase protein proved predictive for cardiovascular events in patients at risk for cardiovascular disease as well as for the outcome of patients that already suffered a cardiovascular event. Other markers of vascular inflammation such as soluble cell adhesion molecules and levels of cytokines have provided insight in pathogenetic mechanisms underlying clinical cardiovascular disease. However, their use in clinical practice is not yet opportune since a lot of controversy on the validation of measurements remains. Also, study results are conflicting and there is doubt about the evidence that these markers add to risk prediction by established risk factors.

In Chapter 2 the relation between two hallmarks of early atherosclerosis, inflammation and endothelial function, has been investigated. We studied a group of, yet clinically unaffected, patients with familial hypercholesterolemia. Although both enhanced inflammation, as defined by elevated levels of CRP, and endothelial dysfunction, as defined by a decreased flow-mediated vasodilatation, were present in these patients, no relation between the two entities could be established. Our findings in this study were not in accordance with reports by others. The
pros and contras of endothelial function testing as tools for cardiovascular risk stratification were discussed. Also, the relationship between inflammation and endothelial function was discussed in the light of the current knowledge. Others have described a relation between inflammation and endothelial function in patients with severe vasculitis or patients that had received a S. Typhi vaccination. A relation between the two aspects of atherosclerotic disease has also been reported in patients with an acute coronary syndrome. We conclude that more outspoken inflammation is required to coincide with endothelial dysfunction in patients without clinically overt atherosclerosis.

Chapter 3 described the results of a small intervention trial in which patients with established atherosclerotic cardiovascular disease were treated with fluvastatin, a HMG CoA inhibitor. We found that upon treatment, besides lipid lowering, there was a decrease of levels of the soluble cell adhesion molecules ICAM-1 and E-selectin as well as a transient reduction in levels of neopterin and a reduction in levels of CRP. These findings support for the hypothesis that statin therapy in patients with cardiovascular disease is beneficial due to a combined effect on monocyte/macrophage activity, endothelial function and inflammatory activity.

Another intervention trial has been described in Chapter 4. Inflammation in cardiovascular disease is thought to be partly the result of an activated renin angiotensin system. Considering the effects of angiotensin II on the immune system and the vessel wall, a qualification for angiotensin II as a pro-inflammatory cytokine is well merited. We demonstrated that treatment with an angiotensin-converting enzyme (ACE)-inhibitor reduced levels of the adhesion molecule s-ICAM-1 but not CRP. Only recently, the first prospective studies confirmed the long-standing thought that ACE-inhibitors are effective in reducing coronary atherosclerotic events. The findings in our study contribute to the hypothesis that this reduction is the result of anti-inflammatory properties of ACE-inhibitors. However, CRP levels were not lowered by the study drug, and, until today, ACE-inhibitors have not been reported to reduce CRP levels. We hypothesised that ACE-inhibitors affect the inflammatory cascade via a different mechanism than other drugs known to reduce CRP levels, such as statins or aspirin. The debate is still out on whether the beneficial effects of ACE-inhibitors could be the result of their blood pressure lowering effects. Other blood pressure lowering therapies, however, have not yet been reported to induce a reduction in inflammation. There is also evolving evidence that treatment with ACE-inhibitors results in a reduced prevalence of diabetes mellitus, probably by a decrease in insulin resistance. This could also account for a reduced inflammatory response, since diabetes and the associated metabolic syndrome are both known to be strong pro-inflammatory conditions. Thus, much of the potential anti-inflammatory capacity of ACE-inhibitors has yet to be elucidated.

In Chapter 5 we addressed the hypothesis that an autoimmune phenomenon may be responsible for the fact that some patients experience so-called premature atherosclerosis. In a large cohort of patients we determined the presence of anti-neutrophil cytoplasmatic antibodies (ANCA). These antibodies are found in patients with vasculitis, where they are supposed to induce the pathophysiological process of vasculitis. We hypothesised that premature atherosclerosis could be a form of low-grade vasculitis and that ANCA may be present in a substantial proportion of patients. This hypothesis could not be confirmed. The prevalence of ANCA positive patients in our cohort was not different from the prevalence found in population-based studies. Moreover, in the ANCA-positive patients we found no evidence for enhanced inflammation,
vasculitis, or other autoimmune phenomena. On the other hand, we confirmed that patients with premature atherosclerosis are exposed to a substantial number of established cardiovascular risk factors.

