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Calcification and C-reactive protein in atherosclerosis : effects of calcium blocking and cholesterol lowering therapy

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Chapter 6

No effect of C-reactive protein on early atherosclerosis development in ApoE*3-Leiden/hCRP transgenic mice

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Abstract

Objective The acute phase protein C-reactive protein (CRP) has been associated with risk of cardiovascular disease (CVD). Atherosclerosis is the most important mechanism underlying CVD. However, it is not clear whether CRP is causally involved in the development of atherosclerosis. Mouse CRP is not expressed at high levels under normal conditions, and increases in concentration only several fold during an acute phase response. Since the dynamic range of human CRP is much larger, apolipoprotein E*3-Leiden (E3L) transgenic mice carrying the human CRP gene offer a unique model to study the role(s) of CRP in the development of atherosclerosis.

Methods and Results Atherosclerosis development was studied in 15 male and 15 female E3L/CRP mice; male and female E3L transgenic littermates were used as controls. The mice were fed a hypercholesterolemic diet for thirty (males) or twenty-five (females) weeks. Cholesterol exposure did not differ between E3L/CRP and E3L mice. Plasma CRP levels were on average 10.2 ± 6.5 mg/L in male E3L/CRP mice, 0.2 ± 0.1 mg/L in female E3L/CRP mice, and undetectable in E3L mice. Quantification of atherosclerosis in the aortic root showed that lesion area in E3L/CRP mice was not different from that in E3L mice.

Conclusion This study demonstrates that mildly elevated levels of CRP in plasma do not contribute to the development of early atherosclerosis in hypercholesterolemic E3L/CRP mice.

Keywords: atherosclerosis, C-reactive protein, inflammation, mouse, transgene

Introduction

Cardiovascular disease due to atherosclerosis is a major cause of mortality and morbidity in Western society. In recent years inflammation has emerged as a key factor in atherosclerosis development.[1] The acute phase protein C-reactive protein (CRP) has been shown to predict the risk of cardiovascular events.[2] Since the plasma concentration of CRP reflects the inflammatory condition, it has been hypothesised that CRP levels reflect the inflammatory condition of the vascular wall. Whether CRP itself contributes to atherosclerosis development is, however, still unknown.

CRP is a member of the highly conserved pentraxin family, and is produced mainly by hepatocytes in response to infection or inflammation. Its production is stimulated by cytokines such as IL-6, IL-1 and TNFα. CRP binds to phosphatidylcholine in cell membranes and plasma lipoproteins, in a Ca^{2+}-dependent manner, and has a role in opsonization of infectious agents and damaged cells.[3] I*n vitro* studies have reported numerous effects of CRP on endothelial cells, smooth muscle cells and monocytes. CRP treatment often elicits proinflammatory and proatherosclerotic effects.[4] For instance, CRP activates endothelial cells to produce adhesion molecules, induces monocyte chemoattractant chemokine-1 (MCP-1) production facilitating leukocyte adhesion and diapedesis, contributes to the migration of smooth muscle cells, enhances uptake of native LDL by macrophages, and activates complement.[5-11] Furthermore, local CRP production by cells in the atherosclerotic lesion has been reported.[12]

Apolipoprotein E*3-Leiden (E3L) mice exhibit elevated plasma cholesterol and triglyceride levels[13], resembling familial dysbetalipoproteinemia in humans. Plasma cholesterol levels in these mice can easily be titrated by diet.[14-16] E3L mice develop various stages of atherosclerosis, varying from mild type I-III foamy lesions to severe type IV-V lesions, depending on plasma cholesterol levels and duration of exposure.[14,16] Since CRP is a major acute phase reactant in man but not in mice, the human CRP gene was introduced into E3L mice to enable us to study the contribution of human CRP to the development of early atherosclerosis in hypercholesterolemic mice.

Methods

Mice

CRP transgenic mice[17,18] carry a 31-kb fragment of human genomic DNA, containing the CRP gene and flanking sequences, on a C57Bl/6 background. $E3L^{+/-}/CRP^{+/-}$ mice were obtained by crossbreeding female E3L mice[13-16] with heterozygous male CRP transgenic

mice. Non-CRP transgenic littermates were used as controls. E3L mice are also bred on a C57Bl/6 background.

Experimental design

Four groups of mice were used. Fifteen male E3L/CRP mice (group I) and 15 female E3L/CRP (group II) were used. As controls, 15 male (group III) and 15 female (group IV) E3L transgenic littermates were used. The presence of the E3L transgene was assessed by an ELISA for human apoE.[16] The presence of the hCRP gene was assessed by PCR genotyping.

