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Animal Models

No effect of C-reactive protein on early atherosclerosis in LDLR$^{-/-}$ / human C-reactive protein transgenic mice

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Summary
The association between increased concentrations of C-reactive protein (CRP) and future cardiovascular events is well established. However, it is currently unclear whether this clinical observation represents an epiphenomenon or whether the pentraxin may actively promote the development of atherosclerosis. Experimental studies with knockout mice with a defect in apolipoprotein E (ApoE$^{-/-}$) have been used to investigate the role of CRP in atherogenesis, but the results obtained have been contradictory so far. Since knockout mice with a defect in low density lipoprotein receptor (LDLR$^{-/-}$) may represent a better model of atherogenesis compared to ApoE$^{-/-}$ animals, we undertook experiments to investigate the atherogenic potential of CRP using LDLR$^{-/-}$ knockout mice. We crossbred CRP transgenic animals expressing the human CRP pentraxin (huCRP) to LDLR$^{-/-}$ mice, fed the resulting double mutants a pro-atherogenic Western type diet (WTD) for four, eight or 12 weeks, respectively, and quantitated atherosclerotic lesion development. Significant differences of lesion size or lesion composition could not be detected between the huCRP-positive LDLR$^{-/-}$ mice and the huCRP–negative LDLR$^{-/-}$ controls corroborating the contention that CRP does not play a pathogenetic role in early murine atherogenesis.

Keywords
LDLR mice, CRP, atherosclerosis

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Introduction

The association between increased concentrations of C-reactive protein (CRP) and future cardiovascular events has been established (1). However, it is unclear whether this clinical observation represents an epiphenomenon or whether the pentraxin may actively promote the development of atherosclerosis (2, 3).

Experimental studies with genetically modified mice lend themselves to the investigation of the role of CRP in atherogenesis, but the results obtained so far have been contradictory. The first publication indicated a pro-atherogenic role of CRP in a double-mutant mouse model, combining a transgene encoding for the human CRP (huCRP) and the ApoE$^{-/-}$ knockout defect (4). In contrast to this study, three other research groups failed to detect an influence of CRP on murine atherosclerosis (5–8). In two of these studies, the discrepancy of results as compared to the study by Paul et al. (4) might have been attributable to differences of the experimental models. Thus, Trion et al. (5) introduced the huCRP transgene to the Apoe*3-Leiden mouse model of atherosclerosis, and Reifenberg et al. (6) have backcrossed a rabbit CRP (rbCRP) transgene to the ApoE knockout strain. However, the studies by Pepys et al. (7, 8) had used the identical murine huCRP ApoE model as Paul et al.

Since knockout mice with a defect in low density lipoprotein receptor (LDLR$^{-/-}$) may represent a better model of atherogenesis compared to ApoE $^{-/-}$ animals, experiments were undertaken in the current study employing this mouse strain.

We crossbred huCRP transgenics (9) to LDLR$^{-/-}$ mice, fed the resulting double mutant animals a pro-atherogenic Western type diet (WTD) for four, eight or 12 weeks, respectively, and quantitated atherosclerotic lesion development. Significant differences of lesion size or lesion composition were not detected between

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the huCRP-positive LDLR$^{-/-}$ mice and the huCRP-negative LDLR$^{-/-}$ controls corroborating the contention that CRP does not play a pathogenetic role in early murine atherogenesis.

Methods

Mouse strains

The huCRP transgenic strain (9) was generously provided by Johan Björkegren (Karolinska Institutet, Stockholm) and LDLR$^{-/-}$ knockout strain (official designation: B6.129S7-Ldlr^{tm1Her}/J, Stock No 2207) was purchased from The Jackson Laboratory (TJL Bar Harbor, Maine, USA). Both strains had been backcrossed to the C57BL/6 strain for more than 10 generations. The huCRP LDLR$^{-/-}$ double mutant mice were generated by outcrossing the huCRP transgene to the LDLR$^{-/-}$ strain and by backcrossing huCRP-positive hybrids to the LDLR$^{-/-}$ parental strain. The LDLR knockout and wild type alleles could be discriminated by PCR as recommended by TJL. The huCRP transgene was amplified by PCR using primer combination 5' CCA TGG AGA AGC TGT TGT G 3' and 5' CAA ATG TGT ACT GGA GCT AC 3'. Mice were housed in the Central Laboratory Animal Facility of the University of Mainz, Germany, under specified pathogen free conditions. Animals were housed in accordance with standard animal care requirements and maintained on a 12 / 12 hour light-dark cycle. Water and food were given *ad libitum*.

