Elevated Serum C-Reactive Protein Levels and Early Arterial Changes in Healthy Children

Mikko J. Järvisalo, Aimo Harmoinen, Maarit Hakanen, Ulla Paakkunainen, Jorma Viikari, Jaakko Hartiala, Terho Lehtimäki, Olli Simell, Olli T. Raitakari

Objective—Elevated serum concentration of C-reactive protein (CRP) predicts cardiovascular events in adults. Because atherosclerosis begins in childhood, we undertook a study to determine whether changes in brachial artery endothelial function and the thickness of the carotid intima-media complex, 2 markers of early atherosclerosis, are related to CRP levels in healthy children.

Methods and Results—Brachial artery flow-mediated dilatation (FMD) and carotid artery intima-media thickness (IMT) were measured with ultrasound in 79 children (aged 10.5±1.1 years). Compared with the children with CRP levels under the detection limit (<0.1 mg/L, n=40, group 1), the children with higher CRP (0.1 mg/L≤CRP=0.7 mg/L, n=20, group 2; CRP >0.7 mg/L, n=19, group 3) had lower FMD (9.0±4.4% versus 7.8±3.3% versus 6.5±2.6%, respectively; P=0.015 for trend) and greater carotid IMT (0.45±0.03 versus 0.46±0.04 versus 0.49±0.06 mm, respectively, P=0.002 for trend). CRP level remained a statistically significant independent predictor for brachial FMD and carotid IMT in multivariate analyses.

Conclusions—These data suggest that CRP affects the arteries of healthy children by disturbing endothelial function and promoting intima-media thickening. The findings support the hypothesis that CRP plays a role in the pathogenesis of early atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:●●●●●●.)

Key Words: atherosclerosis ● endothelial function ● inflammation ● ultrasound

High C-reactive protein (CRP) levels predict coronary events in patients with cardiovascular disease (CVD) as well as in apparently healthy subjects. In adults with CVD or its risk factors, elevated CRP levels are associated with endothelial vasodilator dysfunction and the development of carotid atherosclerosis. Experimental studies have shown that CRP can be found in arterial walls affected by atherosclerosis but not in healthy vessel walls. In a concentration-dependent manner, CRP directly increases endothelial production and the expression of monocyte chemoattractant protein-1 and adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1). CRP also influences monocyte chemotaxis during atherogenesis, and the deposition of CRP precedes and mediates the appearance of monocytes in early atherosclerotic lesions. Recently, it has been shown that CRP mediates the uptake of native LDL into macrophages, which is a novel mechanism for foam cell formation without biochemical modification of LDL.

Thus, together, these data suggest that CRP may have a direct proatherogenic role by disturbing endothelial function and promoting the formation of early atherosclerotic lesions. Because atherosclerosis begins in childhood, we undertook a study to assess whether changes in brachial artery reactivity and the thickness of the carotid intima-media complex (intima-media thickness [IMT]), 2 markers of early atherosclerosis, are related to serum CRP levels in healthy children.

The study of arterial changes in children can provide unique data on early atherosclerosis and its determinants that are not obscured by other chronic diseases or lifestyle habits.

Methods

Subjects

We studied 79 children; all were healthy nonsmokers without any regular medication and with no family history of premature vascular disease. None of the children had had symptoms of infection during the 2 weeks before the study. Subjects were children of the hospital staff members (n=36) and children participating in a risk factor study (n=44). Children’s parents were asked about their smoking (smoking/nonsmoking) on a questionnaire. The study protocol was approved by the local Ethics Committee. Legal guardians gave written informed consent.

Ultrasound Studies

All studies were performed in the morning to fasting subjects with the use of a Sequoia 512 mainframe (Acuson) and 13.0-MHz linear array transducer. All ultrasound scans were performed by an expe-
rienced vascular operator who was unaware of the children’s clinical details. The studies were performed in the morning between 7:30 and 9:30 AM, after the children had fasted overnight. Blood pressure was measured from the brachial artery of the nondominant arm 3 times during the ultrasound study with the use of a standard sphygmomanometer, and the mean of these 3 measurements was used in the analyses. The brachial artery studies and carotid artery studies were performed by using standard techniques for the study of children.\(^\text{15,16}\)

