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Chapter 9

Effects of CRP-infusion on endothelial function and coagulation in normo- and hypercholesterolemic subjects

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(submitted)
Abstract

**Background** Hypercholesterolemia and inflammation are both pivotal determinants in atherogenesis.

**Objective** We assessed the effects of C-reactive protein (CRP)-infusion on endothelial vasoreactivity, inflammation and coagulation in patients with familial hypercholesterolemia (FH) and normocholesterolemic controls.

**Patients and Methods** A bolus of 1-25 mg/kg highly purified recombinant human (rh)CRP was administered intravenously. Endothelium-dependent and –independent vasoreactivity to serotonin and nitroprusside, respectively, were assessed using venous occlusion plethysmography before and after CRP-infusion. For biochemical analyses, blood was drawn at consecutive time-points.

**Results and Conclusions** At baseline, FH-patients were already characterized by blunted endothelium-dependent vasodilation (max 89.2±30.0% versus 117.7±13.1% in controls; p=0.037). Concomitantly, procoagulant activity was higher in FH-patients, showing elevated prothrombin fragment 1+2 (F_{1+2}) levels (p=0.030) and plasminogen activator inhibitor type-1 (PAI-1) activity (p=0.016). Upon CRP-challenge, endothelium-dependent vasodilator capacity further deteriorated in FH-patients (p=0.029), 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 (p=0.009 for F_{1+2} levels; p=0.018 and p=0.003 for PAI-1 antigen and activity, respectively). No difference in inflammatory responses was observed between both groups. Here we show that CRP perturbs endothelial function and evokes procoagulant responses particularly under hypercholesterolemic conditions. These data lend further support to strategies targeting both LDL-cholesterol and CRP to improve cardiovascular prevention.
Introduction

Apart from the detrimental impact of LDL-cholesterol (LDL-C) on atherosclerosis progress­
ion, inflammation has now been recognized as a critical determinant, exerting significant pro-atherogenic activities. Interestingly, interactions have been reported between both mediators. Oxidative modification of LDL-C may evoke a strong inflammatory response\(^1\), whereas, in turn, inflammatory activation causes redox dysregulation and upregulation of lectin-like oxLDL receptor-1 (LOX-1)\(^2\), promoting LDL-uptake within the arterial wall.

The acute phase reactant CRP has emerged as relevant predictor of future cardiovascular events, independently from established risk factors\(^3,4\). CRP constitutes a member of the innate host defense against pathogens, and amongst its activities are regulation of the complement system\(^5\), binding to oxLDL\(^6\) as well as facilitating its clearance, and mediating neutrophil responses and phagocytosis\(^7\). More recently, basic molecular research revealed that CRP itself may also exert proatherogenic actions, particularly at the level of the arterial wall and mononuclear cells\(^8,9\). However, opposing observations in human CRP-transgenic mice have ignited the debate whether CRP is a marker or may truly act as a mediator of atherogenesis. Simultaneously, it has become clear that the mouse model is less suitable to provide final answers\(^10,11\). As a consequence, human studies addressing the concept of CRP adversely affecting atherogenesis are warranted. In this respect, we recently found that CRP-infusion elicits activation of inflammatory and coagulation pathways in healthy volunteers\(^12\).

To further explore the potential proatherogenic function of CRP in humans, we evaluated the impact of CRP-infusion on vascular reactivity, inflammation and coagulation, all key participants in the course of atherogenesis, in subjects with elevated LDL-C levels (familial hypercholesterolemia, FH) as well as in matched, normocholesterolemic controls.

Methods

Study design

Twelve healthy, non-smoking, male individuals, including 6 FH-heterozygotes (mean [±SD] age, 36.3±11.5 years) recruited from Dutch kindreds with known LDL-receptor mutations, and 6 normocholesterolemic controls (35.8±10.9 years) were enrolled in the study. The FH-subjects except for one, were carriers of the N543H/2393del9 combo-mutation. Written informed consent was obtained from all participants. The study protocol was approved by the Institutional Review Board of the Academic Medical Centre in Amsterdam. The FH-patients had no history of overt cardiovascular disease and none of them were receiving any drugs aside statins. Also, none of the controls were receiving drugs. Before inclusion, statin use in the FH-cohort was stable for many years. Lipid-lowering medication and anti-oxidant supple­mentation was discontinued for at least 4 weeks. All subjects were free of febrile illness at least 4 weeks prior to the study. They refrained from alcohol and caffeine-containing drinks
for more than 24 hours prior to the study. During the statin washout-period FH-patients were instructed by the department’s dietician to maintain their lipid-lowering diet, as recommended by the AHA/NCEP-1 (Step one diet). Based on results from the dose-escalation study, as described below, an intravenous bolus of 1.25 mg/kg CRP was administered after an overnight fast. Endothelium-dependent and -independent vascular reactivity was assessed under standardized conditions before and 6 hours after CRP-infusion. Blood was drawn at baseline and 1, 4, and 8 hours post-infusion.