Induction of atherosclerosis

Male huCRP LDLR$^{-/-}$ mice as well as LDLR$^{-/-}$ controls were placed on a Western-type diet (WTD, Ssniff Spezialdiäten GmbH, Soest, Germany) at an age of eight weeks. The WTD diet contained 21% (wt/wt) fat and 0.15% (wt/wt) cholesterol. The pro-atherogenic WTD diet was administered for a time span of four weeks (12 huCRP LDLR$^{-/-}$ mice and 13 LDLR$^{-/-}$ controls), eight weeks (12 huCRP LDLR $^{-/-}$ mice and 13 LDLR$^{-/-}$ controls) and 12 weeks (8 huCRP LDLR$^{-/-}$ mice and 13 LDLR$^{-/-}$ controls), respectively.

Lipoprotein analysis of murine sera

Murine sera were diluted 1:3 before quantitative cholesterol and triglyceride analyses. Quantitative cholesterol determinations were conducted using a colorimetric assay (CHOD-PAP, Roche® Diagnostics, Mannheim, Germany). Triglycerides were determined by quantifying free glycerine originating from hydrolytic cleavage (GPO-PAP, Roche Diagnostics).

huCRP assay

Human CRP was measured by an automated particle-enhanced turbidimetric immunoassay (Cobas®) (10, 11), with a lower limit of detection of 0.2 µg/ml, run on the Roche/Hitachi 917 analyzer.

Tissue preparation and quantitation of atherosclerotic lesions

At the end on the WTD, the mice were sacrificed by exposure to carbon dioxide. Peritoneal cavities were opened and the cadavers fixed in 4 % PBS-buffered formaldehyde. Hearts were resected and sequentially cut into a total of 60 (5 µm thick) sections around the aortic sinus as described (12). Out of 60 sections, every fifth slide was stained with trichrome and computer-assisted (Image Pro Discovery, Media Cybernetics, Silver Spring, MD) measurement of plaque size was performed as described previously (12). The rest of the sections were used for immunohistochemistry. The rest of the aorta was resected *en bloc* down to the iliac bifurcation and carefully cleaned of perivascular adipose tissue under a dissection microscope (Leica MZ6, Leica, Bensheim, Germany), opened longitudinally and stained with freshly prepared Sudan IV as described previously (13). Sudan stained atherosclerotic lesions *en face* were then quantified using Photoshop-based image analysis (Version 8.0.1, Adobe Systems Inc., San Jose, CA) as described (13).

Immunohistochemical and histochemical analyses

Immunostaining of murine tissues with the murine MAbs was performed using the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingham, CA). Serial 5 µm-thick sections of the paraffin-embedded aortic sinus were deparaffinized in xylene. All slides were treated with 3 % H_2O_2 to block endogenous peroxidase activity. Slides were incubated consecutively with 5% normal serum to block non-specific binding, primary antibody for 1 hour, biotin-conjugated secondary anti-mouse or anti-rabbit antibody for 30 minutes and avidin-biotin-peroxidase reagent for 45 minutes at room temperature. The reaction products were identified by immersing the slides in diaminobenzidine tetrachloride to give a brown reaction product. The slides were then counterstained with hematoxylin and mounted. Negative controls included replacement of the primary antibody by irrelevant isotype-matched antibodies or non-immune serum. The following antibodies were used: rat IgG2b anti-mouse F4/80 antigen (monocytes, macrophages, 1:20, Acris Antibodies, Hiddenhausen, Germany), murine anti-smooth muscle a-actin (1A4, 1:100, Sigma, St Louis, MO) and goat IgG anti-mouse complement component C3d antibody (1:200, R&D Systems). Collagen

Table 1: Human CRP transgenesis does not affect serum lipids and lipoproteins. (Values are presented as mean ± standard deviation.)

WTD	Body weight, g		Serum cholesterol, mg/dL		Serum triglycerides, mg/dL	
	huCRP LDLR-/-	LDLR-/-	huCRP LDLR-/-	LDLR-/-	huCRP LDLR-/-	LDLR-/-
4 weeks	31.0 ± 3.9	30.8 ± 3.6	1668 ± 390	1748 ± 391	741 ± 222	839 ± 170
8 weeks	37.1 ± 3.3	37.5 ± 3.6	2244 ± 390	2409 ± 540	970 ± 260	916 ± 284
12 weeks	36.5* ± 3.4	40.9* ± 2.9	2527 ± 374	2555 ± 299	1283 ± 296	1424 ± 250

* $p < 0.05$ (T-test).

