High-density lipoprotein and C-reactive protein, friend and foe in cardiovascular disease
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Chapter 11

Summary
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In this thesis entitled "High-density lipoprotein and C-reactive protein, friend and foe in cardiovascular disease" we set out to clinically examine two novel cardiovascular risk factors for their potential role in mediating atherogenesis. Driving force behind this thesis constitutes the notion of a large proportion of clinical events that cannot be prevented despite established, efficacy-proven strategies including LDL-C reduction. As a consequence, a call for novel drug targets to further improve cardiovascular outcome will not cease to exist over the next few years.

Arterial inflammation has been put forward as a 'pivot' in the pathophysiology of cardiovascular disease. In fact, inflammation has been linked to all consecutive stages in the evolution of atherosclerotic plaque formation, respectively plaque buildup, destabilization and rupture and/or thrombosis that may result in an acute clinical event. By virtue of the disciplines epidemiology and basic molecular research, various anti- and pro-atherosclerotic mediators have been identified and tested for their role in accelerating atherogenesis. Amongst them, a great deal of interest has focussed on high-density lipoprotein (HDL) and C-reactive protein (CRP), as they are both versatile agents deeply involved in systems that control inflammatory activity. Drugs targeted at modulation of their levels and activity hold therefore great promise, but first, integrating their biological activities separately into an unified paradigm of early atherosclerotic events should be the goal for researchers in this area. Available data on both HDL and CRP, mostly derived from in vitro labor, have served to fuel our experiments studying these two seemingly opponents in vivo in humans. We applied various read-out monitors, involving structural and functional assessment of established pathways involved in atherosclerosis progression.

Amongst others, human models of accelerated atherosclerosis were used, ie. familial hypoalphalipoproteinemia and familial hypercholesterolemia. Since these models are characterized by early vascular disease, effects of such mediators are more capable of being perceived presumably in the context of failing compensatory mechanisms and/or higher susceptibility to inflammatory stimuli. In the following, individual chapters, included in this thesis, are briefly described succeeded by final conclusions and future perspectives.

PART I

Chapter 1 & 2 In recent years, both CRP and HDL have exhaustibly been associated with cardiovascular risk. CRP was first discovered in 1930 by Tillett and Francis in sera from patients during early stages of certain infectious diseases, notably lobar pneumonia, in the presence of dilute solutions of the C polysaccharide of Pneumococcus. The role of CRP was then already considered important in modulating inflammatory processes. Last two decades, clinical reports, bringing forward evidence for CRP's designation as an independent predictor
of cardiovascular events, have served to fuel experiments that established its detrimental effects on atherogenesis. Alternatively, HDL is a jack-of-all trades working his way through the body’s smallest blood vessels. Serving as a major component of algorithms worldwide to assess individual cardiovascular risk, HDL and its principal protein apolipoprotein A-I (apoA-I) have been implicated in many anti-atherogenic pathways. In this chapter, we review current literature with reference to CRP and HDL and focus on their causal role during the various stages of atherogenesis.

Chapter 3 There is a great need for the development of reliable surrogate markers to assess the beneficial effects of novel interventions on cardiovascular disease. Anticipating our studies evaluating the in vivo effects of HDL and CRP, we therefore reviewed benefits and limitations of noninvasive imaging modalities to assess early cardiovascular disease, namely intima-media thickness, flow-mediated dilatation, magnetic resonance coronary angiography, and electron-beam computed tomography. In this chapter, we address the benefits and limitations of current available noninvasive imaging modalities to assess early cardiovascular disease, namely intima-media thickness, flow-mediated dilatation, magnetic resonance coronary angiography, and electron-beam computed tomography. We discuss whether these techniques may find applications in clinical research or practice. Also, we review their usefulness for individual risk management and monitoring efficacy of therapeutic interventions (i.e., surrogate endoints). Altogether, these techniques have been shown to carry predictive value for future clinical events, but properly designed studies are needed to further determine their value in clinical practice. Moreover, further technical refinement of all these imaging modalities is mandatory. Over the next years, improvement of these tools may render suitable intermediate endpoints in future trials evaluating the impact of novel mediators and/or therapeutic interventions on cardiovascular disease progression.