**Brachial Artery Physiology**

Brachial artery diameter was measured from B-mode ultrasound images as described earlier.\(^\text{14}\) In all studies, scans were obtained at rest and during reactive hyperemia. The subjects lay quietly for 10 minutes before the first scan. The brachial artery was scanned in a longitudinal section 5 to 15 cm above the elbow. Depth and gain settings were set to optimize images of the lumen–arterial wall interface, and the operating parameters were not changed during the study. When a satisfactory transducer position was found, the position was marked on it and the arm remained in the same position throughout the study. All ultrasound scans were recorded on super-VHS tapes for offline analysis. A resting scan was performed, and arterial flow velocity was measured by use of a Doppler signal. Increased flow was then induced by inflation of an adult-sized (12×44.5-cm) pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 250 mm Hg for 4.5 minutes, followed by release. Subsequent scans were taken continuously between 40 and 180 seconds after cuff deflation. We also included a repeated flow velocity recording for the first 15 seconds after the cuff was released.

Vessel diameter was measured by an experienced reader blinded to the study subjects’ laboratory data. The arterial diameter was measured at a fixed distance from an anatomic marker (eg, a fascial plane) with the use of ultrasonic calipers. Measurements were taken from the anterior to the posterior M line\(^\text{17}\) at end diastole, incident with the R wave on a continuously recorded ECG every 10 seconds between 40 and 120 seconds after cuff deflation and every 15 seconds from 120 to 180 seconds after occlusion (including a total of 13 measurements), to ensure the detection of the peak flow-mediated dilatation (FMD) response. The first scan after hyperemia was taken at 40 seconds because it was the earliest time point practicable, inasmuch as flow velocity was recorded for the first 15 seconds after the cuff release. The maximal proportional dilatation from baseline (FMD, as a percentage) and the total dilatation response, defined as the area under the FMD-versus-time curve during the 40- to 180-second period after hyperemia (area under the curve [AUC], as percentage×seconds), were assessed. Mullen et al\(^\text{18}\) have recently shown that when arterial occlusion times that are not >5 minutes are used, the resulting peak FMD response is mediated by the NO pathway.\(^\text{19}\)

Nitrate-mediated endothelium-independent dilatation capacity was tested 15 to 30 minutes after the FMD test by administering 4 consecutive sublingual 50-μg doses of glyceryl trinitrate 5 minutes apart (cumulative dose 200 μg). The maximum diameter 5 minutes after maximum cumulative nitrate administration was used to calculate the proportional increase in diameter from the baseline value (NMD, as a percentage). In our laboratory, the interobserver variation (coefficient of variation [CV]) of FMD measurements (of the same image data) was 8.6%,\(^\text{19}\) and the between-visit CV (12 subjects studied twice 2 hours between studies) in FMD measurements was 9.0%. A similar methodology has been also used by other groups to study arterial endothelial function in children.\(^\text{15}\)

**Carotid Artery Studies**

Carotid artery IMT measurements were performed with a standardized protocol for the right and left carotid arteries with the use of images of the far wall of the distal common carotid arteries, in a manner similar to that previously described in detail.\(^\text{20,21}\) Briefly, the proximal part of the carotid bulb was identified, and the segment of the common carotid artery 1 to 2 cm proximal to the bulb was scanned. The image was focused on the posterior (far) wall, and the zoom function was used to magnify the arterial far wall. Several images of the common carotid artery segment from 10 to 20 mm proximal to the carotid bulb (a far wall segment of 10 mm in width) were acquired. Two angles were used in each case: anterior oblique and lateral. We have previously shown that the use of these 2 interrogation angles yields results very similar to those found with the use of 15 different interrogation angles covering an ∼120° segment of the carotid wall.\(^\text{21}\) All scans were digitally stored on the ultrasound system internal hard disk for subsequent offline analysis.

Two end-diastolic frames were selected and analyzed for mean IMT, and maximum IMT and the average readings from these 2 frames were calculated, for both right and left carotid arteries. The images were analyzed by 2 independent readers who were blinded to the subject’s clinical details, and the average values were used in the analysis. Maximal IMT and mean IMT values were used in the analysis. The interobserver CV of IMT measurements (of the same image data) was 3.0%, and the between-visit CV (21 subjects studied twice 1 week between studies) of IMT measurement was 3.9%.

**Serum Lipoproteins and Glycosylated Hemoglobin**

Venous blood samples were taken in the morning, after a 10- to 12-hour fast. Serum total cholesterol, HDL cholesterol, and triglyceride concentrations were measured by standard enzymatic methods with the use of reagents from Boehringer-Mannheim GmbH and a fully automated analyzer (Hitachi 704, Hitachi Ltd). LDL cholesterol concentration was calculated by using the Friedewald equation.\(^\text{22}\) Glycosylated hemoglobin was measured with high-performance liquid chromatography (Variant Analyzer, Bio-Rad).