Dose-escalation study
To establish an adequate CRP-concentration for examining the acute effects of CRP under both normo- and hypercholesterolemic conditions, a dose-escalation infusion was performed in healthy volunteers. Thus, 21 men were randomly assigned to one of three dose groups: 7 in the 0.25 mg/kg group (52.1±5.8 years), 7 in the 0.5 mg/kg group (52.0±5.1 year) and 7 in the 1.25 mg/kg group (35.9±17.2 years). All volunteers were free of febrile illness and medication.

Recombinant human CRP
E.Coli-derived rhCRP (BiosPacific, CA, USA) was supplied in 20 mM Tris, 140 mM NaCl, 2 mM CaCl₂, pH 7.5 and 0.05% (wt/vol) sodium azide and revealed a single 23 kDa band (>99%) after CBBR-staining (1 µg; SDS-polyacrylamide gel). The host cell protein concentration was 85 p.p.m. before purification, as determined by a high-sensitive ELISA in accordance with manufacturers’ instructions (Cygnus Technologies Inc., NC, USA). Subsequently, the rhCRP was purified using size exclusion chromatography to remove contaminants including endotoxin and sodium azide (Univalid bv, Leiden, The Netherlands). High purity was demonstrated by high-pressure size-exclusion chromatography (SEC)-HPLC and reverse-phase HPLC, as well as Time-of-Flight mass spectrometry, showing no other protein fractions than rhCRP. As evaluated by the Limulus assay (turbidimetric kinetic method; ACC inc., East Falmouth, Ma, USA), endotoxin levels in the end-product were <1.5 EU/mL at 0.91 mg/mL rhCRP. Cell culture experiments using human umbilical vein endothelial cells revealed no toxicity of the end-product. Single-dose toxicity studies in mice (n=6) reaching CRP-levels over 4-times that obtained in humans, demonstrated no direct effects of the end-product on temperature, blood pressure or heart rate. The end-product was stored under sterile conditions in a CaCl₂ containing buffer (pH 8.5) at 0-4°C degrees and all experiments were performed within 4 weeks after preparation. Stability of the CRP-pentamer was demonstrated by HPLC-analysis at 4 and 8 weeks after preparation. CRP existed in a pentameric form at time of administration.

Vascular function
Assessment of vascular function was performed using venous occlusion plethysmography (EC4; Hokanson Inc) 13. The non-dominant brachial artery was cannulated using a 20-gauge polyethylene catheter under local anaesthesia. At least 45 minutes after cannulation, forearm blood flow (FBF), expressed in millilitres (mL) per minute per 100 mL of forearm tissue
volume (FAV), was measured simultaneously in both arms. During each measurement blood pressure cuffs around both upper arms were inflated (40 mmHg) using a rapid cuff inflator. Synchronously, bilateral wrist cuffs were inflated to above-systolic blood pressure in order to exclude the hand circulation (200 mmHg). Intra-arterial blood pressure and heart rate were continuously monitored. Next, FBF-responses to cumulative doses of serotonin (5-HT; Sigma Chemical, MO, USA; 0.6, 1.8, and 6 ng.100 mL FAV\(^{-1}\).min\(^{-1}\)) and nitroprusside (SNP; Spruyt Hillen, Utrecht, The Netherlands; 6, 60, 180, and 600 ng.100 mL FAV\(^{-1}\).min\(^{-1}\)) were measured. All agents were administered intra-arterially for 6 and 4 minutes at each dose, respectively, using a constant-rate infusion pump. Six measurements during the last two minutes (steady state) were averaged to determine mean FBF. The two different infusion blocks were initiated after a 15-minute rest period, or until FBF had returned to baseline. Six hours after intravenous infusion of CRP, the two infusion blocks were repeated. Additionally, B-mode ultrasound measurement of carotid artery intima-media thickness (IMT) was performed using an Acuson 128XP/10v equipped with a 7.0-MHz linear array transducer, as described previously\(^{14}\).