Part II HDL

Chapter 4 HDL levels have been inversely related to cardiovascular risk. However, the clinical consequences of a low-HDL state for cardiovascular risk, in absence of other risk factors, is poorly documented. In chapter 4, we examined the impact of isolated low-HDL (familial hypoalphalipoproteinemia) resulting from an apoA-I gene defect (L178P) on coronary artery disease (CAD) risk. By using flow-mediated dilation (FMD) and carotid intima-media thickness (IMT) severity of atherosclerosis progression could be estimated. In addition, CAD risk was evaluated for the apoA-I mutation carriers. Principal findings of this study were the impaired FMD and increased carotid IMT in association with the more than 50% lower plasma levels of apoA-I and HDL in the carriers. Multivariate analysis revealed that heterozygotes had a striking 24-fold increase in CAD risk. Thus, heterozygosity for a novel apoA-I mutation underlies a detrimental lipoprotein profile that is associated with endothelial dysfunction, accelerated
carotid arterial wall thickening, and severely enhanced CAD risk. Reciprocally, these data illustrate the pivotal role in humans of HDL and apoA-I in the protection against CAD.

**Chapter 5** Again, we sought to determine the clinical consequences of an isolated low-HDL state but now we also questioned whether the adverse effects coupled to low-HDL are reversible? We assessed the impact of isolated low-HDL on vascular function in familial hypoalphalipoproteinemia, resulting from an ATP-binding cassette (ABCA)-1 gene defect. In addition, we evaluated the effects of an acute increase in HDL levels by infusing apolipoprotein A-I/ phosphatidylcholine discs (apoA-I/PC) on vasoreactivity using venous occlusion plethysmography (proof-of-principle). At baseline, 9 ABCA1 heterozygotes had more than 50% lower HDL levels, whereas their endothelial vasoreactivity, comprising basal and stimulated NO bioactivity, was significantly blunted compared to 9 controls. Infusion of apoA-I/PC discs, rendering plasma HDL levels similar to that of controls, resulted in complete restoration of endothelium-derived vasomotor responses in the ABCA1 heterozygotes. These findings indicate that amongst its many anti-atherosclerotic properties, HDL exerts direct beneficial effects on the arterial wall and constitutes an attractive target for prevention and treatment of cardiovascular disease.

**Chapter 6** As follow up for our findings of acute effects of raising HDL (chapter 5), we set out to examine whether semi long-term HDL increase would translate into beneficial changes on HDL function in regard to pathways related to atherosclerosis progression. HDL exerts multiple anti-atherogenic actions beyond its role in reverse cholesterol transport, comprising anti-inflammatory, anti-oxidative and direct vascular effect. Novel CETP-inhibitors are capable of mediating significant HDL elevation, yet the precise role of CETP activity in the course of atherogenesis has been a matter of debate. In the present study, modest CETP inhibition in subjects with familial hypoalphalipoproteinemia has been found to confer beneficial effects beyond its well recognized HDL increasing action, including a reduced number of small LDL particles as well as augmentation of the plasma anti-oxidant capacity (reduced oxLDL-autoantibodies and enhanced serum PON-1 activity). Taken together, these findings suggest an anti-atherogenic effect of CETP-inhibition in low-HDL patients and precede the results of ongoing trials to corroborate the impact of these beneficial changes on cardiovascular outcome.

**Part III: CRP**

**Chapter 7** Numerous, mostly in vitro observations suggest an active rather than a passive role for CRP in cardiovascular disease. However, there is no solid evidence, related to these data, in man. Weighing the in vitro evidence, cumulated to date, we assessed in this chapter the direct effects of CRP in healthy man on established pathways in cardiovascular disease
progression (proof-of-principle). Seven male volunteers received intravenously an infusion on two occasions, containing 1.25 mg/kg recombinant human CRP (rhCRP) or diluent, respectively. Main results of the study include observations, that after a more than 10-fold rise in CRP-concentrations, pathways of both inflammation and coagulation were activated. We conclude from these findings that CRP besides being an indicator of cardiovascular risk, may also constitute a mediator of atherothrombotic disease. Whereas this chapter was the first-in-human evidence for such an effect, it can be expected to also receive criticism (controversial). The criticism has focused at the potential role of LPS contamination within the CRP prepare as true cause for the pro-inflammatory effects reported by us. To provide a clear overview of the arguments, which convincingly abolish this line of reasoning, we have included two letters-to-the-editor published in Circulation Research, addressing this issue.