**CRP and ICAM-1 Determinations**

The fasting plasma CRP concentrations were analyzed by a particle-enhanced immunoturbidimetric method with the use of a Cobas Integra 700 automatic analyzer (Hoffmann-La Roche Ltd) and reagents (COBAS Integra C-Reactive Protein [Latex]).\(^\text{23}\) The sensitivity is determined by the smallest analyte concentration that can be reproducibly distinguished from a zero sample. The lower detection limit reported for the assay was <0.1 mg/L. The intra-assay CV was 1.8%, and the interassay CV was 2.9%.

The concentration of soluble ICAM-1 was determined by the sandwich enzyme-immunoassay technique according to the manufacturer’s instructions (BBE 1B, R&D System). The microtiter plates were precoated by 100 μL of appropriate diluted monoclonal anti-adhesion molecule IgG antibody. Thereafter, 100 μL of secondary anti-adhesion molecule IgG-horseradish peroxidase–conjugated antibody, standards, controls, and serum (diluted 1:20) were added in duplicate wells and incubated at room temperature for 1.5 hours. After 6 washes, 100 μL of tetramethylbenzidine substrate was added to each well, and the plate was incubated at room temperature for 20 minutes. The reaction was stopped with acid solution, and the optical density of each well was measured at 450 nm within 30 minutes. As a correction wavelength, 620 nm was used. The signals obtained from standards of known concentration were used for the development of a standard curve. The concentrations in samples were calculated by using a 4-parameter logistic curve fit.

**Statistical Methods**

Results are expressed as mean±SD. Because CRP values have skewed distribution, children were divided into predetermined groups according to CRP percentiles. Group 1 included children with CRP values less than the detection limit, 0.1 mg/L (less than the median value). The other half of the children were divided into groups 2 and 3; the 75th percentile was used as cut-point value (group 2 [n=20], 0.1 mg/L<CRP≤0.7 mg/L; group 3 [n=19], CRP >0.7 mg/L). The comparisons of 3 groups were performed by ANOVA. Continuous CRP values were also used in the analysis after a square-root transformation to account for the skewed distribution. Univariate and multivariate regression analyses were used to study the relationships of arterial changes with CRP. Repeated-measures ANOVA was used to test whether the magnitude of FMD responses measured between 40 to 180 seconds after hyperemia
differed between the study groups. All statistical analyses were performed by using the Statistical Analysis System. 

Results

The characteristics of children are shown in Table 1. The CRP levels ranged from 0 to 8.6 mg/L (mean 0.7 mg/L). None of the children had serum CRP levels $>10$ mg/L, indicative of acute infection. Children with higher CRP levels had lower peak FMD and AUC, higher IMT, body mass index (BMI), and serum ICAM-1, but similar NMD, blood pressure, serum lipids, and glycosylated hemoglobin (Table 1). There were no differences in parental smoking rates (data not shown).

Brachial artery peak FMD and AUC were inversely associated, and common carotid artery IMT was directly associated with the CRP group in the univariate regression analyses (Table 2). These relationships also remained significant after adjustment for BMI or ICAM-1 in regression models (Table 2) and were essentially similar if the continuous square-root-transformed serum CRP values were used in the models instead of the CRP group variable. When ICAM-1 and BMI were both forced into the multivariate model, the associations between CRP group and arterial indices remained significant, except for mean carotid IMT ($P=0.097$). ICAM-1 also tended to be correlated with mean carotid IMT ($r=0.22$, $P=0.05$). The Figure shows that the temporal development of FMD responses measured between 40 and 180 seconds after the cuff release followed a similar pattern over time in the CRP groups, but the magnitude of the response was significantly blunted in groups with higher CRP levels.

Discussion

The present study, conducted in a group of healthy children, showed that elevated serum CRP levels are significantly associated with decreased endothelial vasodilatory function and increased carotid artery IMT. Previous studies in children have shown that diabetes and hypercholesterolemia are associated with brachial artery endothelial dysfunction and increased carotid artery IMT. Therefore, these noninvasive functional and structural arterial measurements may be used as indices of the atherosclerotic vascular process in the study of subclinical atherosclerosis in vivo in children.