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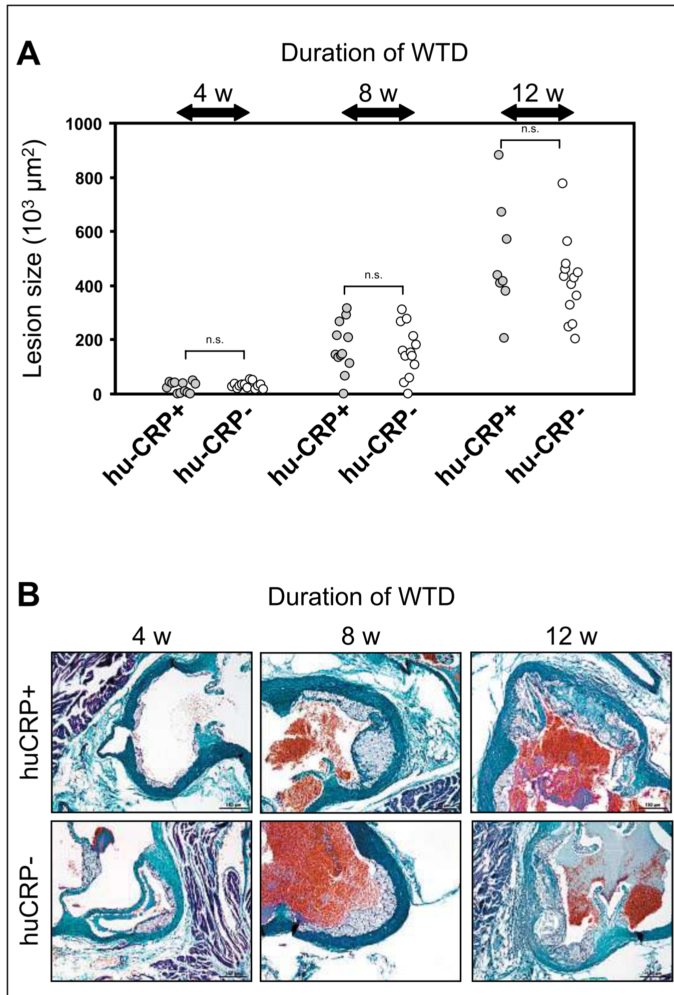

Figure 1: Atherosclerotic lesion development. A) After four weeks (4 w), eight weeks (8 w) and 12 weeks (12 w) on the WTD, respectively, hearts of huCRP LDLR$^{-/-}$ animals (huCRP+, grey circles) and LDLR$^{-/-}$ mice (huCRP-, white circles) were resected, cross-sections of the aortic sinus area were stained with trichrome, and atherosclerotic lesion area were quantified for each animal. Note, that no statistically significant differences of lesion area could be detected between huCRP LDLR$^{-/-}$ and LDLR$^{-/-}$ mice (non-parametric Mann-Whitney *U* test, n.s. = not significant). B) Representative specimens of the aortic sinus area of huCRP LDLR$^{-/-}$ (huCRP+, upper panels) and LDLR$^{-/-}$ (huCRP-, lower panels) mice. Note, that no qualitative differences of gross plaque consistency could be noted between the huCRP-positive and huCRP-negative mice.

content was analysed by picrosirius red and polarized light microscopic imaging. Percent-positive area for immunohistochemical or picrosirius red staining was quantified by Photoshop-based image analysis as described (14,15). Briefly, pixels with similar chromogen characteristics were selected with the "magic wand" tool and the "select similar" command, and the ratio of the positively stained area to the total lesion area studied was calculated with the "histogram" command in Photoshop. All quantitative morphometric and immunohistochemical data were collected independently by two experienced operators blinded to the mice genotypes.

Statistical analyses

Data were analysed with SPSS 14.0 for Windows. Since some of the outcome parameters determined in this study did not follow a normal distribution as judged by Shapiro-Wilk tests, some statistical analyses were performed with the non-parametric Mann-Whitney *U* tests. Serum concentrations of huCRP and lipid contents are presented as mean (± standard deviation). Staining intensities of macrophages, smooth muscle cells, collagen, and C3d are presented as median and interquartile range. Differences between huCRP-positive and huCRP-negative mice were considered as significant when the p-value fell below a limit of 0.05.

