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Chapter 1

C-reactive protein and atherogenesis: from fatty streak to clinical event

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(submitted as review)
Introduction

In recent years, it has become increasingly clear that arterial inflammation is the heart of cardiovascular disease\(^1\). The consecutive stages in the evolution of atherosclerotic lesions are respectively plaque buildup, growth, and destabilization, predisposing to plaque rupture and intravascular thrombosis. This chain of events leading from lesion formation to clinical events has been carefully elucidated during the last 3 decades.

**Pathophysiology of atherosclerosis**

One of the earliest stages in atherogenesis features endothelial dysfunction, characterized by impaired endothelium-derived NO-release and concomitant production of numerous chemo-attracting molecules. Subsequently, activated leukocytes tether and roll along these "dysfunctional" sites, leading to transmigration into the subendothelial compartment\(^2;3\). Within the subendothelium, monocytes transform into macrophages and foam cells via CD36-mediated internalization of oxidized LDL. These activated macrophages interact with T-cells, amongst others through the CD40/CD40L pathway. This culminates into an amplification loop that augments local inflammatory activity with resulting plaque vulnerability and destabilization\(^4\). Apoptosis of various cells in the atherosclerotic lesion, including VSMCs and macrophages, contributes to the formation of a lipid-rich necrotic core and promote adherence of platelets by producing thrombin\(^5;6\). With increasing instability, the plaque may disrupt at the weakened fibrous cap, exposing thrombogenic material with ensuing tissue factor (TF)-mediated thrombus formation and its clinical sequelae.

**Relation between inflammation and atherothrombosis**

A number of circulating inflammatory mediators have been found to independently predict future cardiovascular events\(^7;8\). Amongst them, C-reactive protein (CRP) has emerged as a robust and independent risk indicator. Thus, observational studies as well as recent meta-analyses, including up to 22 prospective studies, have consistently reported that elevated CRP has clear prognostic value for major cardiovascular events and mortality\(^9-15\). Moreover, CRP adds prognostic information to current risk stratification algorithms\(^16\). Postulated from these epidemiological data, individuals at risk for cardiovascular events may be categorized according to their baseline high sensitivity (hs)CRP levels in a low risk (hsCRP <1 mg/L), an intermediate risk (hsCRP between 1 and 3 mg/L), and a high risk group (hsCRP >3 mg/L). The impact of CRP on cardiovascular outcome has been further emphasized by data showing that risk reduction for a first myocardial infarction, as associated with the use of aspirin, seemed directly related to hsCRP levels\(^17\). Accordingly, two secondary prevention trials showed a close correlation between CRP-reduction during statin therapy and decreased atheroma burden (REVERSAL) as well as improved cardiovascular outcome (PROVE-IT), independent from the LDL-lowering effect of statins\(^18;19\). These consistent findings have underscored the concept that CRP may constitute an attractive target for both prevention as well as treatment of cardiovascular events. Combining the clinical findings with experimental observations, a
paradigm shift has occurred in which CRP is no longer merely a marker, but considered a mediator of cardiovascular disease\textsuperscript{20}. In the present review, we will focus on the causal role of CRP during the various stages of atherogenesis.

**CRP and atherosclerotic lesion formation**

Until recently, CRP was thought to be primarily synthesized by hepatocytes, driven by IL-6 with synergistical enhancement of IL-1\textsuperscript{21,22}. A rise in CRP-levels of up to 1000-fold has been observed under many inflammatory and non-inflammatory conditions, including myocardial infarction\textsuperscript{23}. Recent studies have now identified numerous cell-types that may serve as extrahepatic sources for CRP-production, including macrophages of the lung and brain\textsuperscript{24,25}, renal epithelium\textsuperscript{26} and adipocytes\textsuperscript{27}. In line, several studies have reported the presence of CRP in coronary and carotid arterial atheroma\textsuperscript{28,29}. Consistent observations of CRP co-localizing with complement components suggest that such mediators are entangled at the level of the artery wall\textsuperscript{30,31}. Anticipating the origin of vascular CRP-deposition, recent detection of both CRP mRNA and protein within atherosclerotic lesions, predominantly localized to VSMCs and macrophages, have been ascribed to de novo synthesis in the vessel wall\textsuperscript{32}.