**Biochemical analyses**

Blood samples were processed immediately by centrifugation at 1700g for 15 minutes at 20° C and stored at −80° C until batch-wise analysis. Total cholesterol, HDL-cholesterol (HDL-C) and triglycerides were determined enzymatically (Boehringer Mannheim, Mannheim, Germany), whereas the inter- and intra-assay coefficients of variation (CV) were <2% for all three). LDL-C was calculated using the Friedewald equation. Apolipoprotein B-100 (apoB100) and apolipoprotein A-I (apoA-I) were assayed by rate nephelometry (CV<5% for both). CRP was measured by a high-sensitivity method (Roche Diagnostic Corporation, Basel, Switzerland) and (>10 mg/L) by immunonephelometry (P800 analyser, Roche Diagnostic Corporation) (CV<4% for both). IL-6, and IL-8 was assayed by Cytometric Bead Array Analysis (BD Biosciences, CA, USA), whereas ELISA’s were used to measure F\(_{1+2}\) (Dade-Behring, Marburg, Germany), PAI-1 antigen (Monozyme, Charlottelund, Denmark), serum amyloid A protein (SAA; Anogen, Ontario, Canada), type II secretory phospholipase A\(_2\) (sPLA\(_2\); CLB, Amsterdam, the Netherlands), soluble E-selectin (R&D Systems, Abingdon, UK), MCP-1 and D-dimer (Asserachrom D-dimer, Roche, Almere, Netherlands) (CVs<10% for all assays). PAI-1 activity (CVs <6%) was determined on an automated coagulation analyser (Behring Coagulation System) with reagents and protocols from the manufacturer (Dade Behring).

**Statistical analysis**

Unless stated otherwise, data are expressed as mean ± SD. Descriptive statistics between both groups were compared by means of Student’s \(t\) test or Mann Whitney test (nonparametric), as appropriate. Statistical analysis was performed of the FBF responses and clinical parameters between both groups using two-way analysis of variance (ANOVA) for repeated measures (SPSS for Windows 10.0, SPSS Inc., IL, USA). For comparisons within groups the
Wilcoxon signed rank test or the Mann-Whitney U test was used, as appropriate. A probability value of <0.05 was considered significant.

**Results**

**Baseline clinical characteristics (table 1)**

Plasma levels of total cholesterol, LDL-C and apoB100 (all \( p < 0.010 \)) were significantly higher in FH-patients. Concomitantly, HDL-C \( (p = 0.045) \) and apoA-I levels \( (p = 0.004) \) were lower in the FH-cohort. Compared to controls, higher BMI values were found in the FH-patients \( (p = 0.025) \), whereas blood pressure, heart rate and FBF were not different. The IMT-values appeared higher in the FH-subjects compared to controls, however the difference between these small groups did not reach statistical significance.