Chapter 8 Besides its role in atherosclerosis progression, CRP has been postulated to be causally involved in the acute complications of atherosclerosis. Since leukocytes are thought to play a major role in destabilization of the 'culprit lesion', we sought to determine whether CRP may adversely affect the inflammatory profile of circulating leukocytes and thereby may contribute to the occurrence of an acute event. By using quantitative real-time PCR analysis, whole-blood expression profiles were analyzed for 95 inflammatory markers before and after infusion of 1.25 mg/kg rhCRP in 5 male volunteers. Relevant transcript levels were measured at baseline, 4 and 8 hours after rhCRP-infusion. Main findings of the study were mRNA upregulation of MMP9 and MCP-1 of 17- and 11-fold, respectively with concomitant increases in corresponding plasma protein levels. In whole blood culture stimulation assays, CRP induced pro-inflammatory changes that were abolished by heat-inactivation. The latter finding precludes a role for contaminants within the purified CRP-preparation in the absence of optimal control experiments in man. Interpreting these results, CRP beyond its role as risk indicator may be linked to acute cardiovascular events through activation of peripheral leukocytes.

Chapter 9 Since cardiovascular factors are considered to have greater impact in humans, already characterized by early vascular disease, we evaluated the effects of CRP in presence of (genetically determined) hypercholesterolemia. Based on a prior dose-escalation study, CRP was again infused at the dose of 1.25 mg/kg for evaluating its effects on endothelial vasoreactivity, inflammation and coagulation in 6 asymptomatic subjects with familial hypercholesterolemia (FH) and 6 healthy controls. At baseline, FH-patients showed blunted endothelium-dependent vasodilation, as assessed by venous occlusion plethysmography, and higher procoagulant activity as compared to controls. Upon CRP-challenge, endothelium-dependent vasodilator capacity further deteriorated in FH-patients, whereas no change in vascular reactivity was observed in controls. Additionally, the coagulation activation upon CRP-challenge was significantly augmented in FH-patients compared to controls. No difference in inflammatory responses was observed between both groups. Altogether, we show that CRP perturbs endothelial function and evokes procoagulant responses particularly under hypercholesterolemic conditions.
**chapter 10** Based on our findings in combination with data of recent experimental studies, pretreatment with HDL may prevent the adverse effects of CRP on established pathway of atherosclerosis progression. In the last chapter, we assessed the ability of HDL to neutralize CRP-mediated activation of coagulation and inflammation. Fifteen healthy male volunteers received an infusion of highly purified recombinant human (rh)CRP, where eight were pretreated by an infusion of apoA-I/PC discs. Whereas rhCRP infusion elicited a systemic inflammatory response, cytokine levels remained unaltered in subjects who received apoA-I/PC discs prior to rhCRP infusion. Similarly, thrombin generation and activation of fibrinolysis following administration of rhCRP were abolished by HDL pretreatment. In volunteers receiving apoA-I/PC discs, a substantial part of the rhCRP could be found within the HDL fraction. In *in vitro* experiments using human endothelial cells we confirmed that rHDL-preincubation with CRP completely abolished the inflammatory activity of the latter. These findings may lend further support to the use of HDL-increasing strategies in semi-acute, pro-inflammatory settings.

**Final conclusions**

The *in vivo* human studies involved in this thesis provide convincing evidence, that besides a risk factor, CRP in humans may activate pathways involved in atherosclerosis progression, and interacts with known risk factors to exacerbate early (subclinical) cardiovascular disease. By doing so, we confirm previous data from a large pile of *in vitro* studies. Our findings anticipate on the current public debate on the fundamental role for inflammation in mediating all stages of atherosclerosis, and will allow to design strategies to directly inhibit CRP in order to prevent cardiovascular events.

With respect to HDL, we show that isolated low-HDL is a pro-atherosclerotic condition *per se*. Strikingly, IMT progression rate in subjects with isolated low HDL approximated the progression rate in FH patients, illustrating the impact of low HDL in otherwise ‘healthy’ subjects. These data urgently call for more attention and research in the area of low HDL. For the last 2 decades, attention with respect to dyslipidemia has focused on high LDL, leading to wide implementation of statins. Now, attention should focus at low HDL and its causes for therapeutic choices. Noticeably, this state that apparently renders a person more susceptible to atherogenesis, may be partly reversed by normalizing HDL-levels. The latter was realized by HDL-infusion (*proof-of-principle*) and CETP inhibition, emphasizing the clinical importance of HDL-raising strategies in humans. These observations constitute a powerful stimulus for ongoing efforts targeting HDL-increase as a tool in cardiovascular prevention.
Future perspectives