Results

Efficient expression of the huCRP transgene

Serum huCRP concentrations were determined at the end of the experiment after administration of WTD for four, eight or 12 weeks. No huCRP could be detected in the sera of the non-transgenic LDLR$^{-/-}$ control mice. In contrast, efficient huCRP serum expression was observed in the transgenic huCRP LDLR$^{-/-}$ mutants. The mean (± standard deviation) huCRP serum concentrations as determined after four, eight and 12 weeks on the WTD were 12.8 µg / ml (± 4.8 µg/ml), 10.6 µg/ml (± 4.0 µg/ml) and 7.4 µg/mL (± 4.4 µg/ml), respectively. These values were in accord with the huCRP levels previously observed in apolipoprotein E-deficient or apolipoprotein E*3-Leiden mice (5, 7).

Human CRP transgene expression does not affect serum lipids and lipoproteins

As depicted in Table 1, the mean body weights of huCRP-negative and huCRP-positive LDLR$^{-/-}$ mice did not differ significantly in the two experimental groups fed WTD for 4 and 8 weeks. For unknown reasons, LDLR$^{-/-}$ mice sacrificed after 12 weeks on the WTD showed a statistically significant difference of body weights in dependence of huCRP transgenesis ($p < 0.05$; Table 1). As expected, WTD had the potential to significantly increase serum total cholesterol and triglyceride levels in all LDLR$^{-/-}$ mice (Table 1) as compared to LDLR$^{-/-}$ mice on normal chow (16). However, no significant influences of transgenic huCRP expression on serum triglyceride and cholesterol concentrations were noted in any of the three experimental groups (Table 1).

Atherosclerotic lesion development

The mice of the three separate cohorts were sacrificed after four, eight, and 12 weeks on the WTD, respectively. Only minimal lesions could be observed at any time point in the rest of the aorta from the aortic sinus area down to the iliac bifurcation after Sudan IV staining. However, substantial lesions developed in all animals in the aortic sinus area and increased in size with time, but there was no quantitative difference between the groups with and without transgenic human CRP at any time point investigated (Fig 1A). Representative photomicrographs of atherosclerotic lesions observed are shown in Figure 1B.

Phenotypic analysis of atherosclerotic lesions

Because the lesion areas of huCRP-positive and huCRP-negative LDLR$^{-/-}$ mice did not significantly differ, we then ana-

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lyzed whether transgenic huCRP expression may eventually be associated with alterations of lesion composition. We therefore quantitated macrophages, smooth muscle cells (SMCs), and collagen content in the lesions of all huCRP-positive LDLR$^{-/-}$ animals and huCRP-negative controls, fed with WTD for 8 or 12 months. In animals of both groups, about 50 – 70 % of the lesion area consisted of macrophages (Figs. 2 and 3A) and the rest of the atherosclerotic area was comprised of SMCs (Figs. 2 and 3B), and collagen (Figs. 2 and 3C). Importantly, upon statistical analyses of the immunohistochemical data, no significant differences could be observed between the huCRP-positive LDLR$^{-/-}$ mice and LDLR$^{-/-}$ controls with regard to all three parameters investigated (Fig. 3).

Complement activation in atherosclerotic lesions of LDLR$^{-/-}$ mice

C3d reactivity represents a reliable marker of complement activation in tissues (17, 18), and extensive deposits colocalize with insudated lipoproteins in human atherosclerotic lesions. Only small patches of C3d deposits representing less than 8% of the total plaque areas were observed in lesions of all LDLR $^{-/-}$ mice irrespective of the huCRP genotype (Fig. 4, left). No significant differences could be observed between the huCRP-positive and huCRP-negative animals (Fig. 4, right), indicating that transgenic huCRP expression did not augment complement activation in atherosclerotic lesions of LDLR $^{-/-}$ mice.

Discussion

The question regarding the role of CRP in human atherogenesis is potentially clinically relevant. If a role as a proatherogenic factor were documented, therapeutical approaches aimed to inhibit CRP effects in patients with atherosclerosis would obviously be of interest.