In human coronary artery SMCs, inflammatory stimuli such as TNF, endotoxin or the combination of IL-1/IL-6 have been found to induce CRP-production\textsuperscript{33}. Accordingly, upregulation of CRP-synthesis has been demonstrated in human aortic endothelial cells (HAEC) particularly upon exposure to both IL-1 and IL-6\textsuperscript{34}. Further evidence from animal studies has revealed extensive staining for CRP in lipid-laden macrophage foam cells in hypercholesterolemic pigs as compared to normolipidemic animals\textsuperscript{35}. Circulating CRP was found to correlate with the local inflammatory status as well as with the extent of coronary artery disease. In addition, CRP-levels correlated with aortic atherosclerotic lesion size in rabbits\textsuperscript{36}. Strikingly, CRP protein as well as mRNA were found at all stages of atherosclerosis from early to advanced lesions, localized extracellularly and in the presence of apolipoprotein B100. Elegantly, Inoue et al.\textsuperscript{37} provided support for extrahepatic production by showing an translesional CRP gradient across the inflammatory part of coronary plaques in humans. This gradient was higher in unstable angina than in stable angina. Indeed, percutaneous coronary intervention (PCI) gradually increased the transcardiac gradient (between coronary sinus and peripheral blood) of CRP, where CRP was able to predict post-PCI restenosis. Main conclusions of the authors were that the magnitude of these gradients may reflect local CRP production from the inflamed areas and that CRP may represent a pathophysiological relevant player in PCI-mediated injury and repair.

The mechanisms by which CRP affects the vessel wall appear to be multifaceted, affecting several cell-related pathways in non-receptor and receptor-mediated ways. Thus, CRP may bind and activate the complement system via C1q of the classical pathway in a ligand-complexed as well as an aggregated state\textsuperscript{38}. Also, CRP may bind membranes of damaged host cells through its Ca\textsuperscript{2+}-dependent binding specificity for phosphocholine\textsuperscript{39}. By doing so, CRP may play a role in the clearance of apoptotic and necrotic host cells, contributing to resto-
ration of vascular integrity at injured sites. Furthermore, CRP may bind modified LDL via recognition of phosphorylcholine moieties. This may result in enhanced uptake of modified LDL with subsequent foam cell formation. Like in the systemic circulation, CRP may interact locally with phagocytic cells and resident vascular cells to modulate their inflammatory response in a receptor-mediated way. Deveraj et al. recently demonstrated that CRP exerts its biological activities in human aortic endothelial cells (HAEC) via binding and internalization through the Fcγ-receptors, CD32 and CD64.

Using anti-CD32 and MEK inhibitor PD98059, CD32 have further been implicated in CRP-signaling in human monocytes/macrophages through ERK activation. In fact, CRP may affect various cellular functions via activation of NFκB and p38 MAPK signalling pathways and upregulation of the CD40/CD40 ligand signaling dyad. CRP has also been found to affect lesion-related cell proliferation as well as apoptosis, seemingly opposing effects, by activating the p44/42 MAPK (ERK1/2) pathway and GADD 153 gene respectively in ECs and VSMCs. Finally, CRP has been found to synergistically enhance angiotensin II-induced pro-inflammatory effects, involving cellular migration and proliferation, lesion collagen and elastin content as well as ROS production by modulating AT-1R expression in vitro and in a rat carotid artery angioplasty model. Thus, CRP exerts a wide spectrum of activities in vascular tissues that may promote progression of vascular atherosclerotic lesion formation.