**PART II: LEUKOCYTE ACTIVATION IN ATHEROSCLEROSIS**

The pathogenesis of early as well as advanced atherosclerosis is dominated by a constant input of monocytes/macrophages, T-lymphocytes and even mast cells from the circulation to the site of the atherosclerotic plaque. In the second part of this thesis, the role of leukocyte activation in the acute coronary syndromes was investigated.

Histologic studies have reported that, besides activated monocytes/macrophages, T-lymphocytes, smooth muscle cells and endothelial cells, activated mast cells are also present in specimen of atherosclerotic plaque. Mast cells localise especially in the rupture prone shoulder region, albeit in far lower numbers than the other cellular components of the plaque. Given the fact that activated mast cells are capable of producing a large number of pro-inflammatory substances and proteolytic enzymes, it is likely that they also contribute to plaque disruption.

In **Chapter 6** we have investigated if tryptase, a very sensitive marker of mast cell activation, is elevated in patients with acute coronary syndromes. No elevation of tryptase was found in patients with acute myocardial infarction and unstable angina pectoris as compared to patients who presented with atypical chest pain. Also, levels of tryptase did not correlate with those of CRP. Therefore, we concluded that mast cell activation, if any, in acute coronary syndromes is not reflected systemically.

In **Chapter 7**, monocyte/macrophage activity in atherosclerotic disease is reviewed, focussing on the role of neopterin, a marker of monocyte/macrophage activity. Multiple studies have investigated this biomarker in patients with different stages of atherosclerosis. It is concluded that neopterin levels in serum are elevated in patients with acute coronary syndromes as compared to patients with stable disease. Also, it can be assumed that serum neopterin levels reflect the extent of cardiovascular disease. Importantly, it should be noticed that neopterin is elevated in a number of inflammatory diseases and that especially infectious and/or malignant diseases are known to elevate neopterin levels as well. This could also explain the fact that age is an important determinant of neopterin levels. Cross-sectional studies in healthy blood donors, demonstrated that neopterin levels strongly correlated with age.

*Most studies investigating neopterin in cardiovascular disease that were performed to date, were associative and no data were yet available on the prognostic value of neopterin in patients with coronary artery disease. This issue was addressed in Chapter 8. In the acute coronary syndromes, i.e. unstable angina pectoris and myocardial infarction, there is an enhanced inflammatory response accompanying the clinical events. This inflammation is especially important in unstable angina pectoris and non-Q wave myocardial infarctions since the level of inflammation in these disease entities is highly predictive for the outcome of the event. Remarkably, this is not the case in the Q-wave myocardial infarctions, perhaps because of the fact that in the latter there is substantial myocardial necrosis that causes an inflammatory response itself. We investigated levels of neopterin and CRP in a cohort of 210 patients who suffered a non-Q wave myocardial infarction. After one year of follow-up, patients with elevated levels of neopterin or CRP more often met the end point. The risk for an adverse event during follow-up was most outspoken in patients who had both biomarkers elevated. These data confirm that monocyte/macrophage activity is an important feature in the acute coronary syndromes.*
In Chapter 9 we investigated the functional qualities of peripheral monocytes from patients with acute coronary syndromes and stable coronary artery disease. It proved that, upon activation with bacterial endotoxin (lipopolysaccharide) the production of the anti-inflammatory cytokine interleukin-10 was drastically diminished in patients with acute coronary syndromes. On the other hand there was no substantial difference in the production of the pro-inflammatory cytokine TNF-α. Therefore, it is likely that there is a disrupted anti-inflammatory counterbalance in these patients. These results, combined with the finding that IL-10 protects against atherosclerosis in animal models of atherosclerosis, may shed new lights on the pathophysiology of the acute coronary syndromes and suggest that restoration of the anti-inflammatory mechanisms may become a therapeutic goal in the acute coronary syndromes.