### Table 1. Characteristics of the Study Groups

<table>
<thead>
<tr>
<th>CRP</th>
<th>ANOVA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects (boys)</td>
<td>40 (22)</td>
<td>20 (7)</td>
</tr>
<tr>
<td>Age, y</td>
<td>10.5±0.9</td>
<td>10.4±0.9</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>16.8±2.3</td>
<td>17.9±1.9</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>108±9</td>
<td>110±8</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>62±5</td>
<td>63±6</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.3±0.8</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.54±0.33</td>
<td>1.42±0.27</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.69±0.05</td>
<td>0.69±0.26</td>
</tr>
<tr>
<td>Glycosylated hemoglobin, %</td>
<td>5.3±0.3</td>
<td>5.4±0.3</td>
</tr>
<tr>
<td>ICAM-1, ng/mL</td>
<td>238±54</td>
<td>272±72</td>
</tr>
<tr>
<td>Brachial baseline diameter, mm</td>
<td>3.0±0.3</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>Peak FMD, %</td>
<td>9.1±4.4</td>
<td>7.8±3.3</td>
</tr>
<tr>
<td>AUC, %×s</td>
<td>713±475</td>
<td>616±365</td>
</tr>
<tr>
<td>NMD, %</td>
<td>12.6±4.3</td>
<td>12.6±4.5</td>
</tr>
<tr>
<td>Mean IMT, mm</td>
<td>0.41±0.03</td>
<td>0.42±0.04</td>
</tr>
<tr>
<td>Maximum IMT, mm</td>
<td>0.45±0.03</td>
<td>0.46±0.04</td>
</tr>
</tbody>
</table>

### Table 2. The Relationship Between the CRP Groups and Ultrasound Measures of Arterial Function and Structure

<table>
<thead>
<tr>
<th>CRP, mg/L (Adjusted Means)*</th>
<th>&lt;0.1</th>
<th>≥0.1 to ≤0.7</th>
<th>&gt;0.7</th>
<th>P</th>
<th>$P_{1}$</th>
<th>$P_{2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak FMD, %</td>
<td>9.0</td>
<td>7.9</td>
<td>6.8</td>
<td>0.015</td>
<td>0.048</td>
<td>0.043</td>
</tr>
<tr>
<td>AUC, %×s</td>
<td>713</td>
<td>619</td>
<td>444</td>
<td>0.014</td>
<td>0.028</td>
<td>0.037</td>
</tr>
<tr>
<td>NMD, %</td>
<td>12.2</td>
<td>12.5</td>
<td>11.6</td>
<td>0.17</td>
<td>0.988</td>
<td>0.097</td>
</tr>
<tr>
<td>Mean IMT, mm</td>
<td>0.41</td>
<td>0.41</td>
<td>0.44</td>
<td>0.006</td>
<td>0.024</td>
<td>0.051</td>
</tr>
<tr>
<td>Maximum IMT, mm</td>
<td>0.46</td>
<td>0.46</td>
<td>0.49</td>
<td>0.002</td>
<td>0.014</td>
<td>0.016</td>
</tr>
</tbody>
</table>

*Means adjusted for BMI and ICAM-1.
†Multivariate linear regression adjusted for BMI.
‡Multivariate linear regression adjusted for ICAM-1.

The results remained essentially similar when CRP was included in the analyses as a continuous square-root transformed variable.
The relationship between CRP level and endothelial function has not been previously studied in children. The available studies in adults have shown an association between elevated CRP concentration and endothelial dysfunction in patients with coronary artery disease and in subjects with type 2 diabetes. Importantly, these studies have also demonstrated that lowering of CRP levels either spontaneously or by statin therapy leads to improvement in endothelial function, thus suggesting a causal relationship. In the present study, CRP levels were inversely associated with peak FMD and with AUC, which is a measure of total dilatation response during the first 3 minutes of hyperemia. Because there were no differences in endothelium-independent NMD across the CRP groups, the differences in FMD and AUC seem to reflect changes specifically in endothelial function.

Increased carotid artery IMT or carotid plaques have also been associated with elevated CRP levels in most studies in adults but not all. Differences in methodology and study populations could explain these discrepancies. In our experience, the image quality of carotid scans in children compared with adults is more superior. Furthermore, in the present study, we used the latest digital ultrasound technology and a 13-MHz scanning frequency, which yield very-high-resolution images and excellent reproducibility of the IMT measurements. Thus, very subtle changes in the carotid IMT can be reliably measured in children. The findings of the present study complement the observations of these earlier studies by demonstrating that elevated serum CRP predisposes to early structural and functional changes of atherosclerosis in healthy children. Our observations suggest that CRP may have an important role in the pathogenesis of early atherosclerosis.