CRP and endothelial dysfunction

The association between CRP and endothelial dysfunction, one of the earliest stages of atherogenesis, has been corroborated in various experimental settings. Thus, CRP may promote an inflammatory endothelial phenotype that stimulates leukocyte-endothelium interactions via expression of adhesion molecules and chemokines in various cultured endothelia. This inflammatory phenotype as induced by CRP has partially been attributed to the release of endothelin-1 and IL-6. Further, CRP has been shown to promote monocytic adherence to endothelium by virtue of inducing IL-8-secretion and antagonizing eNOS activity. In line, we recently established that CRP evokes an inflammatory endothelial phenotype in humans. Thus, seven male volunteers received an infusion on two occasions, containing 1.25 mg/kg recombinant human CRP (rhCRP) or diluent, respectively. On average, CRP-levels rose from 1.9 mg/L (0.3-8.5) to 23.9 mg/L (20.5-28.1). Among the major findings were endothelial activation as attested by significant elevation in circulating vWFAg and E-selectin. The eNOS pathway represents a crucial factor for endothelial cell function and may be compromised by CRP. In cultured arterial and venous endothelial cells, CRP was capable of decreasing eNOS mRNA and protein as well as (bio)activity within 24 hours. Noticeably, CRP-concentrations known to predict cardiovascular events were used, whereas these effects were time and dose-dependent. Several mechanisms may be involved here. First, CRP-antagonism of eNOS has been attributed to decreased stability of eNOS mRNA. By another group, CRP was suggested to compromise NO-production by blunting eNOS phosphorylation at Ser1179. Alternatively, CRP may undermine endothelial cell function by reducing prostacyclin secretion and stimulating superoxide anion release from NAD(P)H oxidase via p38.
kinase activation\textsuperscript{59,60}. Moreover, CRP was reported to dose- and time-dependently induce LOX-1, crucial for oxLDL's detrimental effects on endothelial function\textsuperscript{61}.

The effects of CRP on endothelial 'vasoreactivity' have been less equivocal. Amongst others, direct vascular effects of CRP involved attenuation of serotonin-induced vasodilation after 1-hour exposure to 7 μg/mL CRP in porcine vessels\textsuperscript{59}, whereas sensitivity to acetylcholine increased after a 4-hour exposure to 200 μg/mL CRP in aortas of Sprague-Dawley rats\textsuperscript{62}. In humans, we recently assessed the direct effects of CRP on endothelial function (manuscript in preparation). Normolipidemic men showed no alteration in endothelial vasoreactivity 6 hours after increasing CRP-levels by infusion up to \( \approx 26 \mu g/mL \). Whereas discrepancies in dose and exposure-time, should be taken into account, this finding implies that a moderate CRP-increase is unlikely to induce endothelial dysfunction in otherwise healthy individuals. In contrast, CRP-infusion caused marked deterioration of endothelial vasoreactivity in patients with clear hypercholesterolemia. Noticeably, these patients already were characterized by early vascular disease (thickened IMT and impaired endothelial vasoreactivity at baseline). In fact, vasoprotective properties constitutively expressed by healthy endothelium may fail in these patients.

Last, endothelial progenitor cells (EPC) that are responsible for vascular regenerative potential and integrity are of vital importance for endothelial cell function\textsuperscript{63}. The numbers and functional capacity of EPCs have been inversely associated with cardiovascular risk\textsuperscript{64}. Recently, Verma et al. showed that CRP inhibited both basal and vascular endothelial growth factor-stimulated angiogenesis, assessing the \textit{in vitro} angiogenic functions such as EC migration and capillary-like tube formation\textsuperscript{65}. Further, CRP promoted EC apoptosis in a NO-dependent fashion. In accord, CRP has been shown to induce EPC dysfunction by attenuating their migration and adherence capacities, predominantly by impairment of the eNOS pathway\textsuperscript{66}. Thus, CRP mediated downregulation of arteriogenic chemo-cytokines (MCP-1 and MIP-1) and angiogenic activity (eNOS and ICAM-1) in EPCs is in part due to the upregulation of suppressors of cytokine signaling proteins.

All together, CRP may hit on several first-line defense mechanisms in the vascular wall with as end-result diminished NO-activity. The ensuing loss of compensatory capacity may become critical under conditions such as chronic ischemia.