Since testosterone is required for constitutive expression of CRP in mice,[19] only males have detectable levels of CRP in their plasma. However, both male and female E3L/CRP transgenic mice will produce human CRP upon stimulation with LPS.

At the start of the study mice were matched on the basis of age (average: 14 weeks) and cholesterol levels. The Institutional Animal Care and Use Committee had approved all animal experiments.

Diets

Before the start of the study, animals were kept on a standard murine chow diet (Hope Farms, Woerden, the Netherlands). At the start of the experiment the diet of the female mice was changed to a semi-synthetic cholesterol-raising diet supplemented with 0.5% ($^{w}/_{w}$) cholesterol [table 1]. Since gender has been shown to affect hyperlipidemia and atherosclerosis development in the E3L mouse model [20], male mice were fed a diet supplemented with 1% cholesterol and 0.05% sodium cholate ($^{w}/_{w}$) [table 1]. In addition, fructose was added to the drinking water of male mice, which results in an additional plasma cholesterol increase of about 3 mmol/L. Female and male mice were kept on these diets for 25 and 30 weeks, respectively, because male E3L mice develop atherosclerosis more slowly than the females.

All animals had free access to water and food. Body weight and food intake were monitored every 4 weeks.

Table 1. Summary of diet characteristics. [21]

	Females	Males
Weeks on diet	25	30
Diet containing:	15% cacao butter 40.5% sucrose 10% corn starch 1% corn oil 20% casein 5.45% cellulose 5.1% mineral 1% choline chloride 0.2% methionine	15% cacao butter 40.5% sucrose 10% corn starch 1% corn oil 20% casein 5.45% cellulose 5.1% mineral 1% choline chloride 0.2% methionine
Supplemented with:	0.5% cholesterol	1% cholesterol 0.05% cholate
Drinking water	tap water	10% fructose in tap water

Analysis of blood parameters

Blood samples were obtained at baseline, at t = 3, 8, 12, 16, 20, 24, 28 weeks and at sacrifice from each mouse by tail incision after a 4-hour fast. Blood samples were collected into pre-cooled EDTA-coated tubes, and centrifuged at 2000 x g for 10 min at 4°C to obtain plasma. Total plasma cholesterol and triglyceride levels were measured enzymatically [kit # 236691 (Roche Diagnostics, Almere, the Netherlands) and kit # 337-B (Sigma, St Louis, MI), respectively].

Lipoprotein distribution was determined for each group in a pooled plasma sample by fast protein liquid chromatography, using the ÄKTA system (Amersham-Pharmacia, Stockholm, Sweden).

Human CRP concentrations in plasma were measured using a high-sensitivity in-house enzyme immunoassay using rabbit anti-human-CRP IgG (DakoCytomation, Glostrup, Denmark) as capture and tagging antibody.[22] Human CRP Standard (Dade Behring, Marburg, Germany) was used as a calibrator.[22] The lower limit of sensitivity was 0.05 mg/L.

Plasma alanine aminotransferase (ALAT) activities were measured enzymatically (Reflotron kit # 745 138, Roche).

Serum amyloid A (SAA) was determined by ELISA, as prescribed by the manufacturer (Biosource International, Nivelles, Belgium).

Endothelial activation was assessed by determination of the plasma levels of von Willebrand factor (vWF) by ELISA, using antisera from DakoCytomation, essentially as described by Ingerslev,[23] and using pooled normal plasma for calibration.

Assessment of atherosclerosis

After 25 or 30 weeks of diet feeding, mice were sacrificed under general fentanyl / fluanison / midazolam anaesthesia, the hearts were dissected, stored overnight in phosphate-buffered 3.8% formalin, embedded in paraffin, and sectioned. Serial cross-sections were obtained from the aortic root area, and stained with haematoxylin-phloxin-saffron (HPS). For each mouse, 4 sections (5 μm thick) at intervals of 50 μm, representing that stretch of the aortic root where the aortic valves are clearly visible, were used for quantification and typing of atherosclerotic lesions. Total lesion area was determined using the Leica Qwin image analysis software. For each mouse, the average lesion area per cross-section was calculated. To determine the severity of atherosclerosis, the lesions were classified into five categories as described before[14,16]: type I) early fatty streak, type II) regular fatty streak, type III) mild plaque, type IV) moderate plaque, and type V) severe plaque.

