Association Between Chlamydia pneumoniae Antibodies and Intimal Calcification in Femoral Arteries of Nondiabetic Patients

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Background: Chlamydia pneumoniae, a gram-negative bacterium, has been suggested to be a risk factor for atherosclerosis. Calcium is a well-known component of atherosclerotic plaques, but it is uncertain whether infectious agents play a role in the calcification process of the arteries.

Patients: To address this issue we investigated the association of Chlamydia antibodies with intimal arterial calcification as assessed by soft tissue radiograms from the thigh region of 1373 nondiabetic Finnish individuals aged 45 to 64 years.

Results: At baseline, radiologically detectable intimal calcification in femoral arteries was found in 172 (27%) of 638 men and 43 (6%) of 735 women (P = .001). The presence of intimal artery calcifications was strongly related to conventional atherosclerotic risk factors and to Chlamydia antibodies. In Cox regression analysis, association of Chlamydia antibodies with intimal artery calcification persisted after extensive adjustment for other cardiovascular risk factors (P = .04). A dose-response relationship was observed between Chlamydia antibodies and intimal femoral artery calcification (P = .006). The presence of intimal artery calcification was strongly associated with an increased risk of future coronary heart disease mortality (P < .001).

Conclusion: Chlamydia antibodies are strongly associated with intimal calcification of the femoral arteries.

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CHLAMYDIA PNEUMONIAE, a gram-negative obligate respiratory pathogen, has been suggested to be a possible trigger or even causative agent in the pathogenesis of atherosclerosis. This hypothesis was first presented by Finnish investigators, who showed that patients with coronary heart disease (CHD) had elevated IgG and IgA titers of antibodies and specific circulating immune complexes to Chlamydia. This result has been replicated in other populations, and, moreover, the presence of Chlamydia particles in atherosclerotic lesions in coronary and carotid arteries, the aorta, and abdominal aortic aneurysms has been directly demonstrated using polymerase chain reaction and immunohistochemical methods. These findings have led to eradication trials with antibiotics. In the rabbit model, Chlamydia infection accelerates the development of atherosclerosis, and treatment with azithromycin prevents it. Also, in human studies, azithromycin treatment may reduce the risk of cardiac events in patients with unstable angina or non-Q-wave myocardial infarction (MI) or in male survivors of MI.

Calcium has been a largely neglected, although well-known, component of atherosclerotic plaques. The presence of calcification in the coronary arteries as evaluated by electron-beam computed tomography has been suggested to be a sensitive, although not specific, marker of CHD that may yield information beyond traditional risk assessment. In the early phase, atherosclerotic lesions are composed mainly of lipids; from the third decade of life onward, the lesions are formed by progressive accumulation of intracellular and extracellular lipids and foam cells. From the fourth decade of life, atherosomatosus lesions may evolve primarily from fibrotic or calcific lesions. These lesions may further progress by surface defects, hemorrhage, or thrombus formation into complicated lesions and clinical manifestations. The early phases and progression of arterial calcification are poorly understood. In atherosclerotic plaques, calcium is found as hydroxyapatite, the form found also in the bone. Further similarities with calcium and bone metabo-
PATIENTS AND METHODS

STUDY POPULATION AT BASELINE

A random sample of nondiabetic individuals born and currently living in the Kuopio University Hospital district (eastern Finland) or in the Turku University Central Hospital district (western Finland) was taken from the population register containing all individuals aged 43 to 64 years. Of the 827 individuals in eastern Finland and 863 in western Finland originally eligible for the study, 651 in eastern Finland and 730 in western Finland participated in the study, giving participation rates of 79% and 85%, respectively. Two participants in eastern Finland and 6 in western Finland were excluded from the final analyses because diabetes mellitus was diagnosed at baseline. The final nondiabetic study population consisted of 649 individuals in eastern Finland and 724 in western Finland.