Inflammation and the immune system in atherosclerosis: future perspectives

The rapid evolution of molecular techniques in medicine has provided us with an enormous amount of information regarding pathophysiological mechanisms in almost all known diseases. Interestingly, diseases with completely different clinical presentations or even etiologies, appeared to have a lot in common. Atherosclerosis has been demonstrated to have striking pathophysiologic similarities with seemingly unrelated diseases such as cirrhosis, rheumatoid arthritis, chronic pancreatitis, pulmonary fibrosis and glomerulosclerosis [1]. Therefore, a lot can be learned from the pathophysiology of other inflammatory diseases. Since inflammation has been identified as a major component of atherosclerotic vascular disease it seems rational to further explore the inflammatory response as 1) a prognostic tool in clinical management and 2) a possible target for intervention.

The inflammatory response as a clinical tool

Abundant evidence has emerged demonstrating that CRP has the potential to be used as a risk indicator in cardiovascular disease [2]. In a recent report by the American Heart Association and the Centers for Disease Control, the first guidelines for the use of biomarkers in cardiovascular disease were provided [3]. Although most of the statements were Class IIa or b evidence based (conflicting evidence) the Writing Group concluded that high sensitivity CRP testing should be performed as an adjunct to major risk factors in patients with an intermediate risk cardiovascular profile. However, a lot of issues concerning CRP testing in cardiovascular disease remain unresolved. Firstly, no clinical trials have been published in which an inflammation guided treatment modality is tested [4]. Since, for example, a post-hoc analysis of the CARE study has suggested that patients with elevated levels of CRP but normal lipid values should benefit from statin treatment [5], this should be investigated in a prospective manner. Also, CRP testing should be further explored in patients with acute coronary syndromes. Several smaller studies have demonstrated that CRP values at admission in patients with unstable angina pectoris are predictive for an adverse outcome [6-8]. However, no inflammation-guided trials have been performed to date in these patients as well. Importantly, known risk factors such as smoking and obesity are associated with elevated CRP levels either. Also, other biomarkers such as neopterin, cytokines, cell adhesion molecules and potential new ones should be further investigated for the picture has not yet been completed. For example, it was demonstrated that pregnancy-associated plasma protein A (PAPP-A), a metalloproteinase, was abundantly present in unstable coronary plaques but not in stable plaques. Although novel, this is not really surprising, since it is thought that metalloproteinases are largely
responsible for degrading the fibrous cap of the atherosclerotic plaque, thus inducing an unstable plaque phenotype [9]. Serum levels of PAPP-A were strongly elevated in patients with acute myocardial infarction and unstable angina pectoris as compared to stable angina and controls [10] and PAPP-A identified patients with unstable angina who had both low levels of troponin I or CRP. Other biomarkers, that are not specifically linked to inflammation may hold promises for the future as well. The neurohormone B-type natriuretic peptide (BNP) has been demonstrated to predict the short- and long-term occurrence of adverse events in patients with different kinds of acute coronary syndromes [11;12]. BNP is a natriuretic and vasodilatory peptide that is regulated by ventricular wall tension. It has proved useful as a prognostic marker in patients with heart failure [13]. The fact that different processes reflected by different markers have proven predictive in patients with acute coronary syndromes has led to the first attempts in defining a multi-marker strategy to predict adverse events in the acute coronary syndromes [14].

The value of CRP testing alone or in combination with other biomarkers to evaluate therapeutic measures needs further exploration, since the degree of inflammation may discriminate between patients who need specific treatments and those who do not. Currently, we are investigating whether statin therapy is able to attenuate the inflammatory response as measured by serum levels of various markers.