Immunohistochemistry

We performed immunohistochemistry using primary antibodies specific for ICAM-1 (monoclonal anti-mouse-ICAM-1, 1:100, R&D, Minneapolis, MN), monocytes and macrophages (AIA31240, 1:1000, Accurate Chemical and Scientific) and CRP (anti-CRP clone 8, 1:500, Sigma). 5 μm thick sections were deparaffinized and rehydrated, and endogenous peroxidase activity was eliminated by treatment with 0.3% H_2O_2 for 15 min. After washing with PBS, sections were heated in 0.01 M citric acid (pH 6.0) for 10 min (when staining for ICAM-1), and incubated with 5% BSA in PBS for 15 min at room temperature. Sections were then incubated overnight with the primary antibody at 4°C. Subsequently, the sections were incubated with a biotinylated secondary antibody. Avidin-biotin conjugated HRP (DakoCytomation) and NovaRed substrate (Vector Laboratories, Burlingame, CA) were used to visualize the antibody complex. Sections were counterstained with haematoxylin.

Statistics

All data are presented as mean ± SD, or median (95% confidence interval). For statistical analysis SPSS 10.0 for Windows was used. Effects of the CRP transgene were tested

statistically for female and male mice separately. Parametric and non-parametric tests were used as indicated. To analyze the differences between groups the Mann-Whitney rank sum test was used. P values less than 0.05 were regarded as significant. Spearman's correlation coefficient was used for calculating correlations.

Results

Body weight and food intake

Body weight [table 2] and food intake (not shown) of E3L/CRP mice did not differ significantly from body weight and food intake of E3L mice, at any time point.

Table 2. Plasma levels of lipids and inflammatory markers, and lesion area at sacrifice.

	Females		**Males**	
	E3L/CRP	**E3L**	**E3L/CRP**	**E3L**
Body weight at sacrifice, g	22.6 ± 2.3	23.9 ± 2.5	29.6 ± 2.2	29.9 ± 2.5
Triglycerides, mmol/L (average)	1.6 ± 0.6	1.7 ± 0.6	1.7 ± 0.8*	1.2 ± 0.7
Cholesterol, mmol/L (average)	13.3 ± 3.8	13.6 ± 3.1	17.2 ± 6.2*	14.7 ± 5.7
Cholesterol exposure, mmol/L*weeks	312 ± 31	325 ± 25	552 ± 61	499 ± 96
Lesion area per section, x10^3 μm^2	35 ± 31	36 ± 27	27 ± 16	24 ± 22
Endpoint measurements:				
SAA, mg/L	16 ± 11	31 ± 27	114 ± 58	145 ± 88
vWF (% of normal pooled plasma)	50 ± 24	57 ± 26	153 ± 94	83 ± 53
CRP, mg/L	0.2 ± 0.1	< 0.05	10.2 ± 6.5*	< 0.05

Data are presented as mean ± SD for 13 mice per group. * $p < 0.05$ E3L/CRP males compared to E3L males (Mann-Whitney rank sum test)

Inflammation markers

CRP could only be detected in the plasma of E3L/CRP transgenic mice. Plasma CRP levels were on average 10.2 ± 6.5 mg/L in male mice. In female E3L/CRP mice, plasma CRP levels were much lower: 0.2 ± 0.1 mg/L [table 2].

Data on SAA and plasma vWF levels are shown in table 2. SAA levels were considerably higher in male mice as compared to female mice. Even though the SAA levels were lower in the E3L/CRP transgenic mice as compared with the gender-matched E3L mice, these differences were not statistically significant. vWF was significantly higher in the male mice as compared to the females, but there were no significant differences between E3L/CRP mice and E3L mice. CRP levels were not significantly correlated with either SAA or vWF levels.

Plasma cholesterol levels, cholesterol exposure and lipoprotein composition

Plasma cholesterol levels, as measured at 8 time points (t = 3, 8, 12, 16, 20, 24, 28, and 30 weeks), were, on average, 13.3 ± 3.8 mmol/L for female E3L/CRP mice, 13.6 ± 3.1 mmol/L for female E3L mice, 17.2 ± 6.2 mmol/L for male E3L/CRP mice and 14.7 ± 5.7 mmol/L for male E3L mice [table 2]. There was no significant difference in cholesterol exposure (plasma cholesterol times weeks of exposure) between the two male groups, nor between the two female groups [table 2, fig. 1A]. Thus, the presence of the human CRP gene did not modify plasma cholesterol levels in E3L mice.

Triglyceride levels did not differ significantly between the female groups, but the male E3L/CRP mice had higher triglyceride levels than male E3L mice [table 2]. Lipoprotein profiles at 20 weeks of high-cholesterol feeding did not differ between groups of the same sex [fig. 1B].

Initially, ALAT activities in plasma were around 50 U/L at t=0. In the female groups plasma ALAT levels gradually increased to approx. 150 U/L at the end of the study. In the male groups plasma ALAT levels increased more, to final values of approx. 350 U/L, presumably due to the use of 0.05% cholate in the diet.