Knockout mice with a defect in apolipoprotein E (ApoE$^{-/-}$) or low density lipoprotein receptor (LDLR$^{-/-}$) develop atherosclerotic lesions and are currently finding wide-spread use as models for investigating pathomechanisms underlying atherosclerosis (16, 19, 20). The LDLR$^{-/-}$ model may be superior for a number of reasons. First, atherosclerotic lesions do not develop spontaneously as in ApoE$^{-/-}$ animals, but are inducible under WTD. Second, the serum lipoprotein pattern of LDLR$^{-/-}$ animals is characterized by high-level LDL rather than chylomicrons and VLDL (as in ApoE$^{-/-}$ mice) (16, 19, 20), and thus more closely mimicks the situation in humans.

Somewhat unfortunately, murine CRP does not represent an acute phase protein but is constitutively expressed in the animals at baseline levels (21). Experimental approaches to investigate the role of CRP in mouse models therefore necessitate the introduction of transgenes overexpressing human (9) or rabbit (22) CRP to murine strains that have been rendered prone to atherosclerosis. While such "chimeric" double-mutant models harbor intrinsic problems (23), their use in atherosclerosis research appears to be warranted given the importance of the underlying medical question and the current lack of alternative animal models.

In the present study, the huCRP transgene was introduced to the LDLR knockout strain. The plasma CRP level in the male mice was around 10 µg/ml, much lower than the extraordinary

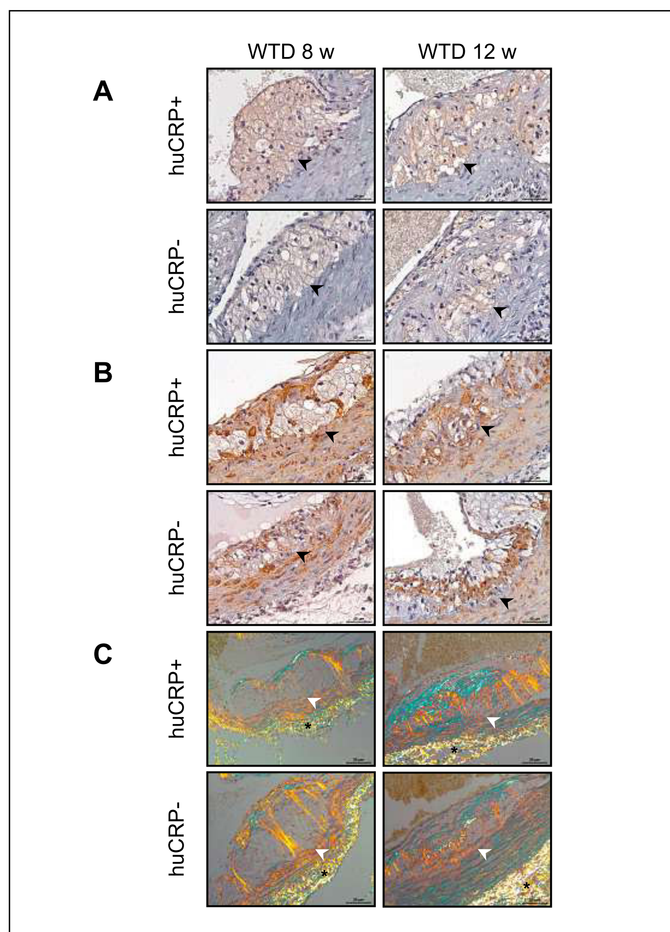

Figure 2: Phenotypic analysis of atherosclerotic lesions. Representative photographs of atherosclerotic lesions of huCRP LDLR$^{-/-}$ animals (huCRP+) and LDLR$^{-/-}$ controls (huCRP-) after eight weeks (8 w, left panels) and 12 weeks (12 w, right panels) on the WTD. Atherosclerotic lesions of the aortic sinus area were stained with rat anti-mouse F4/80 antigen (A) for quantification of macrophages, mouse anti-smooth muscle **a-actin** (B) for quantification of SMCs, and picrosirius red with subsequent polarization (C) for quantification of collagen. Percent-positive area for macrophages (brown colour), SMCs (brown colour) and collagen (yellow, green, orange and red polarized colour) was quantified by Photoshop-based image analysis (see also Fig. 3). The vessel lumen is to the upper left-hand corner. The demarcation beween intima and media is indicated by arrowheads. In C, note that the adventitial tissue (asterisks) also polarizes after picrosirius red staining (internal positive control).

high CRP levels in the mice studied by Paul et al. (4) and more similar to the plasma CRP values associated with increased cardiovascular risk in humans (1). The results concerning atherosclerosis development in the double mutants were clear: we were unable to detect any significant differences between the huCRP-positive LDLR$^{-/-}$ mice and the huCRP-negative LDLR$^{-/-}$ controls, neither with regard to quantity nor quality (macrophages and SMCs, collagen content) of developing murine lesions.