<table>
<thead>
<tr>
<th>Table 1. Demographic and Baseline Parameters of the Study Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FH patients (n=6)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FH patients (Mean (SD))</th>
<th>FH patients (Median (95% CI))</th>
<th>Controls (Mean (SD))</th>
<th>Controls (Median (95% CI))</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>36.3 (11.5)</td>
<td>41.5 (24.2-48.4)</td>
<td>35.8 (10.9)</td>
<td>31.5 (24.4-47.3)</td>
<td>0.870</td>
</tr>
<tr>
<td>BMI, kg/m(^2)</td>
<td>28.3 (4.1)</td>
<td>27.5 (24.1-32.6)</td>
<td>23.2 (2.5)</td>
<td>22.2 (20.5-25.8)</td>
<td>0.025</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>130 (4)</td>
<td>129 (125-134)</td>
<td>131 (8)</td>
<td>133 (123-139)</td>
<td>0.470</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>83 (6)</td>
<td>82 (76-90)</td>
<td>80 (4)</td>
<td>82 (76-84)</td>
<td>0.430</td>
</tr>
<tr>
<td>Basal FBF, ml.100 mL FAV-1.min-1</td>
<td>2.8 (0.9)</td>
<td>2.7 (1.8-3.7)</td>
<td>2.7 (2.4)</td>
<td>2.0 (0.2-5.1)</td>
<td>0.210</td>
</tr>
<tr>
<td>TC, mmol/l</td>
<td>6.4 (0.9)</td>
<td>6.4 (5.5-7.3)</td>
<td>4.0 (0.8)</td>
<td>3.9 (3.1-4.8)</td>
<td>0.004</td>
</tr>
<tr>
<td>LDL-C, mmol/l</td>
<td>4.6 (1.1)</td>
<td>4.6 (3.4-5.7)</td>
<td>2.2 (0.6)</td>
<td>2.2 (1.6-2.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>HDL-C, mmol/l</td>
<td>1.0 (0.2)</td>
<td>1.0 (0.8-1.2)</td>
<td>1.2 (0.2)</td>
<td>1.2 (1.0-1.4)</td>
<td>0.045</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>1.7 (1.4)</td>
<td>1.2 (0.2-3.2)</td>
<td>1.1 (0.9)</td>
<td>0.8 (0.2-2.1)</td>
<td>0.340</td>
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<tr>
<td>Apolipoprotein A-I, g/l</td>
<td>0.8 (0.1)</td>
<td>0.8 (0.7-0.9)</td>
<td>1.1 (0.1)</td>
<td>1.1 (1.0-1.1)</td>
<td>0.004</td>
</tr>
<tr>
<td>Apolipoprotein B100, g/l</td>
<td>1.2 (0.3)</td>
<td>1.1 (0.9-1.4)</td>
<td>0.7 (0.2)</td>
<td>0.7 (0.5-0.9)</td>
<td>0.006</td>
</tr>
<tr>
<td>HsCRP, mg/l</td>
<td>1.8 (1.7)</td>
<td>1.5 (0.3-6.3)</td>
<td>2.8 (3.0)</td>
<td>2.3 (0.3-6.0)</td>
<td>0.340</td>
</tr>
<tr>
<td>IMT, -10-1 mm</td>
<td>8.3 (1.5)</td>
<td>7.9 (6.6-9.9)</td>
<td>6.9 (0.7)</td>
<td>6.9 (6.2-7.6)</td>
<td>0.109</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HR, heart rate; FBF, forearm blood flow; FAV, forearm tissue volume; TC, total cholesterol; TG, triglycerides; hsCRP, high-sensitivity CRP; IMT, intima-media thickness.

**Dose-escalation study (figure 1)**

Upon CRP-infusion, plasma CRP levels acutely increased, peaking after 1 hour, i.e. from 2.5±4.0 to 7.9±4.0, from 2.4±3.3 to 13.1±2.8 and from 1.3±0.9 to 28.1±5.9 mg/L in the 0.25 mg/kg group, 0.5 mg/kg group and 1.25mg/kg group, respectively. Subsequently, \( F_{1+2} \) levels increased from 0.8±0.3 to 1.9±0.8 nmol/L \( (p = 0.018) \) in the 1.25 mg/kg group, and from 0.8±0.2 to 1.1±0.3 nmol/L \( (p = 0.028) \) in the 0.5 mg/kg group, peaking after 4 hours. Noticeably, a dose-dependent activation of thrombin generation was observed at 4 hours.
Figure 1. Dose escalation study with levels of CRP, IL-6 and F1+2 before and after CRP-infusion. Mean (± SD) concentrations of CRP, IL-6 and F1+2 in response to rhCRP-infusion. The rhCRP-infusion was associated with an acute and dose-dependent rise in CRP-levels, peaking after 1 hour. Concentrations >10 mg/L (dose 0.5 mg/kg body weight) were required to activate both the inflammatory (IL-6) and coagulation (F1+2) pathways. Notably, moderate CRP elevations in the physiological range were not associated with activation of either of these pathways.

CRP-levels before and after CRP-infusion

Based on the results from the dose-escalation study, 1.25 mg/kg CRP was administered in the FH-patients and controls. During the infusion studies, none of the subjects experienced adverse effects. Body temperature and hemodynamics remained constant throughout the experiment. At baseline, CRP levels were 1.8±1.7 mg/l in FH-patients and 2.8±3.0 mg/l in controls (p=0.340). After CRP-infusion, CRP levels increased to 30.0±3.5 mg/l and 26.2±2.9 mg/l in FH-patients and controls, respectively.