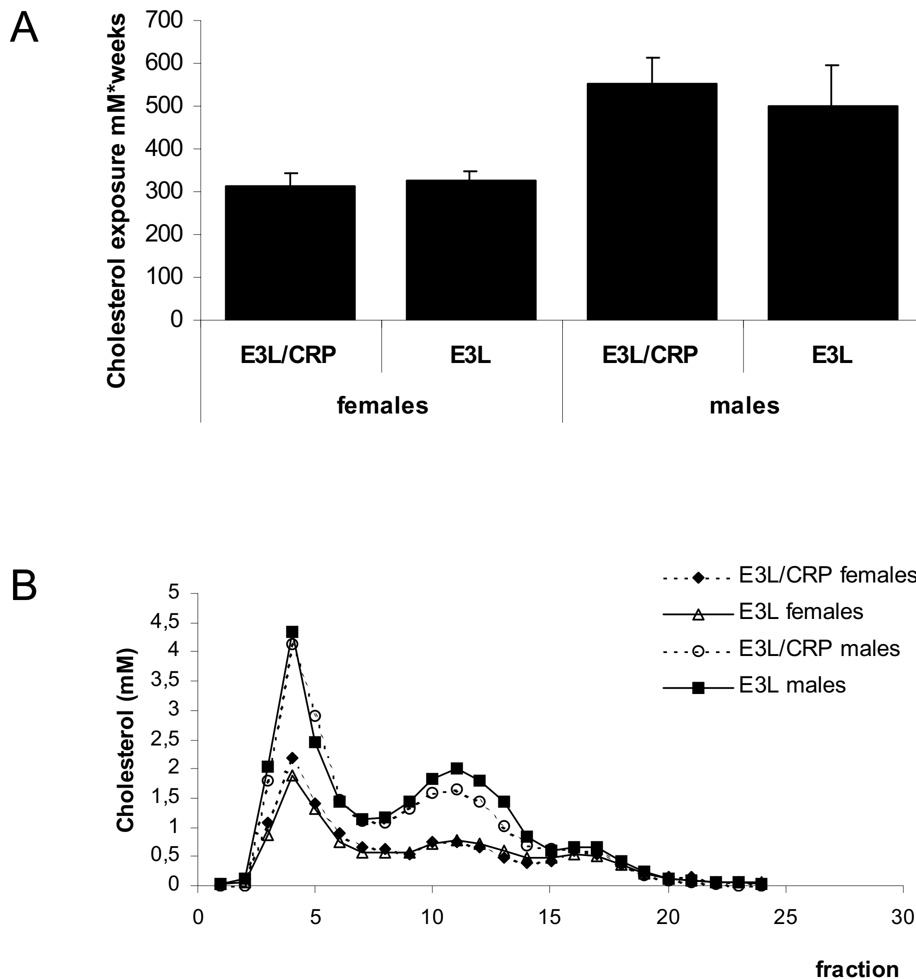


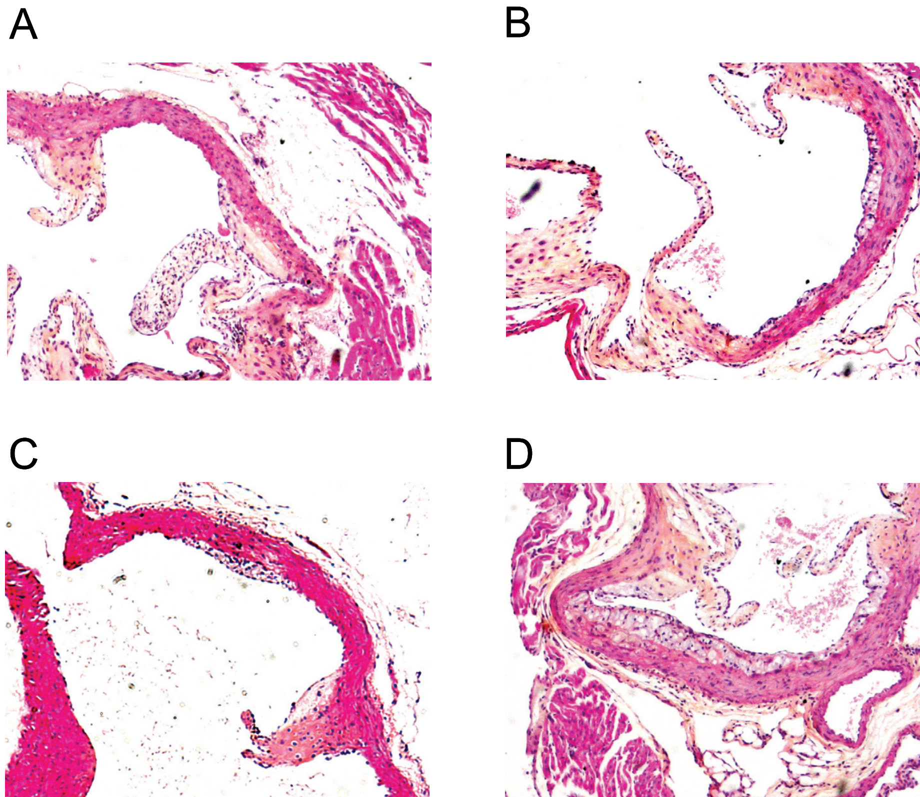
Figure 1. A. Total cholesterol exposure (mM*weeks) of the study groups (n = 13 per group). Data are presented as mean ± SD. There are no significant differences in cholesterol exposure between the two male groups, nor between the two female groups. **B.** Cholesterol profiles of plasma lipoproteins. Lipoprotein distribution was determined for each group in a pooled plasma sample by fast protein liquid chromatography.