Formation of both C3d and C5b-9 neoantigens constitute unequivocal evidence for complement activation (24) and therefore positive stainings for C3d constitute evidence for complement activation in atherosclerotic lesions. Specific immunohisto-

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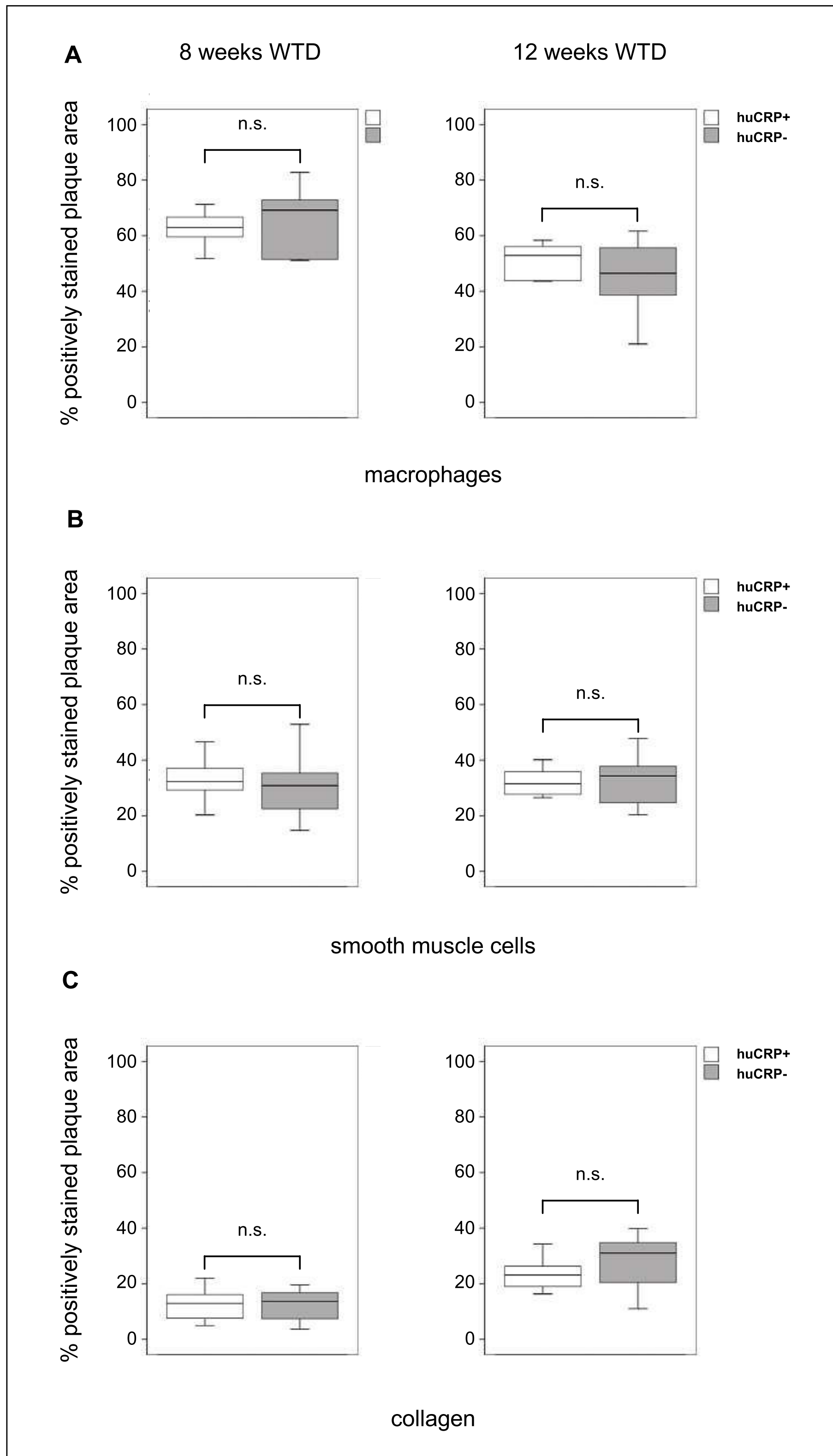