Comparison regarding some background variables was made between participants and nonparticipants by using the central register of the Social Insurance Institution. Participating and nonparticipating groups were similar with respect to clinical characteristics.

STUDY PROGRAM AND METHODS AT BASELINE EXAMINATION: 1982-1984

The study program was carried out during one outpatient visit to the Clinical Research Unit of the University of Kuopio or to the Rehabilitation Research Center of the Social Insurance Institution. The methods have been described in detail elsewhere.22 The visit included an interview on the history of chest pain symptoms suggestive of CHD, smoking, alcohol intake, physical activity, and the use of drugs. All medical records of participants who reported during the interview that they had been admitted to the hospital because of chest pain or symptoms suggestive of stroke were reviewed. Review of the medical records was performed by 2 of us (M.L. in Kuopio and T.R. in Turku) after careful standardization of the methods between the reviewers. The World Health Organization criteria for verified definite or possible MI based on chest pain symptoms, electrocardiographic changes, and enzyme determinations were used in the ascertainment of the diagnosis of previous MI.

Blood pressure was measured with the patient in the sitting position after a 5-minute rest using a mercury sphygmomanometer and was read to the nearest 2 mm Hg. Patients were classified as having hypertension if they were receiving drug treatment for hypertension or if their systolic blood pressure was at least 160 mm Hg or their diastolic blood pressure was at least 95 mm Hg.

BIOCHEMICAL METHODS

All laboratory specimens were obtained at 8 AM, after a 12-hour fast. Fasting plasma glucose concentration was determined using the glucose oxidase method (Boehringer Mannheim, Mannheim, Germany). Serum lipid and lipoprotein levels were determined from fresh serum samples drawn after a 12-hour overnight fast. Serum total cholesterol and triglyceride levels were assayed using automated enzymatic methods (Boehringer). Serum high-density lipoprotein (HDL) cholesterol levels were determined enzymatically after precipitation of low-density lipoprotein (LDL) and very low-density lipoprotein with dextran sulfate and magnesium chloride.24 The LDL cholesterol was calculated using the Friedewald formula as follows:

$$\text{LDL Cholesterol} = \text{Total Cholesterol} - \text{HDL Cholesterol} - \left( \frac{0.45 \times \text{Total Triglycerides}}{1} \right)$$

In patients with a triglyceride value greater than 354 mg/dL (4.0 mmol/L), the LDL cholesterol concentration was not calculated. Chlamydia IgG and IgA antibodies were determined from blood samples drawn during baseline examination. A simplified microimmunofluorescence modification with one spot was used.25 Antigen of the Kajaani 6 strain was used.

Patient characteristics and cardiovascular risk factors in relation to the occurrence of intimal femoral artery calcification are given in Table 1. Patients with intimal artery calcification were more often men, were older, were less obese, had a higher frequency of previously verified MI, and more often had a history of smoking than did those without artery calcification; the prevalence of hypertension was similar between the groups. Furthermore, higher insulin levels and adverse lipid profiles (elevated LDL cholesterol and total triglyceride levels and low HDL cholesterol levels) were also associated with intimal artery calcification. The frequency of medial artery calcification was 22.1% (278/1259) in nondiabetic participants, but, as in patients with type 2 diabetes mellitus,26 no evident association with respect to conventional cardiovascular risk factors was found (data not shown). Chlamydia pneumoniae antibodies were also significantly associated with the occurrence of intimal artery calcification (Table 1). In the following statistical analyses, the IgA and IgG antibodies were combined, but the conclusions were identical whether we used either of these variables.