Atherosclerosis development

Atherosclerotic lesion area and the severity (type I-V) of the lesions were quantified in four cross sections of the aortic valve area for each individual mouse. Representative photomicrographs of atherosclerotic lesions present in the four groups are shown in figure 2.

No significant differences in average lesion size were observed between the E3L/CRP and E3L mice, either in male or in female mice [table 2, fig. 3A]. In the male mice there was no correlation between lesion size and plasma CRP concentration (r_s = 0.56, n.s.).

Figure 2. A+B+C. Representative photomicrographs of mild atherosclerotic lesions (type I-III) as observed in the different study groups and **D.** of a more severe type IV lesion (haematoxylin-phloxine-saffron staining).



Lesions were somewhat larger in female mice (with minimal expression of CRP) than in male mice (which did express CRP), despite a larger cholesterol exposure in the male groups. This sex difference, which differs form that in most strains of mice, is in agreement with previous observations in this strain of mice,[20] and with observations in unpublished studies (J.J. Emeis, data on file) that lesion development in males is slower than in females in APOE*3Leiden mice.

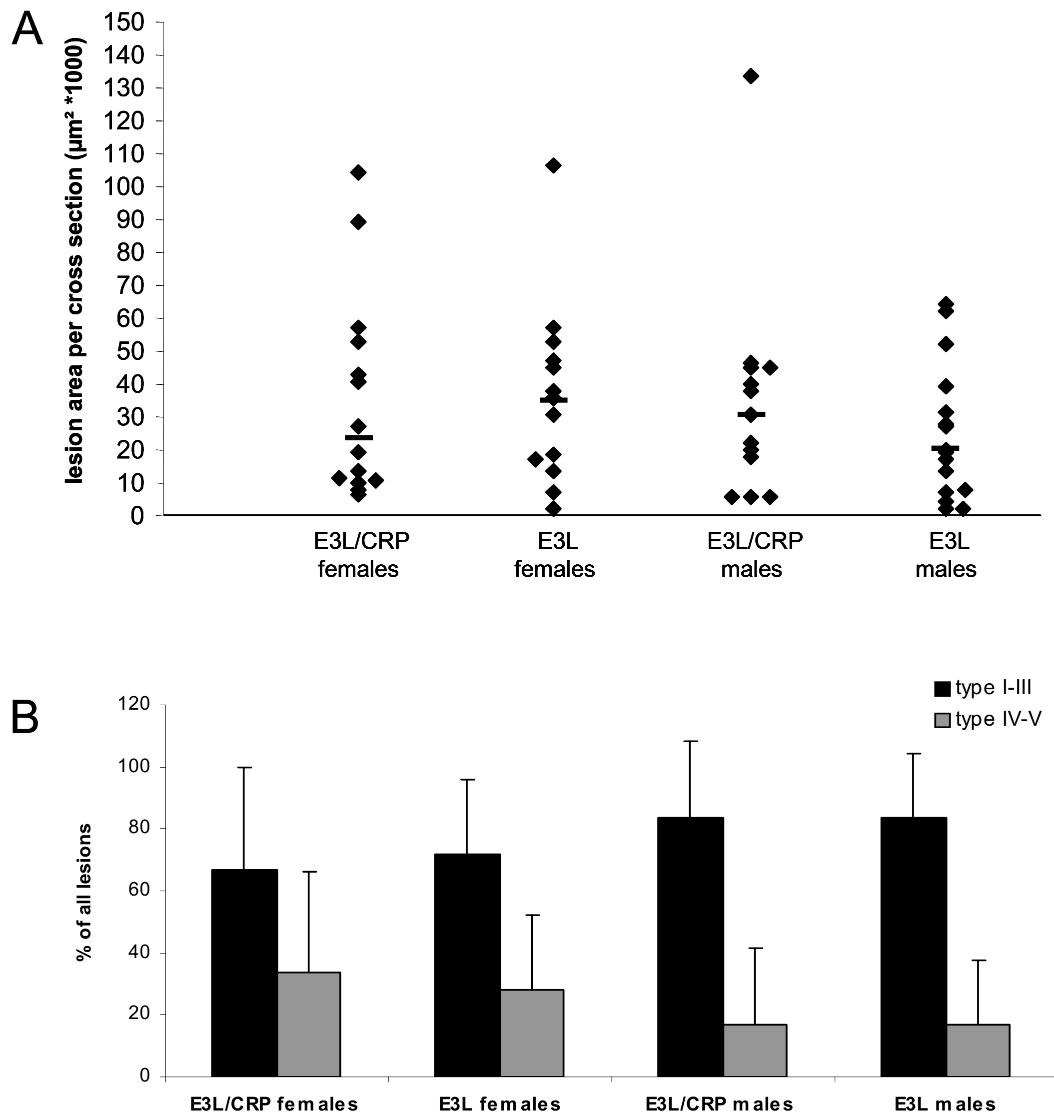


Figure 3. A. Shown are individual values (♦) for average atherosclerotic lesion area per section per mouse; (—) indicates the median value for each study group. There are no significant differences in lesion size between the male groups or between the female groups. **B.** Distribution of the severity of atherosclerotic lesions, as determined by the percentage of lesions classified as type I-III lesions (fatty dots and streaks and mild plaques) and type IV-V lesions (moderate and severe plaques). Data are presented as mean ± SD. See table 3 for a statistical evaluation.