CRP levels and CRP distribution were similar to those previously reported in a population of healthy children. Elevated serum CRP levels were associated with elevated BMI, confirming previous observations in children and adults. Possible mechanisms for the observed association may include increased tumor necrosis factor-α production by adipocytes, which induces the synthesis of interleukin-6, the main hepatic stimulus for CRP production. It has been recently shown that CRP causes expression of ICAM-1 by endothelial cells. Accordingly, we found higher ICAM-1 concentrations with increased CRP levels, a further indication of endothelial activation in these children. Previous studies in adults have associated increased serum levels of soluble adhesion molecules, including ICAM-1, with atherosclerotic diseases and increased IMT. The relationships between CRP group and arterial changes, except for mean carotid IMT, remained significant after adjustment for BMI and ICAM-1 in the multivariate model. ICAM-1 was also univariately weakly associated with mean carotid IMT. Taken together, these results suggest that ICAM-1 may have a role in the CRP-associated increase in arterial wall thickness. Further studies are needed to determine the cellular pathways through which CRP and ICAM-1 induce early arterial changes.

Experimental studies have revealed several potential mechanisms explaining how CRP may exert its proatherogenic effects. CRP mediates the uptake of biochemically intact LDL into macrophages, which is a novel mechanism for foam cell formation in atherosclerosis. CRP accumulates in atherosclerotic lesions, induces monocyte chemotaxis, and mediates the receptor-mediated deposition of monocytes in the arterial wall. Because CRP influences reactive oxygen production by macrophages, it may also facilitate LDL oxidation in the subintimal space. Chronic infections may elevate CRP levels in children, but no antibody titers for infectious agents were determined in the present study. However, none of the children had experienced symptoms of acute infections during at least 2 weeks before the study. The present study examined the relationships between serum CRP and arterial properties by using a cross-sectional setting. A more ideal approach would be a longitudinal study to investigate the progression/regression of
atherosclerotic vascular changes, their association with serum CRP levels, and the influence of CRP-lowering therapy.

Clinical Implications
Increased CRP has been considered an epiphenomenon in CVD and inflammation/infection to account for increased risk. However, recent epidemiological, clinical, and experimental data suggest that CRP could directly participate in atherogenesis. This may have important clinical implications because CRP levels can be lowered either by medical therapy, including statins39–41 and fenofibrates,42 or by weight reduction.43 In a recent clinical trial, lowering of CRP levels by statin therapy in a primary prevention setting was indeed associated with a reduction in coronary events that occurred independently of the lipid-lowering effects.44 Thus, lowering of CRP either by pharmaceutical intervention or by weight reduction might have beneficial antiatherogenic effects.

Increased CRP has been considered an epiphenomenon in atherogenesis. This may have important clinical implications because CRP levels can be lowered either by medical therapy, including statins3 9–4 1 and fenofibrates, 42 or by weight reduction.43 In a recent clinical trial, lowering of CRP levels by statin therapy in a primary prevention setting was indeed associated with a reduction in coronary events that occurred independently of the lipid-lowering effects.44 Thus, lowering of CRP either by pharmaceutical intervention or by weight reduction might have beneficial antiatherogenic effects. Because elevated CRP levels are related with early functional and structural atherosclerotic vascular changes in children independently of conventional risk markers, further studies aiming to reduce the atherosclerotic burden by CRP reduction may be warranted in high-risk young individuals with elevated CRP levels.

In summary, our data suggest that CRP may affect the arteries of healthy children by disturbing endothelial function and promoting IMT. These findings are in line with observations of experimental studies and indicate that CRP may have an important role in the pathogenesis of early atherosclerosis.

Acknowledgments
This study was financially supported by the Academy of Finland, the Research Foundation of Orion Corp, the Research Foundation of Instrumentarium, the Finnish Cultural Foundation, the Turku University Foundation, and the Juho Vainio Foundation. The nitroglycerin preparation used in this study was a gift from Pohj-Boekamp GmbH. The authors would like to thank Hans Helenius, MSc, for statistical advice.

References


Elevated Serum C-Reactive Protein Levels and Early Arterial Changes in Healthy Children
Mikko J. Järvisalo, Aimo Harmoinen, Maarit Hakanen, Ulla Paakkunainen, Jorma Viikari, Jaakko Hartiala, Terho Lehtimäki, Olli Simell and Olli T. Raitakari

Arterioscler Thromb Vasc Biol. published online May 30, 2002;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2002/05/30/01.ATV.0000024222.06463.21.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/