Although the presence of intimal artery calcifications was, as expected, strongly related to conventional atherosclerotic risk factors (Table 1), the association with Chlamydia antibodies remained statistically significant even after extensive adjustment for confounding factors (age, sex, area of residence, total cholesterol level, smok-
of Chlamydia pneumoniae was obtained from Labsystems Oy (Helsinki, Finland). Incubation for 4-fold serum dilutions was 1 hour. For IgG titrations, dilutions starting at 1:32 were used. For IgA titrations, the serum samples were absorbed by Gullisorb treatment (Gull Laboratories, Salt Lake City, Utah), and dilutions were started at 1:10. All titrations were read by the same person (P.S.) using dry magnification (×400) in a Leitz fluorescence microscope with a 100-W mercury vapor lamp. Participants were classified as having elevated titers for Chlamydia if the IgA titer for Chlamydia was 1/40 or greater and the IgG titer was 1/128 or greater, as has been shown previously.

RADIOLOGICAL METHODS

Native soft tissue radiograms of the thigh were taken with the patient in a recumbent position. Radiological findings were analyzed by a radiologist (M.S.) in random order without knowledge of the Chlamydia antibody titers of the patient. The lower limb artery calcifications were divided according to the method of Lindbom into discrete plaque (intimal type) and uniform linear railroad track (medial type) calcifications. Grading of arterial calcifications was carried out separately on both sides by assessing the involvement of arterial trunks or branches. The extent of intimal and medial calcifications was originally graded as follows: 1 indicates none; 2, slight (calcifications just visible involving the arterial trunks or their branches of ≤5 cm long); 3, moderate (intermediate grade, neither grade 2 nor grade 4); or 4, marked (considerable calcification of ≥50% of the length of the arterial trunk with or without involvement of the arterial branches). For statistical purposes, intimal calcifications were divided into absent (grade 1) or present (grades 2-4), except in Figure 1, where they were graded as none (grade 1), moderate (grade 2 and grade 3), or severe (grade 4). The χ² coefficients for intraobserver variation were 0.87 for intimal calcification and 0.88 for medial calcification.

COLLECTION OF FOLLOW-UP DATA

In 1990, a postal questionnaire containing questions about hospitalization because of acute chest pain and symptoms suggesting stroke or lower limb amputation was sent to every surviving participant of the original study cohort. All medical records of participants who died between the baseline examination and December 31, 1989, or who reported in the questionnaire that they had been admitted to the hospital because of the aforementioned symptoms were reviewed. The World Health Organization criteria for definite or possible stroke based on a clinical syndrome consisting of neurological signs or symptoms persisting for longer than 24 hours were used to ascertain the diagnosis of stroke, as in the baseline study. The criteria for MI and stroke were identical to those in the baseline study. Copies of death certificates of patients who had died were obtained from the files of the Central Statistical Office of Finland. In the final classification of the causes of death, hospital and autopsy records were used, if available. The end point evaluated in this study was mortality from CHD (International Classification of Diseases, Ninth Revision, codes 410-414). The study was approved by the ethics committees of the University of Kuopio and the University of Turku.

STATISTICAL METHODS

Data analyses were performed using a statistical software program (SPSS/PC; SPSS Inc, Chicago, Ill). The results of continuous variables are given as mean±SE or percentage. Differences between the groups were assessed using the χ² test or the 2-tailed t test for independent samples, when appropriate. The multivariate Cox regression model and Kaplan-Meier survival curves were used to investigate the association of cardiovascular risk factors with the incidence of fatal CHD events.

The major findings of this large, population-based study were that the presence of chlamydia antibodies was associated in a dose-response fashion with the degree of intimal artery calcification and that this association was not explained by other cardiovascular risk factors. These findings suggest, but do not prove, that Chlamydia may play a fundamental role in the pathogenesis of calcification of atherosclerotic lesions.

In this study, native soft tissue radiographs were used to visualize artery calcifications. Separation of the intimal and medial forms of calcification is in most instances easily done. Chlamydia antibodies were associated with intimal artery calcifications and not with medial artery calcifications. The former type of calcification status, and hypertension (Table 2). Even further adjustment for HDL cholesterol level, total triglyceride level, and body mass index did not change the magnitude of this association (Table 2).