Assessment of lesion severity (scored as type I to type V lesions) showed that in female mice 69% of the lesions found were mild (type I-III lesions) and 29% were more severe (type IV and V lesions) [fig. 3B, table 3]. In the male mice, 83% of the lesions found were of type I to III, and 17% of type IV-V. No differences in lesion severity distribution were observed

between E3L/CRP mice and E3L mice of the same sex, nor between male and female mice [fig. 3B, table 3].

Table 3. Lesion severity in male and female E3L and E3L/CRP mice.

Lesion type	E3L/CRP males	E3L males	E3L/CRP females	E3L females
Type I-III	71	94	42	63
Type IV and V	18	21	15	25

The table indicates the absolute number of lesions of type I-III (fatty spots, fatty streaks and capped fatty lesions) and lesions type IV and V (complicated lesions with and without media involvement). There is no significant difference in the distribution of lesion types between the four groups (Chi-square = 3.67, df = 3, p = 0.230). By Fischer's exact test no differences were found either between the two female groups (p=0.850), or the two male groups (p=0.724), nor between the two male groups combined versus the two female groups combined (p=0.073).

Characterization of vessel wall and lesions

Because no differences in lesion area had been observed, we sought to determine if CRP was associated with any differences in the composition of the lesions. This was determined by assessment of endothelial activation, i.e. ICAM staining, by assessing the number of adhering monocytes and by the determination of the macrophage-containing area of the lesion.

The endothelium of the vessel wall was found to be ICAM-positive. However, no differences in staining intensity of ICAM-1 in the lesions were observed whether mice expressed CRP or not, either in male or in female mice.

The number of monocytes adhering to the endothelium was counted in the same slides used for quantification of atherosclerosis. Monocyte adhesion to the endothelium was not significantly different between the female E3L/CRP and female E3L mice, nor between the male E3L/CRP and male E3L [fig. 4A].