Inflammation as a therapeutic target
All stages of atherogenesis can be considered as an inflammatory response to injury. Many of the underlying mechanisms have been elucidated, providing the basis for potential new therapeutic strategies, such as treatment with antibodies targeted to molecules involved in atherogenesis or modification of genetic processes. But also established therapies are able to affect the atherosclerotic process through modulation of the inflammatory response. It is now clear that therapies such as HMG CoA inhibitors exert their beneficial effects partly through modulation of the immune system [15;16]. Statin therapy is likely to reduce the levels of inflammatory biomarkers [17]. Statins display a broad array of pleiotropic effects and are even reported to influence bone metabolism. There is still a debate whether these effects are the result of lower serum cholesterol levels or the result of other mechanisms. From epidemiologic studies we know that the prevalence of atherosclerotic cardiovascular disease is lowest in those countries were people have the lowest levels of serum cholesterol. It is also known that dietary measures such a lower intake of saturated fats and cholesterol, high intake of polyunsaturated fats and moderate alcohol consumption are associated with a lower rate of cardiovascular diseases [18]. In an animal model it was demonstrated that dietary lipid lowering reduced atherosclerotic plaque inflammation [9]. Other established cardiovascular therapies such as inhibition of the renin-angiotensin system with ACE-inhibitors [19;20] and aspirin [21] are also thought to modulate the inflammatory response. Other drugs that are currently in use for patients with acute coronary syndromes such as clopidogrel [22], a platelet aggregation inhibitor, and abciximab [23], a glycoprotein IIbIIIa blocker, are thought to have anti-inflammatory effects as well, although the mechanisms are not completely clear. The inflammatory response at cellular level is regulated by transcription factors. In atherosclerosis the most important transcription factors are nuclear factor κB (NFκB), and the peroxisome proliferator-activator receptors α and γ (PPARα and PPARγ). NFκB is the most important mediator of inflammation in atherogenesis. Almost all known cardiovascular risk factors are known to activate NFκB transcription. It is activated in the
cytoplasm of different inflammatory cells by binding of angiotensin II, oxidised LDL, cytokines, lipopolysaccharide or advanced glycosylated endproducts to their respective receptors on the cell membrane. From the cytoplasm, NFκB is translocated to the nucleus, where it binds to target genes that are subsequently transcribed. Theoretically, suppression of the NFκB system could prove beneficial in treating cardiovascular disease. On the other hand it is known that NFκB activation leads to the production of anti-apoptotic factors and also the cardioprotective effects of ischemic preconditioning are in part attributed to NFκB activation [24]. Inhibition of NFκB in macrophages of LDL-receptor knock-out mice, paradoxically led to more severe atherosclerosis. Possibly because NFκB inhibition resulted not only in a decrease in pro-inflammatory but also in anti-inflammatory cytokines such as IL-10 [25]. To date, no direct therapies influencing NFκB are available [26].

The PPARs are important mediators of the inflammatory response as well. Both PPARα and PPARγ influence the inflammatory response in atherogenesis, and currently therapies are available that act through activation of PPAR metabolism. PPARα, for example, is activated by fibrates whereas PPARγ is induced by a new group of anti-diabetic medication the so-called glitazones. The beneficial effects of fibrates on preventing cardiovascular events have already been established in large clinical trials [27], whereas those of the glitazones are merely supported by a large amount of pre-clinical studies. Their use as anti-atherogenic medication in clinical practice has not yet been established [28].

The immune system itself may prove an interesting target in anti-atherosclerotic therapy. Especially anti-inflammatory mechanisms provide an interesting target for future developments. In animal studies as well as in clinical studies, a pivotal role for IL-10, has come forward [29]. IL-10 is a pleiotropic cytokine that is produced by Th2 type T lymphocytes, monocytes/macrophages and B cells. It exerts strong anti-inflammatory effects on a broad range of cells by blocking NFκB activity [30]. Studies in patients with inflammatory bowel disease, using recombinant IL-10, were rather disappointing and surprisingly resulted in induction of the pro-inflammatory cytokine IFNγ and higher neopterin levels [31]. But, recently, promising results have been presented in animal models using gene therapy for the induction of IL-10 [32;33]. Another immunological mechanism that holds promises for the future, is the CD40-CD40ligand (CD154) signalling system. The CD40 receptor is expressed on T-lymphocytes and binding activates these cells to promote an inflammatory response [34]. Antibody treatment directed against CD40 ligand in atherosclerotic mice has proven to delay plaque formation [35]. Currently, anti-CD154 therapy is under investigation in patients with systemic lupus erythematosus. Although the therapy proved safe, no beneficial effects have yet been reported [36].