**CRP and atherothrombosis**

Atherothrombosis is critical to both disease progression and clinical events when plaque integrity is compromised and thrombosis risk is increased. Several mechanisms that are potentially affected by CRP may destabilize plaque integrity. Besides its activities in monocyte infiltration, cytokine production, NO-activity and oxidized LDL-uptake by macrophages, CRP is believed to promote matrix degradation and thereby contribute to plaque instability. Supporting evidence comes from a recent autopsy study in 302 men and women, showing that elevated hs-CRP levels relate to sudden deaths attributable to coronary disease, independently of established cardiovascular risk factors\textsuperscript{67}. In detail, elevated hs-CRP levels correlated to thin cap atheromas, as well as to CRP-deposits in macrophages and lipid cores (within plaques).
Although higher in fatal thrombosis, hs-CRP was also elevated in case of stable plaques. According to the study’s main findings, CRP levels were later reported to correlate with the disease activity of plaque rupture in nonculprit lesions, using coronary angioscopy. Recent reports have demonstrated CRP’s ability to increase MMP synthesis with ensuing collagen-degrading activity in monocytes-macrophages as well as HUVECs, without affecting tissue inhibitors of MMP. Montero et al. presented data showing that CRP may increase levels of MMP-1 and MMP-10 from HUVECs and HAECs without affecting MMP tissue inhibitors. Cellular pathways that were implicated are p38 MAPK, MAPK activated ERK kinase, and Jun N-terminal kinase pathways. They further reported that patients with higher hsCRP-levels >3 mg/L, tend to have higher MMP-1 and MMP10 levels even after adjusting for confounding factors. Finally they show that CRP and MMP co-localize in the endothelial layer and macrophage-rich areas of advanced plaques. Noticeably, enzymatic activity of MMPs has been put forward as a central pathway linking inflammation to plaque instability and eventually rupture.

Another key player that may contribute to plaque instability is IL-8, the cytokine that has been found capable of inhibiting local TIMP-1 expression with subsequent imbalance between MMPs and TIMPs at focal sites in the atherosclerotic plaque. In this regard, Devaraj et al. recently showed that CRP mediates IL-8 expression in HAECs and HCAECs by virtue of NFκB activation.

Upon direct contact between lesion content and blood components such as after plaque rupture, thrombotic reactions are triggered. TF is the major initiator of the coagulation cascade and pivotal for thrombus formation. Previous studies have demonstrated that CRP may stimulate TF-release from mononuclear cells. Likewise, CRP has recently been shown to dose-dependently induce TF in endothelial cells and smooth muscle cells, implicating activation of the NFκB pathway. With reference to impaired fibrinolysis, CRP has been shown to time- and dose-dependently induce intracellular plasminogen activator inhibitor-1 (PAI-1) mRNA and protein, and PAI-1 activity in HAECs, particularly under hyperglycemic conditions. The underlying mechanism implicates stabilization of PAI-1 mRNA, where interfering with the function of eNOS, IL-6 and endothelin-1 receptor fail to attenuate CRP’s effect on PAI-1. Recently, Singh et al. demonstrated that CRP may inhibit tissue plasminogen activator (tPA) antigen and activity via stimulation of IL-1β and TNF-α in HAECS. Moreover, higher CRP levels in volunteers were associated with increased euglobulin clot lysis time. These in vitro data could actually be reproduced in a human model.

Upon CRP-challenge, human volunteers showed significant thrombin generation and impaired fibrinolysis, represented by increases in prothrombin F1+2 fragments and PAI-1 antigen/activity respectively. Strikingly, these procoagulant responses upon CRP-challenge were significantly augmented in hypercholesterolemic subjects compared to normolipidemics (manuscript submitted). In this series of studies, we were unable to detect TF-upregulation. However, we did find a progressive decline in factor VIIa levels, which may indirectly indicate activation of the TF-pathway (unpublished data). Noteworthy, there was no indication of impairment of the protein C pathway. Combining these data, CRP may exert devastat-
ing effects on potentially unstable lesions by affecting many critical destabilizing processes. Although other inflammatory molecules have previously been linked to thrombosis, CRP with his many faces may be at the core of the inflammation-thrombosis continuum.