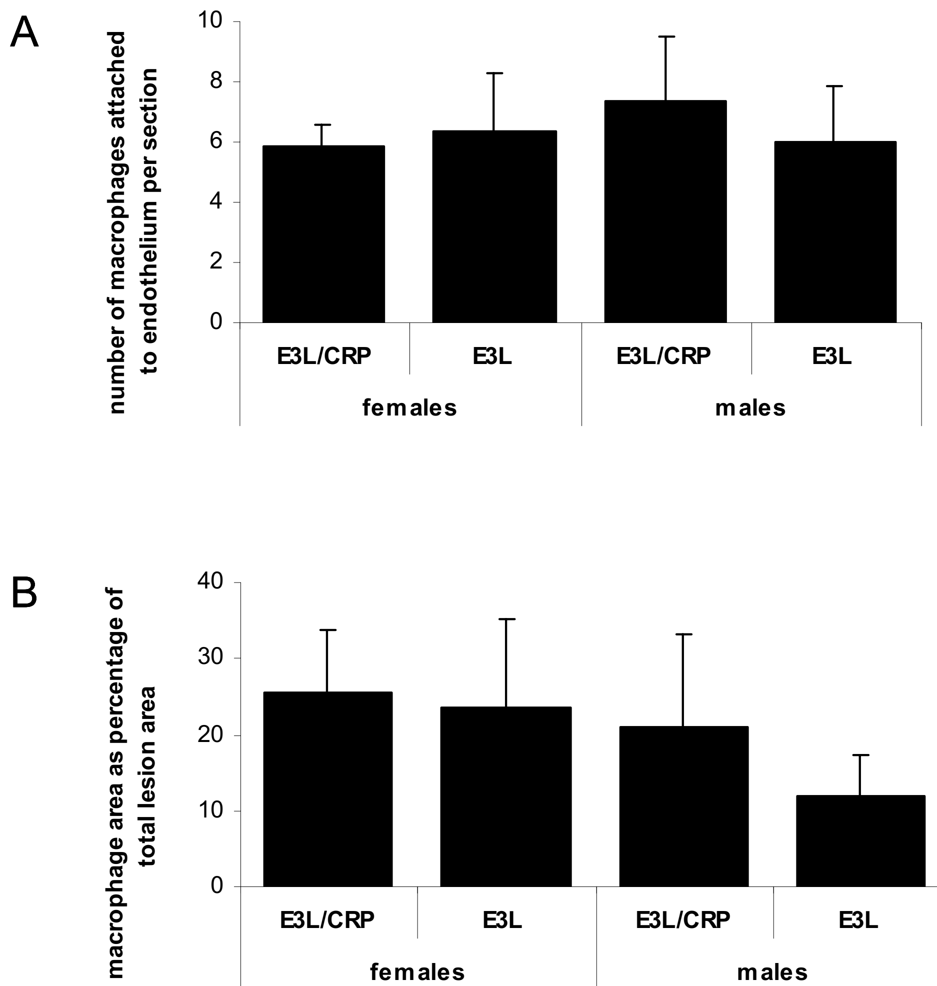


Figure 4. Effect of CRP on monocyte attachment to the endothelium and macrophage area. Adherent monocytes were counted in the same sections used for quantification of atherosclerosis. **A.** Average number of monocytes per section adhering to the endothelium. No significant differences in monocyte adhesion were observed between the E3L/CRP and E3L mice of the same sex (n = 5 per group). **B.** Macrophage area as percentage of total lesion area. No significant differences in macrophage area were observed between the E3L/CRP and E3L mice of the same sex (n = 5 per group).

In general the female mice had a larger macrophage-containing area than the males, which is in agreement with the fact that the lesion area of the females was larger [table 2]. When macrophage area was corrected for total lesion area no differences were observed between female E3L/CRP and female E3L mice [fig. 4B]. In male E3L/CRP mice, macrophage-containing area was 1.8-fold (n.s.) increased when compared to E3L mice of the same sex [fig. 4B].

We were unable to demonstrate the presence of CRP in the atherosclerotic lesions using a the same antibody technique as described by Paul *et al.*[24]

Discussion

Even though numerous *in vitro* studies have suggested a possible causal involvement of CRP in atherosclerosis, *in vivo* evidence for a proatherogenic role of CRP is scarce. In the present study we demonstrated that, in E3L mice, the presence of CRP in the blood did not modify the extent, nor the severity, of atherosclerosis development. Also, the effect of CRP on ICAM-1 expression observed *in vitro* [7] could not be confirmed *in vivo* in the present study.

The E3L transgenic mice are an established model for diet-induced atherosclerosis, and a useful model for drug induced effects on lesion formation.[25,26] The lesions found in this study were mostly mild, as we wanted to study the earlier stages of lesion development. Also, lesions in male mice were smaller than lesions in female mice, in agreement with previous (unpublished) observations that male E3L mice develop lesions more slowly. However, more severe lesions were also present in both male and female mice, demonstrating that these mice have the potential to develop more extensive and more complex lesions, as we have also observed in other studies (compare also van Vlijmen *et al* [20]). Lesion severity did not differ between male and female mice, nor beteen E3L and E3L/CRP mice [table 3].

Inflammation has been implicated in atherosclerosis development, and plasma levels of inflammatory markers can be used as predictors of the risk for cardiovascular events.[27,28] Therefore, we measured, besides CRP, also SAA and vWF at the endpoint of the study, vWF serving as a marker of endothelial cell activation. SAA was used as a second, general murine inflammation marker since it is present in all mice. There were no significant differences in SAA and vWF levels between the E3L/CRP and E3L mice. Male mice had higher levels of SAA and vWF than the females. This is probably a result of the cholate, which had been added to the diet of the males only, and may produce inflammatory responses.[29,30] The difference in plasma CRP level between male and female mice is due to the testosterone-dependence of CRP synthesis in this species.[19] Therefore, there was no evidence of a difference in inflammation status between the two male study groups or between the two female study groups.