Modulation of inflammation can also be expected from therapies that enhance serum levels of HDL-cholesterol. HDL itself has anti-inflammatory activity, possibly through inhibition of NFκB or through scavenging effects attributed to its apolipoprotein A1, but this needs further investigation [30].

A major concern for public health is the evolving epidemic of the metabolic syndrome. This syndrome is defined as the combination of at least three of the following factors: abdominal obesity, hypertension, hypertriglyceridemia, low HDL-cholesterol levels and an impaired glucose tolerance. The syndrome is strongly associated with enhanced inflammatory activity supposedly arising from IL-6 production by adipocytes [37] and patients with the metabolic syndrome are at increased risk for developing diabetes mellitus and cardiovascular
disease. In a large cohort of apparently healthy women it was demonstrated that elevated levels of CRP added to the risk profile of patients with the metabolic syndrome [38]. Thus, besides life-style modification, strategies aimed at reducing inflammation may also prove beneficial in these patients.

Recently, a set of so-called ‘protective’ genes has gained attention. One of them is the gene encoding for hemeoxygenase-1 (HO-1). HO-1 is an enzyme that is upregulated in most human cells upon a large number of (oxidative) stressors. It is believed that it exerts its protective effects partly via the production of carbon monoxide (CO) through the degradation of heme [39]. Very recently it was demonstrated that CO inhibits atherosclerotic lesion formation, possibly through a mechanism involving the generation of cGMP, the activation of p38 mitogen-activated protein kinases and the expression of the cell cycle inhibitor p21(Cip1) [40]. Also, exogenous suppletion of low doses of CO inhibit monocyte activation [41].

The most potent anti-inflammatory and immunomodulating drugs that are in use for a long time are corticosteroids. Historically, they are known to protect the heart from ischemic injury [42]. However, their use in a clinical setting has been hampered by their ability to reduce wound healing and scarring, leading to the development of cardiac aneurysms and potentially fatal cardiac ruptures [43]. New investigations have re-opened this line of research and have shown that corticosteroids protect the ischemic myocardium by activating endothelial nitric oxide synthetase [44]. Moreover, in a clinical trial of patients with stable angina pectoris awaiting revascularisation, prednisone therapy after stent implantation was found to reduce clinical events as well as the angiographic restenosis rate [45]. Thus, it can be postulated that a reappraisal of corticosteroids is warranted in acute coronary syndromes in which myocardial damage is limited, such as unstable angina pectoris and non Q-wave myocardial infarction. Local coronary drug delivery via drug eluting stents, may prove an interesting option for many new therapies, since systemic side effects often limit the use of new drugs.

**Conclusion**

Atherosclerosis and its clinical sequelae are the result of a longstanding, low grade inflammatory response in which there is an interplay between the cells of the immune system, and those of the vessel wall such as endothelial cells and smooth muscle cells. The current insights in the pathophysiology of atherosclerosis have led to new ways of identifying patients at risk by measuring levels of inflammatory markers, such as CRP and neopterin. Today’s knowledge has opened directions for new therapies and it has shed new light on established therapies. Inflammation in cardiovascular disease is the response to multiple injuries by modifiable risk factors such as smoking, dyslipidemia, diabetes mellitus, obesity and hypertension. Therefore, the greatest efforts should still be reserved for prevention programmes, because the Western world is facing an epidemic of obesity and diabetes, whereas the incidence of atherosclerotic cardiovascular diseases is rising global

**REFERENCES**