When the presence of intimal artery calcification was based on 3 categories (none, moderate, and severe), a dose-response relationship was observed. More severe the arterial calcification, the more common the Chlamydia antibodies (Figure 1). In other words, the more severe the arterial calcification, the more common the Chlamydia antibodies. No statistically significant association of medial artery calcification with Chlamydia antibodies was observed (none, 9.5%; moderate, 11.1%; severe, 12.4%; P= .25).

Smoking was markedly associated with intimal artery calcification and Chlamydia antibodies, blunting this relationship in smokers, but in nonsmokers, the presence of Chlamydia antibodies was markedly higher in individuals with intimal artery calcification (Figure 2).

We also investigated the relationship between radiological intimal artery calcification and CHD mortality during 7-year follow-up using the Cox regression model. As shown in Figure 3, the presence of intimal artery calcification was strongly associated with an increased risk of CHD mortality. This relationship remained statistically significant even after adjustment for other cardiovascular risk factors or risk modifiers, including age, sex, body mass index, total cholesterol level, hypertension, and smoking status. However, the risk of CHD mortality in patients with and without intimal femoral artery calcification was not modified by the presence of Chlamydia antibodies (Figure 3).

COMMENT

The major findings of this large, population-based study were that the presence of chlamydia antibodies was associated in a dose-response fashion with the degree of intimal artery calcification and that this association was not explained by other cardiovascular risk factors. These findings suggest, but do not prove, that Chlamydia may play a fundamental role in the pathogenesis of calcification of atherosclerotic lesions.
cation represents obstructive atherosclerosis, whereas the latter is a nonobstructive calcification of the medial layer commonly associated with aging and diabetes mellitus. However, as an index of atherosclerosis, the radiological intimal artery calcifications are rather crude, and radiological examination is likely to underestimate the degree of atherosclerosis. However, this method is sensitive, cheap, readily available, and, as shown in this study, a strong predictor of CHD mortality.

Chlamydia antibody titers may decrease substantially within a few years after seroconversion and may increase with the occurrence of reinfection. This temporal variation implies that any association of vascular disease with antibody titers for Chlamydia measured only once is substantially weaker than associations of vascular disease with long-term antibody concentration or more direct evidence of persistent infection at the relevant anatomical site. Therefore, it is remarkable that in our study the severity of intimal artery calcification increased linearly with the occurrence of Chlamydia antibodies. This association is suggestive of a close association of these processes, although suggestions of causality must be viewed with great caution.

The mechanisms via which Chlamydia can increase the risk of atherosclerosis remain unclear. Potentially it may precipitate acute cardiovascular events (plaque rupture) or may increase the size of the atherosclerotic plaque. This association could be mediated, at least in part, by an indirect effect of an adverse pattern of known or potential cardiovascular risk factors. Indeed, Chlamydia is associated with an adverse profile of serum lipids and lipoproteins, coagulation factors, and oxidative metabolites. Furthermore, smoking is strongly associated with arterial calcification, and as smokers more frequently have Chlamydia antibodies, smoking could account for a significant proportion of this effect. The relationship between Chlamydia antibodies and arterial calcification was not particularly strong among smokers in our study but was most evident in nonsmokers. Therefore, in our study and some previous studies, the association of Chlamydia with atherosclerosis is not explained statistically by conventional risk factors. Other indirect mechanisms behind this link could be mediated by chronic inflammation and/or cross-reactive antibodies. However, there is also in vitro evidence for a more direct association because Chlamydia may infect and multiply in smooth muscle cells, macrophages, and endothelial cells, which may contribute to the formation of atherosclerotic plaques. Our findings that Chlamydia antibodies were associated with intimal calcification but not medial calcification are in accordance with the hypothesis that Chlamydia could be directly involved in the process of calcification.

Although the accumulation of smooth muscle cells is a hallmark of atherosclerosis, the frequency of replication of smooth muscle cells in atherosclerotic plaques is in fact rather low. Therefore, other processes, for example the mechanisms of smooth muscle cell replication as well as plaque rupture, are likely to play an important role.