Recently, Paul *et al*.[24] reported a significant increase in lesion size in CRP/apolipoprotein E knockout (apo $E^{-/-}$) mice. They also detected CRP in the atherosclerotic lesions where it was associated with increased complement C3 deposition, suggesting that CRP stimulated activation of complement within these lesions. Paul *et al*. concluded that CRP has a proatherogenic role *in vivo.* However, the plasma levels of CRP in these mice were extremely high, with a basal expression of 120 ± 77 mg/L. In the general population, presymptomatic, baseline plasma CRP levels greater than 3 mg/L are associated with an increased risk of heart attack and stroke.[31] Among patients with acute coronary syndromes CRP values above 3 mg/L are associated with increased risk of coronary events. However,

CRP values greater than 10 mg/L can reflect a wide range of pathologies, and therefore if patients are presenting with CRP levels exceeding 10 mg/l, CRP can no longer be used in the prediction of risk of atherothrombotic events. In the study reported here, the plasma CRP level in the male mice was approx. 10 mg/L, much lower than the very high CRP levels in the mice studied by Paul *et al*.[24]

We were not able to demonstrate the presence of CRP in lesions described by Paul *et al.*[24], despite the fact that we used exactly the same technique. Maier *et al*.[32] recently suggested that, in unstable human coronary lesions, SAA and IL-6 were locally produced, but CRP was not. If that would also be the case in mice, the lower CRP levels in our mice could explain our inability to demonstrate CRP in lesions, since CRP diffusion into the lesion would be less than in the mice described by Paul *et al.*[24]

In vitro studies have shown numerous effects of CRP on endothelial cells, smooth muscle cells and monocytes, usually eliciting proinflammatory and proatherosclerotic effects.[7-11,33] However, in those studies the concentrations of CRP used were usually very high, ranging up to 100 mg/L, while significant effects were generally observed only at CRP levels exceeding 10 mg/L.

Another point of uncertainty in the *in vitro* experiments relates to the purity of the CRP preparations used. Nagoshi *et al*.[34] have shown that even recombinant CRP preparations can be contaminated with endotoxin. Removal of endotoxin blocked the ability of rCRP to induce the secretion of IL-6 and MCP-1 by human coronary artery endothelial cells. *In vivo*, this problem obviously plays no role.

The results reported here do not support a role of CRP in atherosclerosis development in the E3L mouse. However, the association between CRP and cardiovascular disease is well established, since elevated CRP levels do predict future cardiovascular risk.[2,35,36] Possibly, CRP is implicated in other processes than atherosclerosis development that contribute to the incidence of CVD, such as thrombosis or revascularization after ischemia,[33,37-39] worsening the prognosis of CVD through these mechanisms. Danenberg *et al*.[37] have used human CRP transgenic mice to investigate whether CRP has a prothrombotic effect. They demonstrated that, at 4 weeks after transluminal wire injury, 75% of the femoral arteries was occluded in CRP transgenic mice, compared with 17% in wild-type mice.[37] However, these results can only be extrapolated to the human situation with care, since CRP levels in these mice (18 ± 6 mg/L at baseline to 56 ± 5 mg/L after surgery) were higher than in humans.

CRP has been found to be deposited in the infarcted area together with complement.[40] Griselli *et al*.[41] have demonstrated in an animal model that human CRP and complement activation are major mediators of ischemic myocardial injury. In rats injected with CRP infarct size was increased by 40% as compared to mice not injected with CRP. So, even though

CRP does not appear to be involved in atherosclerosis directly, it may well have other effects that may adversely influence the outcome of a cardiovascular event.

Recently, also protective functions for CRP in atherosclerosis have been reported in *in vitro* studies. Upon incubation with CRP, endothelial cells from human coronary artery or human saphenous vein show increased expression of complement inhibitory factors.[42] However, again the issue of possible artefacts due to contamination of the CRP preparation used is raised since van den Berg and Taylor demonstrated that removal of azide from the CRP preparations used inhibited the CRP-induced decay-accelerating factor (DAF) expression that was reported by Li *et al*.[43] CRP was also reported to protect cells against assembly of the terminal complement complex [44,45], and native CRP (but not monomeric CRP) was shown to inhibit platelet activation.[46,47] However, in the present study we could not demonstrate a protective effect of CRP.

In conclusion, the present study does not support a direct role of CRP in atherosclerosis development in E3L transgenic mice. No differences in lesion area or lesion severity could be observed between mice with and mice without CRP in this study. We conclude that moderately increased levels of plasma CRP do not affect the development of atherosclerosis in hypercholesterolemic E3L/CRP transgenic mice.

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