Vascular function (figure 2)

The intra-arterial infusion of the endothelium-dependent vasodilator 5-HT increased FBF in a dose-dependent manner in both groups. At baseline, maximal 5-HT-induced vasodilation was lower in FH-patients (89.2±30.0%; p=0.037) compared to controls. Six hours after CRP-infusion, the blunted endothelial vasodilator response to 5-HT decreased even further in the FH-patients (max 42.3±25.7%; p=0.029). In controls, maximal FBF-responses to 5-HT were similar before (117.7±13.1%) and after rhCRP-infusion (110.3±22.0%). Maximal SNP-mediated vasodilation at baseline was
not significantly different between FH-patients and controls and remained unaffected upon CRP-infusion within both groups (FH-patients before 284.0±83.3% and after 277.7±61.7%, p=0.110; controls before 334.6±146.3% and after 341.8±94.7%, p=0.550).

![Figure 2](image1)

**Figure 2.** Endothelial vasodilator capacity before and after CRP-infusion. Individual FBF dose-response curves to the endothelium-dependent vasodilator 5-HT before and after rhCRP-infusion in FH patients (before ●; after ○) and normocholesterolemic controls (before ■; after □). At baseline, maximal 5-HT-induced vasodilation was lower in FH-patients (p=0.037) compared to controls. After rhCRP-infusion, the FBF response to 5-HT in the FH-patients significantly deteriorated (p=0.029), whereas no additional effects were seen in controls.

### Coagulation and fibrinolysis (figure 3)

At baseline, $F_{1+2}$ levels were increased in FH-patients compared to controls (0.8±0.2 versus 0.6±0.1 nmol/l; p=0.030). Additionally, PAI-1 activity was higher in FH-patients than in controls (5.8±1.0 versus 3.7±1.4 U/mL; p=0.016). No significant differences regarding baseline values were observed for PAI-1 antigen or D-dimer. 4 hours after CRP-infusion, levels of $F_{1+2}$ increased to a greater extent in FH-patients compared to controls (3.1±0.7 versus 2.0±1.1 nmol/l; p=0.009). In a similar fashion, PAI-1 antigen (185.8±109.3 versus 54.2±25.2 ng/ml; p=0.018) and PAI-1 activity (11.0±3.6 versus 4.7±1.5 U/mL; p=0.003) increased to a greater extent in FH-patients compared to controls, both peaking at 4 hours. Last, patient and control group showed an increase in D-dimer during the 8-hour study period although this was not significantly different.

### Inflammation (figure 4)

CRP-infusion was associated with a transient rise in plasma levels of the cell recruiter MCP-1 (for both groups p<0.01 versus baseline), and the cytokines IL-6 (for FH-patients and controls p=0.022 and 0.088 versus baseline, respectively) and IL-8 (for both groups p≤0.05 versus baseline), all peaking after 4 hours. However, no differences in response was seen between both groups. Moreover, CRP-infusion was followed by a gradual rise in plasma concentration of sE-selectin (for both groups p<0.05 versus baseline), indicating endothelial cell activation, and the acute phase reactants SAA (for FH-patients and controls p=0.008
and 0.054 versus baseline, respectively) and sPLA₂ (for both groups p<0.03 versus baseline). However, no differences in response was noticed between both groups. During the 8-hour observation, activation of these inflammatory pathways was not coincided by alterations in lipid metabolism in any of the groups.

**Discussion**

A single bolus-infusion of 1.25 mg/kg CRP elicited a progressive decline of endothelium-dependent vasodilation in hypercholesterolemic individuals. In contrast, CRP had no effect on vascular reactivity in normocholesterolemic controls. Concomitantly, CRP-mediated procoagulant responses were augmented in FH-patients, whereas inflammatory responses were not different between both groups. Additional studies addressing the potential benefits of specific CRP-antagonism on top of cholesterol-reduction are therefore warranted.

**Dose-escalation study**

The dose-escalation study revealed dose-dependent effects of CRP on inflammation and coagulation, and activation of these pathways was detectable at CRP-concentrations ≥10 mg/L (dose 0.5 mg/kg), at least in an acute setting. Hence, a CRP-dose of 1.25 mg/kg was chosen for evaluating its effects under normo- and hypercholesterolemic conditions.
Figure 4. Effects of rhCRP-infusion on inflammation. Individual responses of sE-selectin, MCP-1, IL-6, IL-8, sPLA2, and SAA after rhCRP-infusion in FH-patients (●) and controls (○). CRP elicited endothelial cell activation and a systemic proinflammatory response in both FH-patients and controls, however there were no differences in response between both groups.
CRP and Vasoreactivity