### Table 1. Patient Characteristics and Cardiovascular Risk Factors in Relation to the Presence of Intimal Femoral Artery Calcification at Baseline

<table>
<thead>
<tr>
<th>Intimal Femoral Artery Calcification</th>
<th>No</th>
<th>Yes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women, No.</td>
<td>466/692</td>
<td>172/43</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age, y</td>
<td>54.1 ± 0.2</td>
<td>57.6 ± 0.3</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.8 ± 0.2</td>
<td>257.5 ± 0.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Previous myocardial infarction, %†</td>
<td>3.6</td>
<td>12.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>31.9</td>
<td>35.8</td>
<td>.26</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>15.1</td>
<td>44.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Insulin, µU/mL‡</td>
<td>12.0 ± 0.03</td>
<td>13.5 ± 0.01</td>
<td>.003</td>
</tr>
<tr>
<td>Cholesterol, mg/dL§</td>
<td>263 ± 2</td>
<td>270 ± 3</td>
<td>.06</td>
</tr>
<tr>
<td>Low-density lipoprotein</td>
<td>181 ± 2</td>
<td>189 ± 3</td>
<td>.04</td>
</tr>
<tr>
<td>High-density lipoprotein</td>
<td>58 ± 0.4</td>
<td>54 ± 1</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Total triglycerides, mg/dL¶</td>
<td>123 ± 2</td>
<td>146 ± 8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IgA antibodies ≥40 for Chlamydia pneumoniae, %</td>
<td>5.6</td>
<td>9.6</td>
<td>.03</td>
</tr>
<tr>
<td>IgG antibodies ≥128 for Chlamydia pneumoniae, %</td>
<td>6.2</td>
<td>11.2</td>
<td>.01</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SE except where indicated otherwise.†Verified at the hospital or by Q/QS change.
§To convert cholesterol from milligrams per deciliter to millimoles per liter, multiply milligrams per deciliter by 0.02586.
¶To convert triglycerides from milligrams per deciliter to millimoles per liter, multiply milligrams per deciliter by 0.01129.


 Although C. pneumoniae is an asymptomatic organism, it has been implicated in several nonspecific clinical conditions, including CVD. Indeed, the presence of antibodies to C. pneumoniae has been associated with atherosclerotic plaque (92-94). This association is also observed in healthy individuals without cardiovascular disease (95,96).  

The association of C. pneumoniae with atherosclerosis may be linked to superantigen production, which is a common feature of other Chlamydia species (97). The superantigen produced by C. pneumoniae is believed to act on the surface of endothelial cells and bone marrow-derived cells (98). It is also postulated that C. pneumoniae superantigens may trigger the release of cytokines that contribute to atherosclerotic plaque formation (99).  

Several studies have demonstrated that C. pneumoniae is associated with more advanced atherosclerotic lesions, including plaque rupture and unstable angina (100,101). However, the relationship between C. pneumoniae infection and atherosclerosis is not always consistent. Some studies have failed to find a significant association between C. pneumoniae infection and atherosclerotic plaque burden (102,103).  

Furthermore, it is not clear whether C. pneumoniae infection is a cause or consequence of atherosclerosis. Some studies have suggested that C. pneumoniae infection may precede the development of atherosclerosis (104,105), while others have found no evidence of a temporal relationship between the two conditions (106,107).  

Despite the controversies surrounding the role of C. pneumoniae in atherosclerosis, the association of C. pneumoniae infection with several cardiovascular outcomes is well established. This association is also observed in healthy individuals without cardiovascular disease (108). The significance of these findings is not clear, but they suggest that C. pneumoniae infection may be a risk factor for atherosclerosis (109).  

In conclusion, the association of C. pneumoniae infection with atherosclerosis is a complex issue, and more research is needed to clarify the role of this organism in the development of atherosclerotic plaque burden.