**CRP and myocardial necrosis**

The inflammatory response after reperfusion is believed to further compromise ischemic tissue during the post-infarct phase\(^\text{23,79}\). The size of the myocardial damage depends on both systemic and loco-regional factors that are triggers of a complex myocardial inflammatory reaction. As far back as in 1954, the value of estimating CRP-levels as measure for myocardial damage in the setting of an acute coronary event was known\(^\text{80}\). In the '60s, Kushner et al. observed that CRP could be detected within and around necrotic myofibers in myocardial infarcts in rabbits. These authors speculated on local production at the inflammatory site as a result of inflammatory or necrotic tissue changes \(^\text{81}\). Later on, CRP-depositions have been found in human hearts during myocardial infarction in co-localization with activated complement \(^\text{30}\). To assess CRP's role in tissue injury of ischemic necrosis, myocardial samples from 56 patients who transpired after an acute coronary event were analysed \(^\text{82}\). As a major observation, infarctions that lasted for 12 hours to 5 days were characterized by more extensive depositions of complement and CRP than 'younger' infarctions. Additionally, circulating CRP-complexes correlated significantly with the local concentration and the extent of the deposition of both CRP and complement. Together with other findings, CRP was believed to enhance local inflammatory reactions particularly in human myocardial infarcts of more than 12 hours duration. More recently, IgM deposits were also found in heart specimens of patients who died from myocardial infarction, showing a similar pattern to that of CRP and complement activation products\(^\text{83}\). Therefore, IgM was concluded to target complement as does CRP. The authors postulated that although these molecules may recognize the same epitopes, both mediators are likely to differ in their relative contribution to complement activation in patients. The relevance of these pathological findings is supported by in vivo animal studies, showing that human CRP results in worse outcome in experimental myocardial infarction\(^\text{84}\). Thus, human CRP injected into rats has been shown to enhance infarct size by approximately 40%, whereas in vivo complement depletion completely abrogated this effect. Complement depletion also markedly reduced infarct size, even when initiated up to 2 h after coronary ligation. From these data, it has been postulated that human CRP and complement activation are major mediators of ischemic myocardial injury. Recently, a small-molecule inhibitor of CRP has been found in rats to attenuate the increase in infarct size and cardiac dysfunction in reaction to human CRP\(^\text{85}\). The authors attribute their findings to inhibition of CRP in tissue damaging conditions in which binding of CRP to ligands on damaged cells may lead to complement-mediated exacerbation of tissue injury. Thus, CRP may significantly contribute to the severity of the clinical presentation after plaque disruption, where myocardial necrosis may propagate inflammation and vice versa, leading to a vicious circle.
In summary

Cumulating evidence suggests that CRP is a pro-atherogenic factor throughout the entire spectrum of this process, ranging from fatty streak formation to atherothrombosis and tissue injury of ischemic regions. In this novel concept, CRP might be considered a long-term risk factor for cardiovascular events, comparable to established cardiovascular risk factors such as hypertension and diabetes. In sharp contrast to the protective action of CRP as part of the innate defense apparatus, particularly vascular CRP may in fact be harmful by promoting progression of atherosclerotic vascular disease and eventually cardiovascular events. The concept of CRP as a risk factor over life is fully compliant with the data from large epidemiological surveys, in which CRP correlates with both atherosclerosis progression as well as with acute cardiovascular events. Pathophysiologically, such a relation is corroborated by a wide spectrum of pro-atherogenic effects of CRP, in inflammation as well as in thrombosis. Consequently, it is safe to conclude that inhibition of the activity or level of CRP might represent an attractive goal as a novel strategy to prevent the atherothrombotic complications of atherosclerosis.

Reference List