In cultured endothelial cells, CRP was capable of decreasing eNOS mRNA and protein as well as (bio)activity within 24 hours\textsuperscript{15,16}. Recent reports describing CRP's actions 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{17}, whereas the sensitivity to acetylcholine increased after a 4-hour exposure to 200 μg/mL CRP in aortas of Sprague-Dawley rats\textsuperscript{18}. In the present study, CRP increasing up to 26 μg/mL did not alter endothelial vasoreactivity in normocholesterolemic men after 6 hours. Whereas discrepancy in dose and exposure-time, as well as species differences should be taken into account, the present finding implies that a moderate CRP-increase is unlikely to induce endothelial dysfunction in otherwise healthy individuals. In fact, these data are in accordance with findings by Clapp et al, who showed normalization of endothelial dysfunction several hours after typhoid vaccination in spite of still increasing CRP levels\textsuperscript{18}.

FH is characterized by early loss of NO-activity coinciding with accelerated atherogenesis\textsuperscript{13}. FH-patients in our study showed mildly elevated LDL-C levels compared to controls and did not exhibit overt cardiovascular disease. Furthermore, their IMT-values, as surrogate marker for subclinical atherosclerosis, did not statistically differ from that of controls. Nevertheless, their baseline endothelial vasodilator response to serotonin was evidently impaired. Underlying mechanisms include oxLDL-mediated displacement and uncoupling of eNOS, producing superoxide anions rather than NO\textsuperscript{19}. In clear contrast to controls, CRP-infusion in FH patients caused marked deterioration of endothelium-dependent vasodilation. Apparently, the potent vasoprotective properties that are constitutively employed by healthy endothelium, clearly fail in FH-patients\textsuperscript{13}. Several mechanisms may be involved here. First, CRP-antagonism of eNOS has been attributed to decreased stability of eNOS mRNA\textsuperscript{16} and, in a non-genomical manner, blunted eNOS phosphorylation at Ser1179\textsuperscript{20}. These mechanisms are suggested to be mediated by FcγRIIB via protein phosphatase 2A. Second, CRP may reduce prostacyclin secretion and stimulate superoxide anion release from NAD(P)H oxidase via p38 kinase activation\textsuperscript{17,21}. Last, CRP was reported to dose- and time-dependently induce LOX-1, crucial for oxLDL's detrimental effects on endothelial function in human endothelium\textsuperscript{22}. Noticeably, these experimental studies were all carried out with the pentameric configuration of CRP. Monomeric CRP, on the contrary, has recently been suggested to exhibit vasoprotective properties\textsuperscript{23,24}. Thus, anticipating the reported divergent activities of the 2 different conformational shapes of CRP, our study implicated only the pentameric form, as demonstrated by HPLC.

The authors acknowledge that further research is warranted to address the role of above-mentioned pathophysiological mechanisms responsible here. Summarizing, CRP-elevation in these hypercholesterolemic patients, that already are characterized by early vascular disease, may have resulted in further deterioration of endothelium-dependent vasoreactivity by enhanced vascular oxLDL-uptake and/or decreased NO-bioavailability.
CRP and Coagulation
We found higher procoagulant activity at baseline in FH-patients. Moreover, stimulation of thrombin generation and PAI-1 by CRP in these individuals was more pronounced. CRP has been reported to promote tissue factor expression on mononuclear cells, and, in addition, endothelial PAI-1 expression and activity\textsuperscript{25,26}. Given the prominent role of endothelium for counteracting procoagulant pathways, the augmented responses in FH-patients can be linked to decreased NO-activity, deteriorating upon CRP-infusion. Elevated LDL-C and reduced NO-activity, both features of FH, are associated with a higher state of coagulation activation. In turn, interventions aiming at lipid-lowering and stimulation of eNOS-activity, favourably affect ‘blood thrombogenicity’\textsuperscript{27,28}. Further, endothelial PAI-1 expression that correlates with the degree of atherosclerosis is partly regulated by NO\textsuperscript{29}. Collectively, these data indicate that CRP-induced activation of procoagulant pathways may be linked to decreased NO-activity, and may increase risk for arterial thrombotic events particularly under hypercholesterolemic conditions.