Given the plethora of anti-atherosclerotic activities of HDL to temper atherosclerosis progression, strategies aiming at improving HDL levels hold great promise. Currently, 3 main classes of drugs are available to increase HDL levels, involving statins, fibrates and (extended release) niacin. While the results of ongoing trials evaluating the effects of another agent, that is the CETP inhibitor, on hard cardiovascular end points are to be awaited, parenteral administered apoA-I-related therapies are in early clinical development. Recently, 5 weekly infusion of recombinant apoA-I Milano complexed with phospholipids induced significant reduction in coronary atheroma volume, as measured by intravascular ultrasonography.

Our findings of HDL rapidly restoring endothelial vasoreactivity and neutralizing CRP's adverse effects provide support for reconsidering HDL-raising strategies as ‘acute induction therapy’ rather than targeting long-term regression of atherosclerosis per se. ApoA-I mimetic peptides, that are smaller than full-length apoA-I, exert similar properties as those of apoA-I, where their potential clinical use as ‘acute induction therapy’ may be broadened once orally administered apoA-I are not longer recognized by gut peptidases and renders appropriate bioavailability. Like vitamins and therapeutic lifestyle changes, oral apoA-I mimetic peptides in the feature may be recommended as first line therapy for the prevention of cardiovascular disease.

Alternative therapeutic interventions that emerge in this field involve PPAR agonists, which are compounds that may promote macrophage RCT and have anti-inflammatory effects, modulators of the nuclear receptor liver X receptor, and phospholipids such as phosphatidylinositol. A complete new ball game concerns evidence to show that HDL levels may not necessarily reflect HDL functionality. Indeed, we have observed that after CRP challenge, HDL is less able to protect LDL from oxidative injury (data on file). Whether raising circulating HDL levels must be preferred over improving HDL function is still matter of debate. It will be a challenge to compare the impact of emerging compounds, directed at raising HDL levels and/or HDL function, on atherosclerosis or cardiovascular risk.

A substantial amount of evidence gathered in the past two decades, now completed by our recent findings in vivo, directly implicates CRP as a partaker in cardiovascular disease. In contrast to the protective nature of CRP as part of the innate defense apparatus, local CRP may be harmful in subjects, particularly those already characterized by early vascular disease, by promoting atherosclerotic lesion formation with ensuing clinical complications. The idea of CRP as a life-time risk factor is fully compliant with data from large epidemiological surveys, where CRP correlates with both atherosclerosis progression as well as acute events. Consequently, it is safe to conclude that antagonizing CRP represents an attractive goal to retard atherogenesis and prevent atherothrombotic complications.

For that matter, 4 strategies of CRP inhibition have been proposed (1) transcriptional inhibition of hepatic CRP synthesis (2) anti-sense strategies (3) blockage of CRP-mediated complement activation, and (4) blockage of cellular CRP receptor(s). Recently, a small-molecule inhibitor of CRP, known as 1,6-bis(phosphocholine)-hexane and abbreviated as Bis(PCI)-H,
has been found to inhibit incremental myocardial damage resulting from human CRP in a rat model of coronary-artery occlusion. Targeted research for this compound is as yet restricted to acute damage models, where its mechanism of action has been related to attenuation of complement-driven cell damage. Further studies before testing in humans must address issues including clinical applicability, timing of delivery and, finally, the compound's chronic effects on atherosclerosis progression. Concomitantly, antisense oligonucleotides (ASOs) have been developed to selectively reduce CRP mRNA levels in the liver. In preclinical studies, CRP-ASO produced suppression of liver and serum CRP levels in transgenic mice and in monkeys in which CRP was introduced (www.isispharm.com). With reference to adverse effects, a potential ASO-mediated innate immune response are a particular concern. Ongoing studies with apoB antisense (isispharm 2006), however, have shown promising results for this type of intervention in humans. Given the long half life of these ASOs (single injection once every 2-4 weeks is reasonable), these compounds are extremely suitable to be used in chronically elevated CRP in high risk groups. When research on these compounds progresses and human studies come insight, particular consideration should be given to in-depth safety evaluation because of the more protective role of CRP in innate immunity. Before commencing such a therapy, precautions should be taken to rule out coexisting diseases particularly inflammatory or infectious conditions.

Reference List