Figure 3: Phenotypic analysis of atherosclerotic lesions. Quantitative analysis of macrophage (A), smooth muscle cells (SMCs, B), and collagen (C) specific staining of atherosclerotic lesions of huCRP LDLR$^{-/-}$ animals and LDLR $^{-/-}$ control mice after eight weeks (8 w, left panels) and 12 weeks (12 w, right panels) on the WTD by Photoshop-based image analysis (see also Fig. 2). Data are presented as boxplots with median, interquartile range, minimum and maximum. Note, that no significant (n.s. = not significant) influences of huCRP transgenesis on staining reactions could be detected (non-parametric Mann-Whitney *U* test).

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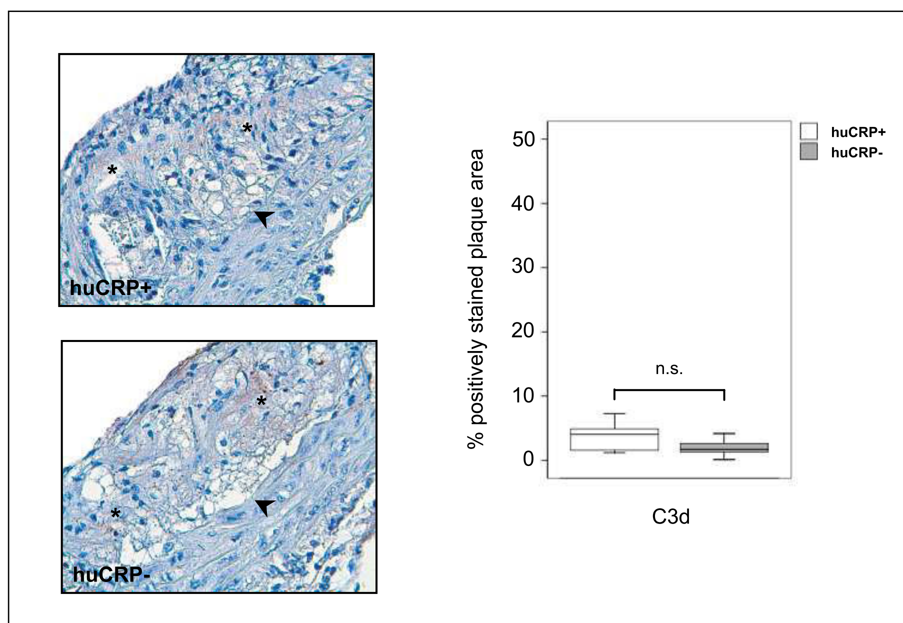
chemical reactivity for mouse C3d was found in all of the atherosclerotic lesions, but the areas stained were rather small and constituted only 4% – 8% of total lesion areas. Again, no significant differences were observed between CRP$^+$ and CRP$^-$ animals. These observations conform to the previous findings that LDLR deficient mice have a normal complement activity (25,26) and that neither free human CRP nor human CRP complexed to modified LDL does activate mouse complement (6).

In conclusion, this study corroborates and extends the results of previous work in ApoE-deficient mice and negates a pro-athe-

Figure 4: Complement activation in atherosclerotic lesions. After 12 weeks on the WTD, atherosclerotic lesions of the aortic sinus area of huCRP LDLR$^{-/-}$ mice (huCRP+) and LDLR$^{-/-}$ controls (huCRP-) were stained with a goat anti-mouse complement component C3d-specific antibody. The panels on the left show respresentative staining reactions (brown color deposits, asterisks). The lumen is to the upper left-hand corner. The demarcation beween intima and media is indicated by arrowheads. The diagram on the right shows the percentage of positively stained area after quantification by Photoshop-based image analysis. Data are presented as boxplots with median, interquartile range, minimum and maximum (non-parametric Mann-Whitney *U* tests, n.s. = not significant).



rogenic role of CRP on murine atherosclerosis. After completion of this work, a report appeared in which CRP slightly suppressed late lesion development (27). The two studies stand in perfect accord with each other with regard to the total lack of CRP on early atherogenesis in LDLR$^{-/-}$ animals.

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