CRP and inflammation
Among its proinflammatory effects, CRP has been shown to promote monocyte-endothelium interaction by virtue of stimulating the release of chemoattractant IL-8 and antagonizing eNOS activity\textsuperscript{16,30}. Further, CRP can induce an inflammatory phenotype of venous endothelium, including upregulation of adhesion molecules and MCP-1, which in part has been attributed to the release of endothelin-1 and IL-6\textsuperscript{31}. In addition, CRP elicits an inflammatory phenotype in artery endothelial cell\textsuperscript{9} and phagocytes\textsuperscript{8}. For that matter, binding and internalization of CRP by Fcγ receptors, activation of the NFκB and p38 MAPK signalling pathways and upregulation of the CD40/CD40 ligand signaling dyad have been implicated in the proinflammatory effects of CRP on cellular functions\textsuperscript{7,23-34}. In agreement with these \textit{in vitro} data, we observed an inflammatory response upon infusing CRP in human subjects, that overall did not significantly differ between both groups. During the 8-hour observation we also didn’t notice any change in lipid profile in either groups. Given that, the differences between FH-patients and controls in regard to CRP-mediated endothelial dysfunction and activation of coagulation cannot be explained from the release of proinflammatory mediators or altered lipid metabolism.

Study limitations
The mean LDL-C levels in this FH-cohort appear lower than may be expected from untreated FH-patients. Of note, all FH-subjects, except for one, were carriers of the N543H/2393del9 combo-mutation, that is characterized by mildly elevated LDL-C levels\textsuperscript{35}. The current study results may thus be extrapolated to non-genetically determined hypercholesterolemia. Moreover, the FH-patients exhibited a significant higher BMI and lower levels of HDL-C/ apoA-I than controls. Considering the close ties between obesity and low-grade inflammation, where inflammatory mediators or other pathways more easily can be initiated, we cannot preclude that obesity may have contributed to the augmented responses seen in FH-
patients. Given the growing evidence of HDL modulating vascular reactivity, a confounding role of low HDL-C in the endothelial response in the FH-patients cannot be excluded.

We were unable to perform control experiments in humans using a placebo containing inactivated CRP, for further differentiation of CRP-mediated effects from that of potential contaminants, i.e. sodium azide and bacterial compounds. Nonetheless, several observations clearly argue against a major role for contaminants in our experiments. First, recent studies indicate that CRP itself has the capacity to stimulate mononuclear and endothelial cells to release IL-6 and IL-8. That, in combination with the absence of stigmata of endotoxemia and distinct patterns of cytokine inductions makes a contributing role for contaminants unlikely. Addressing potential vasodilator effects due to sodium azide contamination, no vasodilator effects were observed upon infusion of purified CRP in our cohort. Moreover, relevant amounts of bacterial compounds within commercial CRP, including endotoxin, would result in activation of the Toll-like receptor family, associated with TNF-release and inflammatory changes, further attenuating impaired endothelial vasoreactivity. Markedly, in contrast to E.Coli-endotoxin, we were unable to detect TNF-release upon rhCRP-infusion.

In concordance, infusion of E.Coli-endotoxin at a dose, that equaled the mean co-infused dose during the CRP-infusion experiments, was not accompanied by any inflammatory effect in healthy volunteers, once more excluding a prominent role for trace amounts of LPS in mediating the inflammatory responses observed after infusion of rhCRP. Finally, absence of any effect whatsoever on vascular function in healthy volunteers, in contrast to clear effects in FH-patients, provides further arguments for a selective, rather than contaminant-driven response. Noticeably, recent evidence suggest that CRP-mediated effects on vascular endothelium may be receptor-mediated.

Clinical implications

There is growing evidence that CRP may participate in atherosclerosis progression, although its precise role has not been fully elucidated. At present, CRP induces endothelial dysfunction and procoagulant activity, predominately in hypercholesterolemic subjects. In light of the REVERSAL and PROVE-IT trials, indicating that lower CRP levels after statin-therapy are associated with better cardiovascular outcome regardless of LDL-cholesterol reduction, further support is now provided for aggressive therapeutic strategies targeting CRP on top of cholesterol-lowering in order to improve cardiovascular prevention. Promising targets for CRP-antagonism involves blocking its biological effects at receptor level as well as its synthesis by antisense oligonucleotide therapies. Recently, a small-molecule inhibitor of CRP has been found to attenuate the increase in infarct size and cardiac dysfunction in reaction to human CRP in rats undergoing experimental myocardial infarction.
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Reference List


